A broad-selective enzyme immunoassay for non-invasive stress assessment in African Penguins (*Spheniscus demersus*) held in captivity

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ABSTRACT

We applied a direct competitive immunoassay for measuring corticosterone and glucocorticoid metabolites in feces (FGMs) as a non-invasive tool for monitoring the stress response of African Penguins (*Spheniscus demersus*) held in captivity in a zoological facility. The developed assay, validated in-house, proved to be rapid (the test could be completed in 90 minutes), sensitive (LOD for corticosterone 0.2 µg l^{-1}, dynamic range 0.75-75 µg l^{-1}) and broad-selective, as it cross-reacted with the major corticosteroids, thus allowing the detection of excreted FGMs resulting from a biological stressor. Matrix interference, due to components of faecal samples, was overcome by diluting sample extracts (1+4 or 1+9, depending on sample features).

The assay enabled us to investigate the response to stress in five animals- three adult males and two adult females- over a period of 30 hours, and to identify the peak of FGM production as being 7-10 hours after the stressful event.

KEYWORDS Corticosterone, glucocorticoid, faecal metabolites, biological validation, class-selectivity

INTRODUCTION

Animals held in captivity are subject to a variety of physical, social, dietary, and ecological limitations that affect their welfare and behavior.[1,2] In recent years, several zoos and aquaria have intensified efforts to develop approaches and tools for assessing the well-being of captive animals, due to increasing public concern about animal welfare and ethical issues.[3] The monitoring of animal welfare has been regulated by national and international provisions aimed at assuring biological and conservation requirements of individual species (*e.g.* Italian Legislative Decree 73,[4] Council Directive 99/22/EC [5]). In addition, several zoo associations operate at national and international levels in promoting excellence in animal care and welfare, and in maintaining a high standard of animal husbandry, *e.g.* UIZA (Unione Italiana Giardini Zoologici e Acquari),[6] EAZA (European Association of Zoos and Aquaria),[7] and WAZA (World Association of Zoos and Aquariums)[8].

A feasible way to monitor the welfare of animals held in captivity is represented by the evaluation of their response to stress caused by major constraints imposed by living in zoos. Exposure to stress usually results in an increased secretion of glucocorticoid hormones (GCs) from the adrenal cortex, and GCs are therefore commonly used as stress markers in human and vertebrate animals.[9] The predominant avian GC is corticosterone (B), a C-21 hormone produced by the adrenal glands, involved in diverse regulatory mechanisms, including: immune reactions, protein synthesis and degradation, and metabolic reactions. The corticosterone plasmatic level is considered a reliable marker of stress levels in birds.[9] Nevertheless, measuring corticosterone and related GCs in the plasma requires handling the animals for blood collection, which could elicit, in itself, a substantial rise in GC concentrations in the blood due to the stress caused by the restraint and taking blood.[9] *blood collection, maybe?*

An accepted alternative method for the evaluation of adrenocortical activity is the measurement of GC metabolites excreted in feces.[10] Although GCs are not actually present in faeces as they are metabolized in the liver, their metabolic products are excreted into the gut via the bile. Faecal glucocorticoid metabolite (FGM) levels have been demonstrated to reflect plasmatic GC concentrations, although as an integrated measure over the time, and after a variable time delay compared to the plasmatic GC response to the stressful event.[10,11] A major benefit of measuring FGMs is represented by the fact that faeces collection is a non-invasive practice~~, thus~~ which allows ~~allowing~~ extensive sampling campaigns, sample collection from small animals as well, and prevention of bias due to the sampling itself. Faeces can be easily collected, because there is no need to capture or handle the animals; therefore, repeated samplings from the same individual are possible without affecting the animal's behavior.[11] However, as metabolic pathways involved in GC degradation are numerous,[12] and are influenced by several factors (including, but not limited to:

species, gender, age, reproductive status, season, etc.), predicting the nature and the chemical structure of targets is almost unachievable. For the same reason, developing specific antibodies for the excreted metabolites of each species, in order to set dedicated immunoassays, is often impractical.[13,14] Nevertheless, it has been argued that knowing the chemical identity of faecal GC metabolites in each species and for each condition is unnecessary.[15]

The most widely accepted method to assess the stress response in animals by FGM measurement is a practical approach, based on the development of immunoassays that exploit so-called broad-selective antibodies (namely polyclonal antisera able to bind a group of related substances rather than a defined compound), and the demonstration of the capability of these assays to reflect adrenocortical activity by a physiological and/or biological validation.[11] ~~An increase of the response of the assay~~ An increased response of the assay (expressed as an increase of FGM concentrations), following an appropriate stimulus, such as adrenocorticotropic hormone (ACTH) challenge or a recognized biological stressor, is assumed to demonstrate the capability of the assay to reflect changes in the activity of the hypothalamic–pituitary–adrenal axes[11,12] and thus to ascertain stress. The physiological and/or biological validation legitimizes the application of the assay for a certain species, and for those individuals and stressful conditions for which it has been tested.[11,16]

The pre-requisite for developing a useful immunoassay is, therefore, the availability of antibodies able to bind the wider variety of GCs (*i.e.* having a broad selectivity). However, sensitivity is also crucial, mainly when FGMs have to be detected in minute quantities of faeces, such as those belonging to small avian species.[14,17] FGM assays have been validated for a multitude of species, including several birds.[9,17-19] Most of these studies tended to employ commercially-available radioimmunoassays or enzyme immunoassays primarily developed to measure cortisol or corticosterone, which usually only cross-reacted with a few other GCs (Table 1), not unexpectedly, as they were designed to selectively measure the target compound.

Specially developed immunoassays have also been described, based on antibodies aimed at measuring a specific faecal GC metabolite (*e.g*. tetrahydrocorticosterone[20]) or designed to be group-specific (*e.g.* 11,17-dioxoandrostanes[11]). Despite their selectivity profiles, all these immunoassays were shown to be able to measure an increase in FGMs that were artificially stimulated in physiological validation experiments. In addition, they have occasionally been applied for non-invasive investigation of the stress response induced by a specific constraint[9,21,22]. However, the conclusions drawn about the effect of a supposed stressful event on animals also depended on the responsiveness of the employed assay, or rather, the capability to detect the increased adrenocortical activity. A higher assay sensitivity (i.e. lower detection limits) would, of course, be desirable.

Therefore, the aim of this work was to establish a sensitive and rapid enzymatic immunoassay in the direct competitive format that exploits a broad-selective antibody towards GCs. Assay optimization was conducted to identify experimental conditions aimed at maximizing sensitivity, and the developed assay was subjected to in-house analytical validation. The assay was used to measure FGMs from African Penguins (*Spheniscus demersus*) held in captivity in a biopark (ZOOM Torino, Italy), to and test its suitability for non-invasive monitoring of stress levels in these animals.

The African Penguin is a marine bird endemic of to South Africa and Namibia. The current conservation status of this species is "Endangered", and WHO/WHAT? is indicated in the Red List of Threatened Species of the IUCN (International Union for Conservation of Nature) because the wild population has dramatically decreased in recent years to less than 75-80,000 mature individuals.[23] Therefore, the African Penguin faces a great risk of extinction, and *ex-situ* conservation programs are becoming increasingly crucial. The African Penguin is a monogamous species with a complex behavioural repertoire,[24] and is exhibited in large groups in zoos and aquaria all over the world. To improve the health and general well-being of African Penguins held in captivity, the identification of stressful conditions is required, in order to develop mitigating strategies. To successfully achieve conservation of endangered species, it is important that captive facilities focus their efforts on welfare and health, which [*riferito a chi?]* involves minimizing and reducing stressful stimuli facing animals in captive environments.[25] Measuring glucocorticoids as an indicator of adrenal activity can help conservation biologists and animal managers understand the causes of poor welfare.[26-29]

Matrix interference due to the variability of the faeces collected from five adult African Penguins (three males, and two females) over 30 hours was studied and surmounted through appropriate sample dilution. Immunoreactive FGM concentrations, measured by the developed assay, were also compared to those obtained by means of a reference enzyme immunoassay, previously developed and validated for a different species of penguin, the Adélie Penguin (*Pygoscelis adeliae*).[30]

MATERIALS AND METHODS

Materials

Steroids (S, Table 1) were purchased from Steraloids (Newport, RI, USA), except for cortisol (F), which was obtained from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA), N,N'-diisopropylcarbodiimide (DIC), N-hydroxysuccinimide (NHS), and 3,3'5,5'-tetramethylbenzidine liquid substrate (TMB) were also purchased from Sigma- Aldrich. Horse-radish peroxidase (HRP) was from Roche Diagnostics (Milan, Italy). Dimethylformamide (DMF), methanol, Tris(hydroxymethyl)aminomethane (TRIS)

and all other chemicals and microtiter plates were obtained from VWR International (Milan, Italy). Rabbit polyclonal anti-3-(O-carboxymethyl)oxime-BSA antibodies were kindly supplied by G. Bolelli (Servizio di Fisiopatologia della Riproduzione, Policlinico S. Orsola, Bologna, Italy).

The hapten used for enzyme labelling was cortisol-3-(O-carboxymethyl)oxime (F-3-cmo, Figure 1) and was synthesized as previously reported.[31] F-3-cmo was then conjugated with HRP by the carbodiimide ester method.[32] The obtained F-HRP conjugate was stored at 4°C, with the addition of 33% (v/v) of glycerol. The diluted working solution was prepared daily in TRIS buffer (20 Mm, pH 8.5, with 0.3M NaCl, 1% BSA, w/v, 0.1% Tween 20, v/v).

Steroid stock solutions were prepared by dissolving the powders in absolute ethanol and stored at -20°C. Standard solutions were prepared by daily diluting the stock solutions with methanol:water (35:65, v/v).

Competitive Enzyme-Immunoassay (EIA)

The immunoreactive solid phase was obtained by coating wells with 150 µl of the antiserum directed towards cortisol diluted 1:10,000 (v/v) with carbonate/bicarbonate buffer (50 mM, pH 9.6), followed by overnight incubation at 4°C. Uncoated well surfaces were blocked with 300 µl PBS supplemented with 0.5% of BSA for 1 hour at room temperature. Wells were then washed using a 0.05% Tween 20 solution.

Calibration curves were constructed by adding 150 µl of F-HRP (1.5 mg L^{-1}) to 50 µl of B diluted in aqueous methanol (35%, v/v) at concentrations ranging from 0 to 50 µg L^{-1}. The mixture was incubated for 1 hour in immunoreactive wells, followed by washing, and colour development was then obtained by a 30 min incubation with TMB (200 µl per well). A volume of 50 µl of sulphuric acid (2M) was used as a stop solution, and absorbance was recorded at 450 nm. Unknown sample concentrations were measured by replacing the B standard solution with sample extracts diluted 1+1 with water as well as further dilutions of 1+4 or 1+9 with aqueous methanol (35%, v/v). All standards and samples were measured in duplicate.

Unknown FGM concentrations were determined by interpolation on the calibration curve, where the signal was plotted against the log of analyte concentration. For each experiment, a calibration curve was determined by nonlinear regression analysis of the data from the standards, using the four-parameter logistic equation.

Relative cross-reactivity (CR) was evaluated by carrying out standard curves of the investigated compounds (S) in the same experimental conditions as B, except for the concentration interval, which was in the range of 0-5000 µg L^{-1}, and was calculated as follows:

$$CR\% = (IC_{50}\ B / IC_{50}\ S)*100$$

where IC_{50} is the S concentration that causes 50% inhibition of the maximum observed signal.

Samples and sample preparation

A total of 28 faecal samples from three adult males and two adult females were collected. The colony was observed from a distance (>5 m), to avoid disturbing the animals, by a researcher standing motionless outside the exhibit. After a defecation event, the researcher entered into the exhibit and gathered the expelled faeces. As urinal and faecal excretion are combined in birds, we only collected the faecal portion from droppings, which was distinguishable by color.[33] Faecal samples were collected into cryovials and stored immediately after collection at -20°C.

Fortified samples were prepared by adding 2.5, 10, and 40 µg l^{-1} of B to three sample extracts, which had previously been tested as containing low levels of FGMs.

FGM extraction was carried out as reported in the literature[32] with the following modifications. Briefly, penguin faeces (which were contaminated with sand of the exhibit) were transferred to a 15 ml tube and extracted with 5 ml of methanol: water (70:30, v/v), by shaking on a rotary shaker for 30 min.

After centrifugation for 5 min at 3000 x g to remove sand and particulate matter, 3 ml of the clear supernatant was transferred to a weighted tube, and the amount of the extracted sample was obtained as 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.

RESULTS AND DISCUSSION

Competitive Enzyme-Immunoassay analysis

The polyclonal antiserum used to develop the assay was developed by stimulating an immune-response using an F conjugate. Nevertheless, cross-reactivity towards B was preliminary demonstrated to be 100% (Table 2); therefore this antiserum was deemed to be suitable for measuring GCs in general, and thus exploited to set the immunoassay.

Checkerboard assays using various combinations of antibody and enzyme tracer concentrations were carried out to select appropriate F-HRP and antibody dilutions for the direct competitive assay. A dilution of 1:10,000 (v/v) of antiserum, and a concentration of 1.5 mg L^{-1} of F-HRP were selected as being the most

199 suitable based on the lowest IC_{50} value . B standards were diluted in aqueous methanol, as FGM extraction
200 from faecal samples typically involves a high percentage of this solvent, according to the literature.[11] The
201 assay proved to be robust for methanol contents lower than 40%, whereas sensitivity decreased for higher
202 solvent percentages. Dilution of B standards in TRIS buffer also negatively affected assay sensitivity and
203 precision. Therefore, the ideal diluent for B standards was established to be methanol: water 35:65 (v/v).

204 Figure 2 shows a typical inhibition curve obtained under optimized conditions. The IC_{50} value of the assay
205 was 4.5 µg l^{-1}. The limit of detection (LOD) was calculated at 90% inhibition of the maximum signal (A_{max}),
206 and the dynamic range as the interval between 20 and 80% of A_{max}[34], and were estimated to be 0.2 µg l^{-1}
207 and 0.75-75 µg l^{-1}, respectively.

208

209 ***Selectivity of the assay***

210 According to the literature, tetrahydrocorticosterone (THB) is thought to be the main B metabolite;[33]
211 however, this point is still debated, and several other possible metabolic products, characterized by very
212 different chemical structures, have also been shown to be excreted in faecal samples of birds.[12,15] Möstl *et*
213 *al.*[12] suggested at least seven possible metabolic pathways starting from B and resulting in the production
214 of: 3-hydroxycorticoids, 11-oxocorticoids, 21-deoxycorticoids, 21-acid corticoids, 17-oxoandrostanes, and 6-
215 hydroxycorticoids. However, faecal metabolites of GCs in birds have not been positively identified, and no
216 data are available on this subject in the literature. Most authors used competitive immunoassays (RIAs,
217 Radio Immuno Assays or EIAs, Enzyme Immuno Assays), developed for measuring corticosterone or
218 tetrahydrocorticosterone, as tools to assess FGM levels[9,17,30], on the basis of a demonstration that the assay
219 is capable of detecting an increase in FGMs, when artificially induced by an appropriate biochemical or
220 biological stimulus. An increased immune-response of the assay is interpreted as a consequence of the
221 increased FGM concentration, regardless of the identification of the chemical compound responsible for
222 the increase.[15]

223 Although responsiveness to induced stress of validated immunoassays for measuring FGMs is
224 controversially related to cross-reactivity of the assay itself (*i.e*. the capability of detecting several different
225 GCs), as is evident from Table 1, achieving broad-selectivity should be a major requirement for the
226 analytical method to be applied for detecting FGMs. Therefore, the selectivity of a polyclonal antiserum,
227 obtained by immunizing with a cortisol derivative modified in position 3 (Figure 1), was tested in response
228 to a large number of steroid structures, which varied according to the substituents in position 3, 11, 6, and
229 17, and according to the presence of insaturations at position 1-2 and 4-5, consistent with the hypothetical
230 metabolic modifications which corticosterone can undergo. Relative cross-reactivities compared to B are
231 shown in Table 2. Most steroids were recognized by the antiserum, at levels between 7 and 38%; among

these, surprisingly, testosterone demonstrated a high cross-reactivity (30%) despite substantial modification of the substituent at position 17 compared to B and F. Oxidation of the 11-hydroxyl to form 11-oxosteroids determined a sharp decrease in binding, as manifested by the relatively low cross-reactivity of cortisone compared to cortisol, and prednisone compared to prednisolone; on the contrary, the substitution of the 11-hydroxyl with hydrogen did not seem to negatively influence the binding (as evident by comparing P and 11-hydroxyl-P). The addition of a substituent at position 6 slightly affected the recognition (CR of metil-prednisolone and prednisolone were 26% and 38%, respectively).

Otherwise, THB and THF showed absolutely no cross-reactivity. The lack of recognition of these compounds was mainly attributed to the hydroxyl substituent at position 3, whereas the saturated ring partially contributed to decrease cross-reactivity, as demonstrated by comparing CR% for the couple adrenosterone/androstanedione, differing in the insaturation of the A ring, which determined a limited CR% decrease (from 1% to 0.2%). Furthermore, androstenediol and androstan-3,17-diol, both having a hydroxyl at position 3, were not recognized, independently from the saturation state of the A ring. The applied extraction procedure could not exclude the presence of conjugates glucocorticoid metabolites, i.e. glucoronides at position 3. However, the conjugation of the hydroxyl substituent could contribute to reverse the decreasing of recognition.

Penguin faeces analysis: analytical validation of the EIA

Collection of faecal samples from African Penguins held in the exhibit of a park creates two main concerns, namely the limited amount of the sample available, and the presence of exogenous materials belonging to the exhibit, such as sand and pebbles. To address the first issue, the developed assay should be as sensitive as possible, and matrix interference should be counteracted without excessive sample dilution. To take into account the presence of spurious materials, quantitation of faeces was obtained by weighing a fixed volume of sample extracts, after centrifuging,, to remove undesired components, rather than weighing the samples themselves. To relate the quantity of measured FGMs to the sample amount, the contribution due to solvent weight was subtracted from the extract weight. The obtained sample weight was, in fact, the weight of the soluble or extractable portion of the sample.

The extraction protocol was taken from the literature[30] and applied without further optimization. Since samples were extracted with methanol/water 70/30, a 1+1 dilution with water was carried out to match the organic solvent content of samples with that of the B standards, and to preserve the sensitivity of the assay..

Furthermore, we observed that collected samples were very variable, not only in terms of the recovered amounts of faeces, but also in terms of the aspect of the extracts. Some extracts were intensely coloured (from pale yellow to dark green); some were transparent, while others were turbid~~, independently from~~ regardless of the colour. The variable appearance of extracts was thought to be connected to faeces composition, and could depend on individual biological variability, individual circumstances at the time of collection, time elapsed from feeding, urea content, etc. Therefore, matrix interference on the assay was evaluated by carrying out recovery experiments on four representative samples: a turbid white (TW), a turbid orange (TO), a limpid light green (LG), and a limpid dark green sample (DG). Extracts were fortified at three levels with B (2, 10, and 50 µg l^{-1}) and non-fortified and fortified samples were analysed using the developed EIA. All samples were strongly overestimated, as testified by recovery rates that were two to ten-fold greater than the expected values (data not shown). The same samples were also tested after being diluted with water or with methanol/water 35/65 as follows: 1+0, 1+1, and 1+4. In addition, two buffered solutions (TRIS buffer at pH 8 and 9) were evaluated as the F-HRP diluent. The pH of the buffering solution and the methanol content did not significantly affect the results obtained on faecal samples (data not shown); nevertheless, dilution factors were demonstrated to strongly influence FGM estimation, mostly for samples that displayed green coloured extracts (Figure 3). The TW sample showed very low levels of FGMs, which resulted as undetectable at higher dilution factors, and were related to the scarce faecal material present in the sample, as confirmed by calculated weight (5 mg). Turbidity, likely associated to urea content, seemed to have a lesser effect on the reliability of results, compared to colour. Green coloured samples were more prone to matrix interference than yellow-orange samples.

To limit the matrix effect, and to establish a unique sample treatment, which possibly did not depend on the characteristics of the sample, an overall 1+9 dilution of faecal sample extracts was chosen, with the following diluents: 1+1 with water to reduce the organic solvent content and match conditions of the greatest sensitivity of the assay, followed by a further 1+4 dilution with methanol/water 35/65 (total sample extract dilution: 1:10). Nonetheless, very dark extracts were also analysed in a dilution of 1:20 (1+1 with water and a further 1+9 with methanol/water), and FGM concentrations were calculated from the mean result of the two dilutions, when agreeing, otherwise from the value given by the higher dilution factor.

The accuracy of the optimized EIA method was investigated by means of recovery experiments on three faecal sample extracts, which were previously assessed to contain low levels of FGMs (< 500 ng/g), and were fortified at three B levels: 2.5 (low), 10 (medium), and 40 µg l^{-1} (high). Within and between-assay precision was established by testing three faecal samples, which were shown to contain three concentration levels of FGMs (low, medium and high), in eight replicates from the same day, and on four different days, respectively (Table 4). Accuracy was between 83 and 116% (Table 3); within-assay precision

was measured to be in the range of 7-8% (n=8); and between-assay precision was measured to be in the range of 5-16% (n=4). The figure of merits of the optimized assay demonstrated that the developed EIA is accurate and precise enough to allow FGM determinations in penguin faecal samples, regardless of sample composition.

Biological validation of the EIA to assess adrenocortical response to stress

To demonstrate the usefulness of the developed EIAas a non-invasive tool for detecting adrenocortical response to stress in African penguins, a biological validation was carried out. Faecal samples from three adult males and two adult females were collected after a well-known cause of stress, namely the capture and immobilization of animals.[35,36] Sample collection started immediately after this stressful event, and continued until about 30 hours following the first collection, except at night. Sample frequency and numerosity depended on the individual, and ranged from three samples, from the animal named "G", to seven samples from the animal known as "S". The FGM content of each sample was measured by the developed v. The same samples were also analysed by the method validated by the group of Möstl and co-workers[30] for measuring FGMs in the faeces of Adélie Penguins (*Pygoscelis adeliae*) and Wilson's storm petrels (*Oceanites oceanicus*),[20] as a reference method. FGMs measured by both analytical methods are shown in Table 5, together with the time elapsed from the stressful event, and the amount of the sample available for analysis. As is evident, for some samples, a reasonable amount of faeces could be collected, whilst in other cases the available amount was lower or absent; FGMs were therefore only measured in the samples for which at least 20 mg of faeces were available (as recommended[17]).

Despite individual variability, results from all five animals qualitatively agreed in suggesting a peak of FGM production between 7 and 10 hours after the stressful circumstance. This observation is in good agreement with results previously reported for other birds. For example, Nagakawa *et al.* reported a profile of FGM excretion after ACTH administration which showed a peak after 6-18 hours in Adélie Penguins;[30] Denhard *et al.* observed a significant increase in FGM levels at 5.5-8 hours after ACTH administration to chickens (*Gallus domesticus*).[13]

In addition, the qualitative behaviour is in good agreement with results obtained through the reference EIA. Nevertheless, from a quantitative point of view, the developed EIA yielded higher levels of FGMs for all samples. The reference EIA, which used an antibody developed towards tetrahydrocortisone, generally gave lower FGM concentrations, and undetectable levels of FGMs in 12 out of the 22 samples analysed. The discrepancy between the two assays could be attributed to the different cross-reactivity profiles of the antibodies employed. The antibody used in the reference assay was decidedly more selective, as it only cross-reacted with 11-hydroxyandrosterone, tetrahydrocortisol, and cortol, while all other tested steroids

(Table 1) showed cross-reactivity values lower than 1%. Since FGMs are a group of unknown compounds that are structurally variable at different positions, as they could belong to several metabolic pathways, the broader the selectivity of the assay, the higher the probability of detecting a larger number of compounds and, therefore, the higher the sensitivity of the assay, or rather the capacity to identify the presence of FGMs.

CONCLUSIONS

We developed An enzyme immunoassay to detect glucocorticoid metabolites was developed, based on a broad-selective antibody. The assay was shown to be accurate, precise and decidedly more rapid than previously reported radio and enzyme immunoassays intended for measuring FGMs. Thus, the time needed to complete the analysis was 90 minutes, rather than overnight incubations, as required by existing immunoassays. The assay was applied to determine FGM levels from African penguins held in captivity, and demonstrated a reliable assessment of FGM increase solicited by an artificially induced biological stressor with high sensitivity. Indeed, ACTH infusion, which is the most commonly employed strategy to validate assays for FGMs, is a more efficient means to provoke the physiological increase of adrenocortical activity, and integrates the biological stress (capture, handling, injection) with the biochemical stimulus. However, as ACTH challenge is a potent stressor, the capacity of a proposed assay to detect the physiological response to stressful events could be overestimated by using this kind of inducement, thus limiting the reliability of conclusions drawn where less intense environmental, biological, or behavioural causes of stress are being investigated. The enzyme immunoassay developed in this study allowed the detection of an adrenocortical response to a biological stress (animal capture) in African penguins and demonstrated that the maximum physiological response (increase of FGMs) was reached after 7-10 h from the stressor. Therefore, this assay can be suggested as a reliable tool to evaluate the effect of potential stressful circumstances that these animals may undergo in captivity, such as, for example: visitor flow, excessive noise, and inappropriate weather. By identifying stressful stimuli, efforts can be made to reduce their effect and prevent their occurrence, in order to improve the general welfare of captive animals and increase breeding success. Nowadays, stress is one of the major issues facing zoological institutions around the world, and identifying and reducing sources of stress should therefore be a key factor for conservation programs of threatened species.[25]

We propose the use of the African Penguin as a model species, and the application of the same methodology to evaluate the well-being of other endangered species kept in captivity. Groups of African Penguins are housed in zoos and aquaria worldwide; these colonies are formed by a high number of birds,

enabling analysis of differences in age, gender and individuality. Finally, the ability to monitor adrenocortical activity in a non-invasive manner in African Penguins, and in general in endangered species held in captivity, is of major value in welfare management strategies, as prolonged periods of elevated GC concentrations interfere with numerous physiological processes, including immune and reproductive functions.

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REFERENCES

1 C. Draper, S. Harris, *Animals.* 2012, **2**, 507-528.

2 S.W. Margulis, C. Hoyos, M. Anderson, *Zoo Biol*. 2003, **22**, 587-299.

3 J.C. Whitham, N.Wielebnowski , *Appl Anim Behav Sci*. 2013, **147**, 247-260.

4 Italian Legislative Decree 73 of 21/3/2005, Attuazione della direttiva 1999/22/CE relativa alla custodia degli animali selvatici nei giardini zoologici, http://www.parlamento.it/parlam/leggi/deleghe/05073dl.htm, (accessed April 2014).

5 Council Directive 1999/22/EC of 29/3/1999 on the keeping of wild animals in zoos, http://europa.eu/legislation_summaries/environment/nature_and_biodiversity/l28069_en.htm, (accessed April 2014).

6 UIZA (Unione Italiana Giardini Zoologici e Acquari), http://www.uiza.org/home.asp, (accessed April 2014).

7 EAZA (European Association of Zoos and Aquaria), http://www.eaza.net/Pages/European%20Association%20of%20Zoos%20and%20Aquaria.aspx, (accessed April 2014).

8 WAZA (World Association of Zoos and Aquariums), http://www.waza.org/en/site/home (accessed April 2014).

9 J.L. Blickley, K.R. Word , A.H. Krakauer , J.L. Phillips, S. N. Sells, C. C. Taff , J.C. Wingfield , Gail L. Patricelli , *PLoS ONE.* 2012, **7**, e50462.

10 R. Palme, S. Rettenbacher, C. Touma, S.L. El-Bahr, E. Möstl, *Ann Ny Acad Sci*. 2005, **1040**, 162-171.

11 C. Touma, R. Palme, *Ann Ny Acad Sci*. 2005, **1046**, 54-74.

12 E. Möstl, S. Rettenbacher, R. Palme, *Ann Ny Acad Sci*. 2005, **1046**, 17-34.

13 M. Dehnhard , A. Schreer , O. Krone , K. Jewgenow , M. Krause , R. Grossmann , *Gen Comp Endocr* . 2003, **131**, 345-352.

14 J.J. Millspaugh, B. E. Washburn , *Gen Comp Endocr* . 2004, **138**, 189-199.

15 S.K. Wasser , K.E. Hunt , J.L. Brown , K. Cooper , C.M. Crockett , U. Bechert , J.J. Millspaugh, S. Larson , S.L. Monfort, *Gen Comp Endocr*. 2000, **120**, 260-275.

16 M.J. Sheriff , B. Dantzer, B. Delehanty , R. Palme , R. Boonstra , *Oecologia*. 2011, **166**, 869-887 .

17 A.M. Young, D.M. Hallford, *Zoo Biol*. 2013, **32**, 112-116.

18 A.M. Staley, J.M. Blanco, A.M. Dufty Jr , D.E. Wildt , S.L. Monfort , *J Comp Physiol B.* 2007, **177**, 609-622 .

19 N.E. Cyr , L.M. Romero , *Gen Comp Endocr* . 2008, **158**, 20-28.

20 P. Quillfeldt, E. Möstl, *Acta Ethol*. 2003, **5**, 115-122 .

21 D. Frigerio , J. Dittami , E. Möstl, K. Kotrschal , *Gen Comp Endocr*. 2004, **137**, 29-36.

22 C.M. Bauer, L.W. Glassman, N.E. Cyr, L.M.Romero, *Comp Biochem Phys A*. 2011, **160**, 390-399.

23 The Red List of Threatened Species of IUCN (International Union for Conservation of Nature), http://www.iucnredlist.org/details/22697810/0, (accessed April 2014).

24 P. Eggleton, W.R. Siegfried, *Ostrich*. 1977, **50**, 139-167.

25 E. J. Narayan, K. Webster, V. Nicolson, A. Mucci, J.M. Hero, *Gen Comp Endocr*. 2013, **187**, 39-47.

26 B.G. Walker, P.D. Boersma, J.C. Wingfield, *Integr. Comp. Biol*. 2005, **45**, 12-18.

27 M. Wikelski, S.J. Cooke, *Trends Ecol. Evol*. 2006, **21**, 38-46.

28 F.E. Clark, M. Fitzpatrick, A. Hartley, A.J. King, T. Lee, A. Routh, S.L. Walker, K. George. *Zoo Biol*. 2011, **30**, 1-16.

29 R. Watson, C. Munro, K.L. Edwards, V. Norton, J.L. Brown, S.L. Walker, *Gen Comp Endocr*. 2013, **186**, 16-24.

30 S. Nakagawa, E. Möstl, J.R. Waas, *Polar Biol*. 2003, **26**, 491-493.

31 S.C. Janovsky, F.C. Shulman, G.E. Wright, *Steroids*. 1973, **23**, 49.

32 G. Giraudi, L. Anfossi, I. Rosso, C. Baggiani, C. Giovannoli, C. Tozzi, *Anal Chem*. 1999, **71**, 4697–4700.

33 C.E. Ninnes, J.R. Waas, N. Ling, S. Nakagawa, J.C. Banks, D.G. Bell, A. Bright, P.W. Carey, J. Chandler, Q.J. Hudson, J.R. Ingram, K. Lyall, D.K.J. Morgan, M.I. Stevens, J. Wallace, E. Möstl, *Gen Comp Endocr*. 2011, **173**, 139-147.

34 Q. Wang, S.A. Haughey, Y.M. Sun, S.A. Eremin, Z.F. Li, H. Liu, Z.L. Xu, Y.D. Shen, H.T. Lei, *Anal Bioanal Chem*. 2011, **399**, 2275-2284.

35 M. Dehnhard, A. Schreer, O. Krone, K. Jewgenow, M. Krause, R. Grossmann , *Gen Comp Endocr*. 2003, **131**, 345-352.

36 B. Jordan, *Rev Sci Tech Oie.* 2005, **24** (2), 515-528.

37 S. Rettenbacher, E. Möstl, R. Hackl, K. Ghareeb, R. Palme, *Brit Poultry Sci*. 2004, **45**, 704-711

435 **TABLES**

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