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SHORT NOTE

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Validation of an enzyme immunoassay to measure faecal glucocorticoid metabolites from Adélie penguins (*Pygoscelis adeliae*): a non-invasive tool for estimating stress?

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Abstract To monitor adrenocortical activity in Adélie penguins (*Pygoscelis adeliae*), we validated an enzyme immunoassay (EIA) for faecal glucocorticoid (GC) metabolites. An adrenocorticotropin hormone (ACTH) challenge was conducted on a paired female and male. The EIA for tetrahydrocorticosterone showed a clear response to the ACTH challenge in both birds. After high-performance liquid chromatography using pooled samples generated from the ACTH challenge, and analysing each individual fraction, three immunoreactive peaks were detected. Both biological and chemical validations strongly suggest that the EIA can be a useful tool for non-invasively measuring GC metabolites in faeces of Adélie penguins.

Introduction

As human activities such as scientific research and recreational visits increase in Antarctica (Enzenbacher 1992), human effects on Antarctic organisms become a matter of increasing concern. For the Adélie penguin, *Pygoscelis adeliae*, accumulating evidence suggests that human activities have serious impacts on breeding populations (Giese 1996 and references therein). For some penguin species, stress responses caused by human disturbance may have contributed to declines in breeding populations (Fowler 1999).

Exposure to stress usually results in an increased secretion of glucocorticoids (GCs; mainly corticosterone in birds) from the adrenal cortex. Although GC concentrations in plasma have been measured for many animals (for Adélie penguin, McQueen et al. 1999; Vleck et al. 2000), blood-sampling procedures alone can cause substantial changes (Romero and Romero 2002). As a result, non-invasive faecal GC analyses have recently become popular (e.g. Wasser et al. 2000; Möstl et al. 2002). However, because most native GCs are metabolised before excretion and resulting GC metabolites can differ between species, the development of assays for GC metabolites has proved difficult (Goymann et al. 1999; Wasser et al. 2000).

Despite these difficulties, non-invasive methods are urgently required to investigate relationships between human disturbance, physiological stress, reproductive success and population declines in species like Adélie penguins that may be highly sensitive to sampling stress. A non-invasive method for quantifying changes in GC metabolites of Adélie penguins has potential for assessing stress associated with, not only human activities, but also other environmental disturbances such as climatic changes.

In this study, an enzyme immunoassay (EIA) for faecal GC metabolites from Adélie penguins was successfully validated using adrenocorticotropin hormone (ACTH) challenges and a high-performance liquid chromatography (HPLC) analysis.

Materials and methods

Sample collection occurred at colonies on Cape Bird, Ross Island, Antarctica (77°13′S, 166°29′E) in late January 2001. A pair of non-breeding birds in good condition were subjected to an ACTH challenge (sex was assigned by observations prior to the capture; see Williams 1995). The two penguins were kept in separate pens, installed next to their original colony. Supplementary food was not provided during the 3-day ACTH challenge (Adélie penguins fast for up to 30 days during the breeding season; Williams 1995). The pens (100×100×80 cm high) were wooden with a plastic-mesh roof (0.8×0.8 cm squares); the wire-mesh bottom (1.2×1.2 cm squares) was also covered with plastic mesh (0.8×0.8 cm squares) to protect the penguins' feet. The pen was suspended 3 cm above clean plastic sheets so any faeces discharged were easily collected.

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Each bird was placed into its pen within 5 min of capture (11.00 hour) and sample collection was initiated (we attempted to sample faecal material not urine). The female (4,250 ± 25 g) and male (4,200 ± 25 g) each received an intramuscular injection with 200 IU of a synthetic ACTH preparation (Synacthen, Novartis New Zealand, Auckland) into left thigh muscles, 5 h after their introduction to the pens. We checked the pen and collected faeces every 3 h for the subsequent 57 h. The average ambient temperature during the ACTH challenge was around −5°C so that, we think, exposure effects (a maximum of 3 h) on pellets were minimal. Twenty samples for the female and 18 samples for the male were obtained.

To establish natural levels of GC metabolites, single faecal samples were also collected from 16 randomly chosen individuals.

All samples (scooped into separate 2-ml vials) were stored in a liquid nitrogen container immediately after collection, and subsequently transferred to the laboratory and stored at −76°C.

The samples (in vials) were freeze-dried (for 96 h) and pulverised. A proportion of the resulting powder (0.05 ± 0.001 g) was weighed and extracted with 4 ml methanol and 1 ml double-distilled water. After vortexing for 30 min, samples were centrifuged (2,500 *g*, 15 min). The supernatants were transferred to new tubes. These extracts were used for the EIA.

In a preliminary experiment, various EIAs for corticosterone and corticosterone metabolites were assessed; the EIA for tetrahydrocorticosterone (5β-Pregnane-3α,11β,21-triol-20-one; Quillfeldt and Möstl 2003) showed the highest amounts of immunoreactive substances in the extracts. For this EIA (see Quillfeldt and Möstl 2003 for details on the assay characteristics), tetrahydrocorticosterone (Steraloids, Wilton, N.H.) was converted into the carboxymethyloxime derivative, linked to bovine serum albumin and then used as an antigen; biotinylated tetrahydrocorticosterone-21-hemisuccinate served as the label (the antibodies and labels are available in limited amounts free of charge from E. Möstl). The working dilution of the antibody was 1:40,000 and 1:8,000 for the label. The standard curve ranged from 0.82 to 200.00 pg/well. This assay was used to measure immunoreactive substances in the extracts from faecal samples of the 2 ACTH-injected penguins and the 16 non-ACTH-injected penguins. Because tetrahydrocorticosterone in faeces might be present in a conjugated form, the immunoreactive substances were measured with and without enzymatic hydrolysis using β-glucuronidase/arylsulphatase (Merck 4114; Kotrschal et al. 1998). All samples for the EIA were run in duplicate. Because of a small sample size in this study, we were unable to calculate inter- and intra-assay coefficients for the assay, but in Quillfeldt and Möstl (2003) where the same assay was used, the intra-assay variation was 10.9% and the inter-assay variation was 17.7%.

To characterise immunoreactive substances in the faeces, 1 ml of the methanolic phases from the pooled extracts obtained from the ACTH-challenged penguins was diluted in 10 ml water, and re-extracted using Sep-Pak C_{18} cartridges (following manufacturer's instructions). The dried extract was dissolved in 20% methanol and separated by HPLC (Novapac C_{18} column 0.39×15 cm, Fa. Waters, Milford, Mass.; solvent: water/methanol; 0–5 min: 20% methanol; 5–35 min linear gradient from 20% methanol to 100%; flow 1 ml per min; 3 fractions per minute were collected). The use of the hydrolysis procedure led to an increase of immunoreactive material in the samples (see below and Fig. 1). However, enzymatic hydrolysis was not performed for HPLC analysis.

Results and discussion

ACTH administration resulted in an increase in faecal GC metabolites for both the female and male (Fig. 1). Enzymatic hydrolysis generally showed higher amounts of immunoreactive material (though the percentage increase in immunoreactive substances varied among samples). For both birds, the ACTH-induced peak of

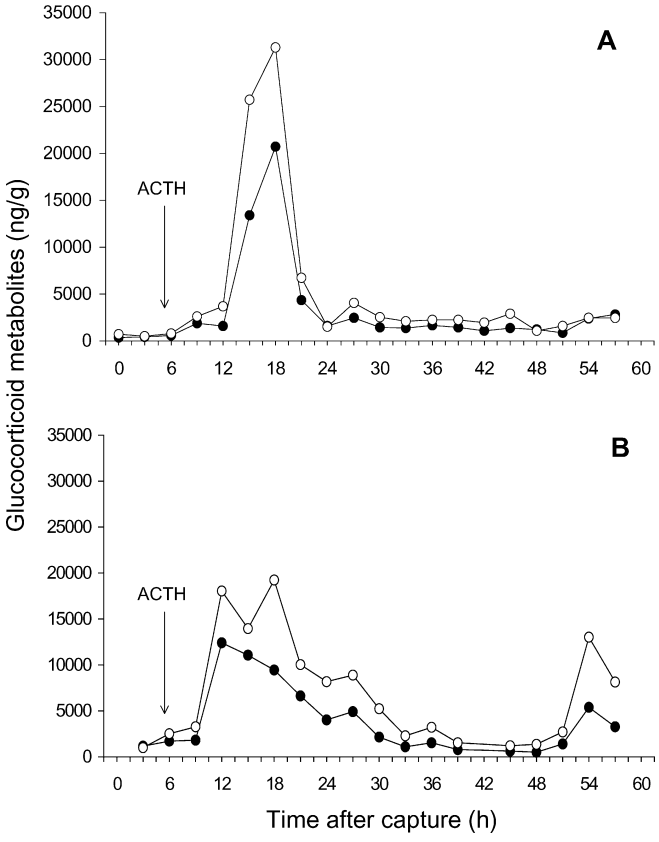

Fig. 1A, B Glucocorticoid metabolites from non-hydrolysed (*filled circles*) and hydrolysed (*unfilled circles*) extracts of dried faecal samples during an ACTH challenge with 40 IU ACTH in **A** female and **B** male penguin

GC metabolites in hydrolysed extracts occurred 13 ± 3 h after the ACTH injection (Fig. 1). Fasting (which may cause reduced faecal mass) is unlikely to explain the increase in immunoreactive material, as the values of GC metabolites decrease within the fasting period. The processes of capture, handling and isolation might have affected the excretion of GC metabolites; the resulting changes may contribute to changes associated with the ACTH injection (Romero and Romero 2002). For the male, there was a peak 54 h after capture that was unlikely to be induced by the ACTH injection. Whatever the reason for this peak, it implies that the GC metabolites detectable with the EIA can measure responses to environmental events unassociated with an ACTH challenge.

GC metabolites from non-hydrolysed and hydrolysed extracts of dried faecal samples from the 16 non-ACTH-challenged penguins ranged from 132.1 to 3,862.6 ng/g (mean ± SE, 1,177.6 ± 241.8 ng/g; median, 906.0 ng/g) and from 160.0 to 14,550.1 ng/g (mean ± SE, 2,427.0 ± 886.0 ng/g; median, 1,121.1 ng/g), respectively. In 14 of 16 cases, hydrolysed extracts contained higher immunoreactive GC metabolites than corresponding non-hydrolysed extracts, but there were no consistent patterns in percentage increases in immunoreactive metabolites between non-hydrolysed samples and corresponding

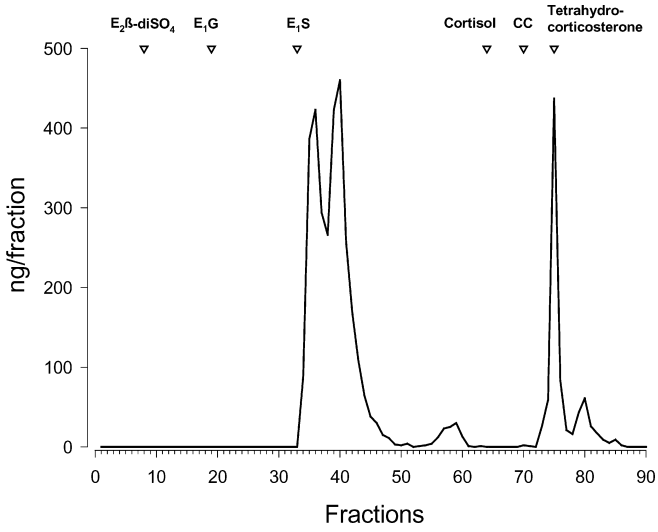

Fig. 2 Elution pattern of immunoreactive substances (tetrahydrocorticosterone equivalents), measured after reversed-phase chromatography using the assay for tetrahydrocorticosterone. The elution of oestradiol 17β-sulphate ($E_2\beta$-diSO_4), oestrone glucuronide (E_1G), oestrone sulphate (E_1S), cortisol, corticosterone (CC) and tetrahydrocorticosterone are marked with *triangles*

hydrolysed samples (see also Fig. 1). The results suggest that faecal GC metabolites can vary greatly depending on the individual (e.g., individuals may be subject to, or experience, different amounts of stress) or the activities they were engaged in (e.g. agonistic behaviour or sighting a predator). The EIA appears to be able to detect variation in natural settings.

The HPLC analysis revealed that the immunoreactive substances eluted in three peaks (Fig. 2). The first two were in the range of steroid monosulphates. As the antibody is quite specific as far as ring A and B of the steroid molecule are concerned, these peaks may represent conjugated forms of tetrahydrocorticosterone. The third peak showed the same chromatographic mobility as unconjugated tetrahydrocorticosterone.

The results of our biological and chemical validations strongly suggest the EIA for tetrahydrocorticosterone can be employed to measure GC metabolites in faeces of Adélie penguins. We recommended this EIA be used without hydrolysis for Adélie penguins because: (1) the non-hydrolysed method yielded very similar information (Fig. 1), (2) the hydrolysis of samples did not create a consistent percentage increase in immunoreactive metabolites under the assay (e.g. Fig. 1), creating additional assay variations, and (3) the hydrolysis procedure incurs extra time and costs.

Quillfeldt and Möstl (2003) have found the same EIA can be used to detect excreted GC metabolites in Wilson's storm-petrels (*Oceanites oceanicus*). Because penguins and petrels are closely related (Sibley and Ahlquist 1990; Paterson et al. 1995), the EIA for tetrahydrocorticosterone may have wide application in these taxonomic groups, and also for other associated species.

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References

Enzenbacher DJ (1992) Tourists in Antarctica: numbers and trends. Polar Rec 28:17–22

Fowler GS (1999) Behavioral and hormonal responses of Magellanic penguins (*Spheniscus magellanicus*) to tourism and nest site visitation. Biol Conserv 90:143–149

Giese M (1996) Effects of human activity on Adélie penguin *Pygoscelis adeliae* breeding success. Biol Conserv 75:157–164

Goymann W, Möstl E, Van't Hof T, East ML, Hofer H (1999) Noninvasive faecal monitoring of glucocorticoids in spotted hyenas, *Crocuta crocuta*. Gen Comp Endocrinol 114:340–348

Kotrschal K, Hirschenhauser K, Möstl E (1998) The relationship between social stress and dominance is seasonal in greylag geese. Anim Behav 55:171–176

McQueen SM, Davis LS, Young G (1999) Sex steroid and corticosterone levels of Adélie penguins (*Pygoscelis adeliae*) during courtship and incubation. Gen Comp Endocrinol 114:11–18

Möstl E, Maggs JL, Schrötter G, Besenfelder U, Palme R (2002) Measurement of cortisol metabolites in faeces of ruminants. Vet Res Commun 26:127–139

Paterson AM, Wallis GP, Gray RD (1995) Penguins, petrels and parsimony: does cladistic analysis of behavior reflect seabird phylogeny? Evolution 49:974–989

Quillfeldt P, Möstl E (2003) Resource allocation in Wilson's storm-petrels determined by measurement of glucocorticoid excretion. Acta Ethol. DOI 10.1007/s102110030074

Romero LM, Romero R (2002) Corticosterone responses in wild birds: the importance of rapid initial sampling. Condor 104:129–135

Sibley CG, Ahlquist JE (1990) Phylogeny and classification of birds: a study in molecular evolution. Yale University Press, New Haven

Vleck CM, Vertalino N, Vleck D, Bucher TL (2000) Stress, corticosterone, and heterophil to lymphocyte ratios in free-living Adélie penguins. Condor 102:392–400

Wasser SK, Hunt KE, Brown JL, Cooper K, Crockett CM, Bechert U, Millspaugh JJ, Larson S, Monfort SL (2000) A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. Gen Comp Endocrinol 120:260–275

Williams TD (1995) The penguins: Spheniscidae. Oxford University Press, New York