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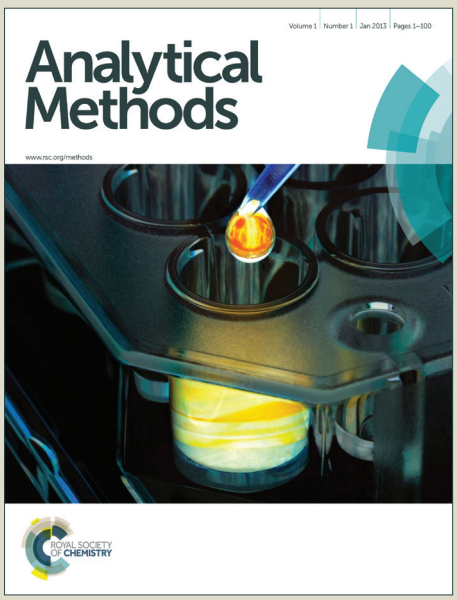

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A broad-selective enzyme immunoassay for non-invasive stress assessment in African Penguins (*Spheniscus demersus*) held in captivity

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ABSTRACT

We applied a direct competitive immunoassay for measuring corticosterone and glucocorticoid metabolites in faeces (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 overcomes by diluting sample extracts (1+4 or 1+9, depending on the sample).

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 ethical issues and public concern about animal welfare.[3] The monitoring of animal welfare has been regulated by national and international provisions aimed at assuring 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 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 it, a substantial rise in GC concentrations in the blood due to the stress caused by the restraint and bleeding.[9]

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, which allows 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 increased response of the assay (expressed as an increase of FGM concentrations) which follows an appropriate stimulus, such as the 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 cross-reacted with a few other GCs (Table 1). This is not unexpected, 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 the supposed stressful event on animals depended on the responsiveness of the employed assay, namely, the capability of the assay to detect the increased adrenocortical activity. A high 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 underwent 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), with the aim of testing its suitability for non-invasive monitoring of stress levels in these animals.

The African Penguin 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) 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. In addition, to successfully achieve conservation of such endangered species, it is important that captive facilities focus their efforts on its welfare and health. Therefore, stressful stimuli facing animals in captive environments should be minimized.[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 obtained 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 suitable based on the lowest IC_{50} value. B standards were diluted in aqueous methanol, as FGM extraction from faecal samples typically involves a high percentage of this solvent according to the literature.[11] The assay proved to be robust for methanol contents lower than 40%, whereas sensitivity decreased for higher solvent percentages. Dilution of B standards in TRIS buffer also negatively affected assay sensitivity and precision. Therefore, the ideal diluent for B standards was established to be methanol: water 35:65 (v/v).

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

Selectivity of the assay

According to the literature, tetrahydrocorticosterone (THB) is thought to be the main B metabolite;[33] however, this point is still debated, and several other possible metabolic products, characterized by very different chemical structures, have also been shown to be excreted in faecal samples of birds.[12,15] Möstl *et al.*[12] suggested at least seven possible metabolic pathways starting from B and resulting in the production of: 3-hydroxycorticoids, 11-oxocorticoids, 21-deoxycorticoids, 21-acid corticoids, 17-oxoandrostanes, and 6-hydroxycorticoids. However, faecal metabolites of GCs in birds have not been positively identified, and no

data are available on this subject in the literature. Most authors used competitive immunoassays (RIAs, Radio Immuno Assays or EIAs, Enzyme Immuno Assays), developed for measuring corticosterone or tetrahydrocorticosterone, as tools to assess FGM levels[9,17,30], on the basis of a demonstration that the assay is capable of detecting an increase in FGMs artificially induced by an appropriate biochemical or biological stimulus. An increased immune-response of the assay is interpreted as the consequence of the increased FGM concentration, regardless of the identification of the chemical compound responsible for the increase.[15]

Although responsiveness to induced stress of validated immunoassays for measuring FGMs is controversially related to cross-reactivity of the assay itself (*i.e*. the capability of detecting several different GCs), as is evident from Table 1, achieving broad-selectivity should be a major requirement for analytical methods to be applied for detecting FGMs. Therefore, the selectivity of a polyclonal antiserum, obtained by immunizing with a cortisol derivative modified in position 3 (Figure 1), was tested in response to a large number of steroid structures, which varied due the presence of different substituents in position 3, 11, 6, and 17, and to insaturations at position 1-2 and 4-5, according to the hypothetical metabolic modifications of corticosterone. Relative cross-reactivities compared to B are shown in Table 2. Most steroids were recognized by the antiserum at levels comprise 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 the binding of antibodies, 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 a hydrogen did not seem to negatively influence the binding (as can be argued 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, which also differ for the absence/presence of the insaturation at the A ring: the loss of the insaturation 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 conjugated glucocorticoid metabolites, i.e. glucoronides at position 3. However, the modification of the hydroxyl substituent could contribute to reverse the decrease of recognition due to the hydroxyl itself.

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, 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 $\mu g\ l^{-1}$) 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-folds 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 the calculated weight (5 mg). Turbidity, presumably associated to the urea content, seemed to have less 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 1:10 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. 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^{-1}), 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 diverse 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 EIA as 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 belonging to the animal named "G", to seven samples collected from the animal known as "S". The FGM content of each sample was measured by the developed EIA. 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 the 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

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 an overnight incubation, as required by existing immunoassays. The assay was applied to determine FGM levels in 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 investigated. The enzyme immunoassay developed in this study allowed the detection of the 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 could be made to prevent their occurrence and/or reduce their effect 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. Indeed, 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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TABLES

Table 1 Comparison of performances of immunoassays employed to measure FGMs from birds reported in the literature

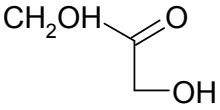
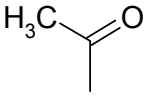
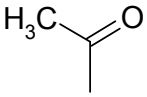
Reference	Bird	Sensitivity	Target compound [a]	Cross-reactivity	Comm [b] or Es Dev [c]
Wasser 2000, Wasser 2005	Northern spotted owl (*Strix occidentalis caurina*)	LOD = 0.2 ng	B	F, P, desoxy-B, T, aldosterone, androstenedione, DHT<1%, other 15 steroids < 0.01%	Comm
Quillfeld 2003	Wilson's storm-petrel (*Oceanites oceanicus*)	IC_{50} = 18 pg/well	THB	11-hydroxyandrosterone 43.9% tetrahydrocortisol 25.7%, cortol 2.7%, F, other 9 tested steroids < 1%	Sp Dev
Nakagawa 2003, Ninnes 2010	Adélie penguin (*Pygoscelis adeliae*)	The same as Quillfeld 2003			
Denhard 2003	Chicken (*Gallus domesticus*)	LOD = 0.2 pg/well	B	Desoxy-B 24.4%, F 13.4%, P 21.8%	Sp Dev
Rettenbacher 2004	Chicken (*Gallus domesticus*)	LOD = 2 pg/well	Immunogen: B, standard for calibration: cortisone	Adrenosterone 30%, 5-androstane-3,11,17-trione 20%; P, 5β-androstane-3,11,17-trione, and 4-pregnene-11β,21-diol-3,20-dione 2%; 4-androstane-3,17-dione and 5α-androstane-3,17-dione 1%	Sp Dev
Frigerio 2004	Greylag goose (*Anser anser*)	IC_{50} = 30 pg/well	11-hydroxy etiocholanolone	B 14.6%, 5-pregnane-3,11,21-triol-20-one 20%, 11-ketoetiocholanolone 3.5%, other 7 tested steroids < 1%	Sp Dev
Staley 2007	Golden eagle (*Aquila chrysaetos*) and peregrine falcon (*Falco peregrinus*)	The same as Wasser 2000			
Cyr 2008, Bauer 2011	European starling (*Sturnus vulgaris*)	The same as Wasser 2000			

Blickley 2012	Greater sage-grouse (*Centrocercus urophasianus*)		The same as Wasser 2000		
Young 2013	Budgerigar (*Melopsittacus undulatus*)	2 pg/mg feces	B	Not determined. According to manufacturer of the antibody: < 0.5% for 17 tested steroids)	Antibody: Comm, Assay: Sp Dev

[a] the chemical compound from which immunogen and enzymatic tracer were synthesized and which has been used for calibration. [b] Commercial kit. [c] Specially developed antibody and/or assay.

Table 2 Cross-reactivity of GCs and foremost steroids as determined by direct competitive ELISA

Compound	**Structural variation**[a]						**Cross-reactivity (%)**
	3	Δ4	11	17	Δ1	6	
Corticosterone, B	=O	=	OH	CH_2OH, =O	—	H	100
Corstisol, F	=O	=	OH	CH_2OH, =O, OH	—	H	100
Tetrahydrocorticosterone, THB	OH	—	OH	CH_2OH, =O	—	H	<0.04
Tetrahydrocortisol, THF	OH	—	OH	CH_2OH, =O, OH	—	H	<0.04
Cortisone	=O	=	=O	CH_2OH, =O, OH	—	H	3
Prednisone	=O	=	=O	CH_2OH, =O, OH	=	H	1
Prednisolone	=O	=	OH	CH_2OH, =O, OH	=	H	38

Methyl-prednisolone	=O	=	OH		=	CH_3	26
Progesterone, P	=O	=	H		—	H	8
11 α-hydroxyprogesterone	=O	=	OH		—	H	1
Testosterone, T	=O	=	H	OH	—	H	7
5β-dihydrotestosterone	=O	—	H	OH	—	H	3
5α-androstan-3,17-dione	=O	—	H	=O	—	H	0.2
4-androsten-3,11,17-trione	=O	=	H	=O	—	H	1
5-androsten-3β,17β-diol	OH	=	H	OH	—	H	<0.04
5α-androstan-3β, 17β-diol	OH	—	H	OH	—	H	<0.04

[a] The chemical structure of B, which is the reference compound, is depicted in Figure 1.

448 **Table 3** Recovery of B from artificially contaminated fecal samples as determined by ELISA detection

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Sample	Estimated FGM content in the sample extract (µg l^{-1}) ± SD	Added B (µg l^{-1})	Measured FGM in the sample extract (µg l^{-1}) ± SD	Recovery[a] (%)
R01	0.4 ± 0.2	2.5	3.1 ± 0.5	108
		10	10.8 ± 1.5	104
		40	36.4 ± 1.8	90
R06	2.1 ± 0.2	2.5	4.5 ± 1.2	96
		10	12.0 ± 1.4	99
		40	46.5 ± 3.4	111
S01	< LOD	2.5	3.2 ± 0.9	115
		10	12.0 ± 1.1	116
		40	33.7 ± 1.5	83

450 [a] calculated as: (measured FGM - estimated FGM content in the sample / added B) *100

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Table 4 Intra and inter-day precision of the developed ELISA on three samples of penguin feces.

Sample	Intra-day		Inter-day	
	Estimated FGM (ng/g)[a]	RSD % (n = 8)	Estimated FGM (ng/g)[a]	RSD % (n = 4)
R06	670	7	670	16
S07	1300	7	1430	13
S03	2010	8	2100	5

[a] FGM amount per gram of the soluble fraction of fecal samples

Table 5 Biological validation of the developed dc ELISA for measuring FGMs in African Penguins held in captivity compared to a reference EIA

Sample	Collected amount (mg)[a]	Time elapsed from the stressor (hours)	FGM (ng/g)[b] estimated by the dc ELISA	FGM (ng/g)[b] estimated by the reference EIA [30]
K01	115	5	1600	700
K02	48	7.5	3200	900
K03	nd[c]	23.5	na[d]	na[d]
K04	68	24.5	1500	400
K05	43	25	500	<LOD
R01	35	4	300	<LOD
R02	63	5.5	2800	840
R03	261	20	2000	250
R04	5	22.5	na[d]	na[d]
R05	nd[c]	24.5	na[d]	na[d]
R06	77	27	700	520
R07	27	27.5	300	<LOD
S01	148	5	200	<LOD
S02	68	6	300	<LOD
S03	32	6.5	2100	190
S04	44	8	1500	<LOD
S05	25	27.5	2000	<LOD
S06	nd[c]	28	na[d]	na[d]
S07	31	29.5	1400	<LOD
G01	23	8	6600[e]	4400
G02	51	23.5	1900[e]	1600
G03	51	27.5	1600[e]	530
Z01	55	5	1300	<LOD
Z02	nd[c]	6	na[d]	na[d]
Z03	25	8.5	2400	<LOD
Z04	nd[c]	20.5	na[d]	na[d]
Z05	37	23.5	1200	<LOD
Z06	207	28.5	200	<LOD

[a] calculated from the weigh of the extract, as described in the experimental section. [b] FGM amount per gram of the soluble fraction of fecal samples. [c] lower than 1 mg. [d] not analysed. [e] dark green sample, diluted 1:20 before analysis

FIGURE CAPTIONS

Fig. 1 Chemical structure of corticosterone (A) and of the hapten (F-3cmo) (B) used to obtain antibodies and the enzyme labelled competitor for the assay.

Fig. 2 A typical calibration curve for corticosterone

Fig 3. Study on matrix interference: effect of diluting sample extracts with aqueous methanol (35% v/v) on four samples representative of the variability of extract appearance: TW (turbid white ◆), TO (turbid orange, ■), LG (limpid light green, ▼), and DG (limpid dark green, ●).

FIGURES

Figure 1

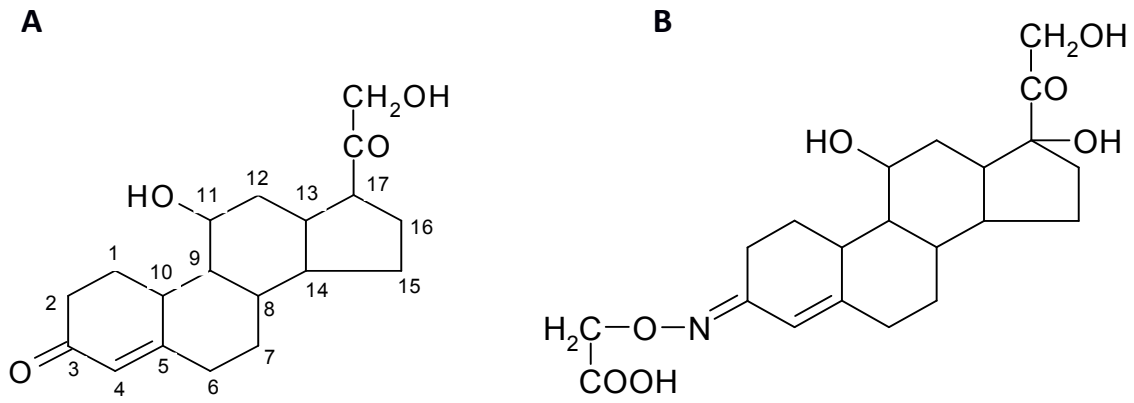

Figure 2

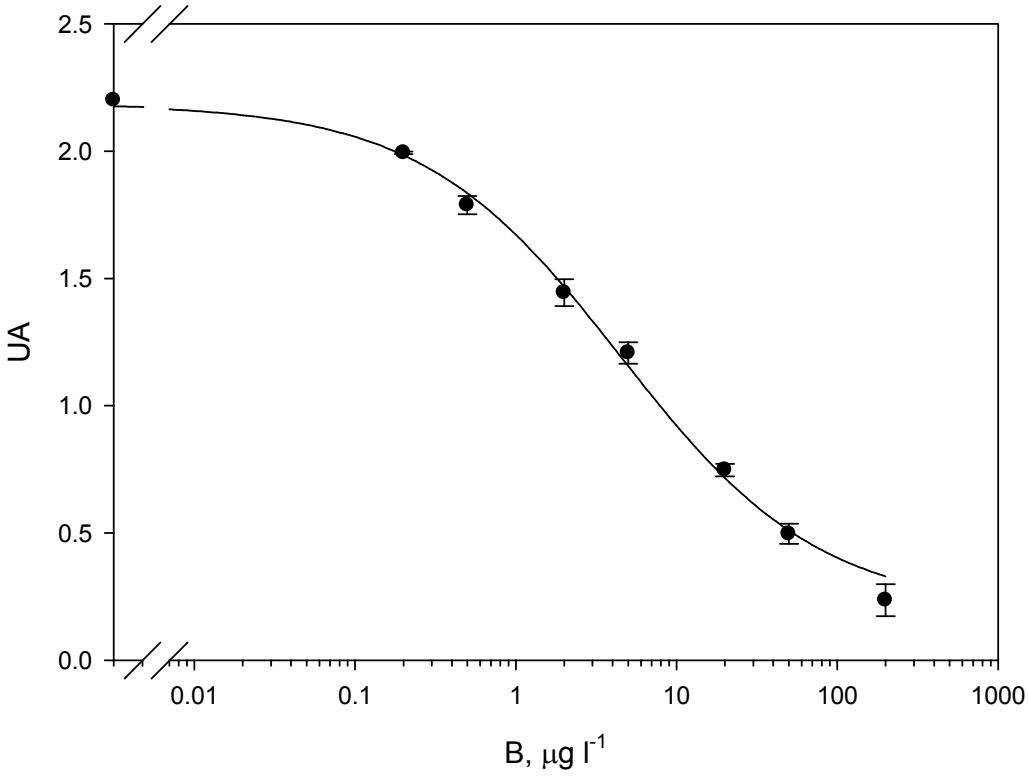

Figure 3