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Short Communication

# Development of a versatile enzyme immunoassay for non-invasive assessment of glucocorticoid metabolites in a diversity of taxonomic species

Rebecca Watson [a,b], Coralie Munro [c], Katie L. Edwards [a,d], Vicki Norton [a], Janine L. Brown [e], Susan L. Walker [a,*]

[a] North of England Zoological Society, Chester Zoo, Chester CH2 1LH, UK
[b] University of Glasgow, Glasgow G12 8QQ, UK
[c] University of California Davis, CA 95616, USA
[d] Institute of Integrative Biology, University of Liverpool, Liverpool L69 7ZB, UK
[e] Smithsonian Conservation Biology Institute, Center for Species Survival, VA 22630, USA

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ABSTRACT

Endocrinology is a useful tool for conservation biologists and animal managers, and measuring glucocorticoids can help understand biological mechanisms associated with species decline and animal welfare. The current study describes the development and optimization of a glucocorticoid enzyme immunoassay (EIA) to non-invasively assess adrenal activity in a variety of taxa. The antiserum (CJM006) was raised in rabbits to a corticosterone-3-CMO-BSA immunogen and used in a standard competitive EIA system. However, the EIA initially produced results with unacceptably high inter-assay variation, attributed to consistent patterns observed within the optical density of developing plates. To determine the cause of this variability, a number of factors were examined using synthetic corticosterone standard and endogenous faecal extract, including: plate type (Nunc MaxiSorp® II versus Immulon IB plates); the use of non-specific secondary antibody; type (artificial versus natural) and presence (light versus dark) of light during incubation; plate loading temperature (4 °C versus room temperature); and substrate reagent temperature (4 °C versus room temperature). Results indicated that variability was associated with plate location effects, which were not initially detected because control samples were always run in the same positions across plates. Light and temperature were the two major factors that affected EIA reliability. For this assay, the standard protocol required slight modification, with the optimal protocol using Nunc MaxiSorp® plates, room temperature substrate reagents and dark incubation conditions. Following optimization, this EIA was then validated biochemically for 38 species, through parallel displacement curves and interference assessment tests of faecal and urine samples. Additionally, biological validation was performed opportunistically in a subset of species, with use of this EIA demonstrating significant elevations in faecal glucocorticoid metabolites following potentially challenging events. In summary, this glucocorticoid EIA cross-reacts with excreted glucocorticoid metabolites across a wide range of taxa, including ungulates, primates, felids, birds, rodents and amphibians. We conclude that when used with optimal reagent and incubation conditions, this EIA will be useful for non-invasive monitoring of adrenal activity in a wide range of wildlife species.



## 1. Introduction

Through the use of hormones as biometric markers, endocrinology is a popular and vastly growing area of research, especially with regards to non-domestic species (Schwarzenberger, 2007; Wielebnowski and Watters, 2007). Measuring glucocorticoids as an indicator of adrenal activity can help conservation biologists and animal managers better understand causes of species decline and poor welfare (Clark et al., 2011; Walker et al., 2005; Wikelski and Cooke, 2006). Although glucocorticoids increase in response to both positive and negative events (Buwalda et al., 2012), persistently high concentrations due to chronic stress can result in immunosuppression and poor reproductive output (Hofer and East, 1998; Mostl and Palme, 2002), and lead to the expression of abnormal behaviours in both captive (Hill and Broom, 2009) and wild animals (Wielebnowski and Watters, 2007). However, monitoring adrenal activity through blood sampling can be problematic if the collection procedure itself induces a stress response, so far many species non-invasive sampling techniques are preferred (Cooke and O'Connor, 2010; Hofer and East, 1998; Wielebnowski

* Corresponding author. Address: North of England Zoological Society, Chester Zoo, Caughall Road, Chester CH2 1LH, UK.
*E-mail address:* s.walker@chesterzoo.org (S.L. Walker).

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and Watters, 2007). Both faecal and urine analyses are routinely used with wildlife species, since they avoid the confounding variables associated with capture (Keay et al., 2006), are easier to collect longitudinally for longer-term endocrine profiling (Touma and Palme, 2005), and are often more applicable to field studies (Freeman et al., 2010), making them more suitable from a conservation perspective (Hofer and East, 1998).

When utilizing non-invasive immunoassay techniques, antibody selection is an important consideration, since hormones excreted in faeces and urine are not always in their native form (cortisol; corticosterone), but rather include a range of metabolites that can vary both between sexes and across species (Palme et al., 1996; Touma and Palme, 2005). A number of metabolite-specific antibodies are available, but these may only be applicable for a sub-set of species due to differences in excretion profiles; therefore, the development of a more broad-spectrum antiserum that is group-specific to a range of glucocorticoid metabolites and therefore suitable across several different taxa is often desired. A decade ago, a corticosterone radioimmunoassay (RIA) was developed and shown to be valid for assessing faecal glucocorticoid metabolites in a diverse array of species (Wasser et al., 2000). However, although widely used, RIAs present challenges to some institutions because of the costs and regulations associated with using radioactive ligands. EIA's by contrast are non-isotopic and therefore more convenient, particularly for laboratories in range countries (Hodges et al., 2010).

With these requirements in mind, one of the co-authors (CJM) developed a corticosterone EIA to assess adrenal activity non-invasively in a variety of taxa. The polyclonal antiserum (CJM006) was produced in rabbits using an established protocol (Munro and Stabenfeldt, 1984) and a standard direct competitive enzyme immunoassay system was developed (Kurstak, 1985). However, during initial validation procedures in our laboratory for the black rhinoceros (*Diceros bicornis*), the assay delivered unacceptable inter-assay coefficients of variation (CV) ranging from 50–60% during repeated analysis of the same faecal extract (K.L. Edwards; unpublished data). In the published literature, acceptable intra and inter-assay CVs are 5–10% and 10–15%, respectively (Kurstak, 1985; Munro and Stabenfeldt, 1984). Therefore, the primary objective of this study was to optimize an EIA method for this antibody by manipulating a number of factors, including microtitre plate type, the use of a non-specific secondary antibody, light exposure during incubation, plate loading temperature and substrate reagent temperature. The second objective was to biochemically validate this EIA for measuring excreted glucocorticoid metabolites in urine and/or faeces from a wide range of taxa.

## 2. Materials and methods

### 2.1. Preparation of antibody and enzyme conjugate

Following the methods of Munro and Stabenfeldt (Munro and Stabenfeldt, 1984), antiserum against corticosterone-3-CMO-BSA (Steraloids, Wilton, New Hampshire; Q1559-000) was produced in four male New Zealand White rabbits according to an immunization protocol described by Vaitukaitis et al. Vaitukaitis et al. (1971). All procedures were reviewed and approved by the Animal Welfare Committee of the University of California, Davis, CA. Each rabbit received 1 ml (1 mg/ml) of the emulsified corticosterone-3-CMO-BSA immunogen (Sigma–Aldrich, St. Louis, MO) at 0, 2, and 4 weeks, with a booster administered approximately every 3–4 weeks thereafter until no further increase in titre was obtained. Rabbit CJM006 produced high antibody titres and therefore was chosen for purification and use in the EIA. The gamma globulin fraction of the anti-corticosterone antiserum was purified by ammonium sulphate precipitation, and the non-specific anti-BSA antisera were removed by equivalence zone adsorption as described previously by Munro and Stabenfeldt (Munro and Stabenfeldt, 1984). The polyclonal CJM006 antibody was found to cross-react with: corticosterone 100%, desoxycorticosterone 14.25%, progesterone 2.65%, tetrahydrocorticosterone 0.90%, testosterone 0.64%, cortisol 0.23%, prednisolone 0.07%, 11-desoxycortisol 0.03%, prednisone <0.01%, cortisone <0.01% and oestradiol <0.01%. Horseradish peroxidase (Sigma–Aldrich, St. Louis, MO; Cat# P8375) was coupled to corticosterone-CMO (Sigma–Aldrich, St. Louis, MO) using the mixed anhydride method described by Munro and Stabenfeldt (Munro and Stabenfeldt, 1984).

### 2.2. Faecal and urine sample collection and processing

Faecal samples (minimum $n = 20$/species) were collected opportunistically from male and/or female individuals from a variety of taxa, including: ungulates, primates, big cats, birds, small mammals and amphibians (Table 1). Urine samples (minimum $n = 19$/species) were collected from female black rhino (*Diceros bicornis michaeli*) and male house mouse (*Mus musculus*). Samples were collected as soon as possible after defecation or urination and stored at −20 °C until processing. Hormones were extracted from faecal material following thawing and manual homogenization, using a wet-weight shaking extraction adapted from Walker et al. Walker et al. (2002). In brief, 0.50 g of faecal material was combined with 5 ml 90% methanol, shaken overnight at room temperature and centrifuged for 20 min at 598*g*. The methanol fraction was decanted and evaporated to dryness before being re-suspended in 1 ml methanol (0.25 ml for macaw species) and stored at −20 °C until analysis. Faecal extracts and urine samples were thawed and diluted as necessary prior to analysis on the EIA.

### 2.3. Enzyme immunoassay standard protocol

The variability in optical density across EIA plates was determined using the following standard competitive EIA protocol, adapted from Munro and Stabenfeldt (Munro and Stabenfeldt, 1984), with each well containing either the same concentration of synthetic corticosterone standard or black rhino faecal extract. The polyclonal antibody CJM006 was diluted (1:15,000) in coating buffer (0.05 M $NaHCO_3$, pH 9.6), loaded 50 μl/well on a 96-well Nunc-Immuno MaxiSorp® microtitre plate (Thermo-Fisher Scientific, UK), covered with a microplate sealer and incubated overnight at 4 °C. Plates were washed five times (0.15 M NaCl, 0.05% Tween 20), and the entire plate loaded with 50 μl/well of corticosterone standard (1.25 ng/ml; C2505 Sigma–Aldrich, Dorset, UK) in EIA buffer (0.1 M $NaPO_4$, 0.149 M NaCl, 0.1% bovine serum albumin, pH 7.0), or black rhino faecal extract (diluted 1:70 in EIA buffer) immediately followed by 50 μl/well of horseradish peroxidase conjugate (diluted at 1:70,000 in EIA buffer). Following incubation in full light (on the laboratory bench top, exposed to artificial ceiling and natural window light) for 2 h at room temperature (RT), plates were washed 5 times and incubated with 100 μl/well of 4 °C substrate [0.4 mM 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt (ABTS), 1.6 mM $H_2O_2$, 0.05 M citrate, pH 4.0), in full light, until average optical density reached 0.8 to 1.0. The resulting optical density of all individual wells was then measured at 405 nm. This method was considered the control method for subsequent experiments A–D, which manipulated a number of elements of this standard protocol, and was aimed to reduce the variation seen both within and between plates.