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Research article

# Validation of an assay to measure glucocorticoid metabolites in the droppings of little penguins (*Eudyptula minor*)

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**Abstract**
Monitoring adrenal activity can provide insight into an animal's physiological state and can be assessed non-invasively via excreta (e.g. bird droppings). However, before this technique can be used, it needs to be validated for each species. In this study, we biologically validated an assay for monitoring adrenocortical activity via faecal glucocorticoid metabolites (FGM) in little penguins (*Eudyptula minor*). The validation incorporated a pre-scheduled husbandry event that is potentially stressful (annual veterinary health check). Four penguins were randomly selected a priori and immediately following their veterinary examination, they were transferred to off-display housing to facilitate individual collection of droppings over the following five days. All penguins exhibited a peak in FGM concentrations within 24 h of the start of the health check and on average FGM concentrations increased six-fold above an individual's mean baseline value. This validated FGM assay provides a valuable addition to the suite of tools available to monitor little penguins in zoos and in the wild.

## Introduction

Animal welfare is a key focus of zoos, with considerable effort dedicated to monitoring and improving welfare conditions (Whitham and Wielebnowski 2013). Assessment of adrenocortical activity is useful for understanding an animal's condition or perception of its environment. The adrenal gland secretes glucocorticoids (GCs), and plays a key role in mediating the physiological stress response (Sapolsky 2002). Measuring the concentration of GCs can therefore provide insight into the stress physiology of animals.

Plasma GC concentrations are widely used to assess stress responses in various domestic species. However, there are constraints associated with blood sampling as it involves capture and handling of animals to collect samples. This procedure is invasive and affects circulating GC levels in response to the stress of handling and restraint (Touma and Palme 2005). Furthermore, there is marked variation in plasma GC concentrations because of the pulsatile secretion patterns and circadian rhythms (Keay et al. 2006).

Faecal glucocorticoid metabolites (FGM) provide a non-invasive tool for monitoring adrenal activity and also provide a pooled estimate of circulating GC concentrations, thereby dampening effects of pulsatile secretion (Keay et al. 2006). However, since GCs are metabolised during excretion, biological validation of an assay is critical in order to demonstrate that it detects FGM reflective of biologically relevant changes in adrenal activity for a given species (Sheriff et al. 2011).

The aim of this study was to biologically validate an enzyme immunoassay for monitoring patterns of adrenal activity in droppings of little penguins (*Eudyptula minor*), which has not previously been done.

## Methods

### *Study animals and validation*

Melbourne Zoo, Australia houses a breeding group of 24 little penguins. Two males and two females were randomly selected *a priori* for this study. Individuals experienced two potential stressors: (1) an annual health check, and (2) transfer to a

novel enclosure, which was associated with a change in social environment. All procedures were approved by the Zoos Victoria animal ethics committee.

The annual health check was performed for the entire group. All penguins were rounded up into two large crates, then, one at a time, birds were weighed, checked for physical abnormalities, and released. This process took a maximum of three minutes per bird. Instead of being released after their examination, the four study subjects were transferred to off-display housing.

The off-display housing was a large, sanded outdoor area (15 m by 10 m) with a pool. In one corner, there were four separate pens (2 m by 0.5 m) adjacent to each other. Each pen had a nest box for shelter and a plastic sheet covering the sand to facilitate sample collection. For the first 24 h following the health check, the four penguins were housed individually. Pens were checked at 1.5, 3.5, 5.5 and 18 h post-transfer and all droppings were collected separately. For the next four days, penguins had access to the full enclosure during the day (0900 until 1600) but were housed in their separate pens overnight to facilitate individual sample collection. Droppings were collected twice per day (0800–0900 and 1600–1700) by observing individuals in their separate pens and collecting samples as soon as they were deposited. All droppings that were deposited overnight were also collected (this is why some data points in Figure 1 have the same x value). Samples were placed in polypropylene vials labelled with ID, date, and time and stored in a freezer (-20 °C) until extraction.

***Steroid extraction and analysis***

Penguin droppings were analysed at Deakin University. Samples were dried in a lyophiliser (TFD series, ilShin Biobase, Amsterdam, Netherlands) at -80 °C for 24 h. They were then pounded and sifted to remove debris. One ml of 60% methanol was added to 0.1 g of dried droppings in borosilicate test tubes. Samples were mixed on a multivortex for 30 min and centrifuged for 15 min (Hettich Universal 320 R, Tuttlingen, Germany; RCF = 2,500). Supernatant was decanted into 1.5 ml microcentrifuge tubes.

FGM were measured using a cortisone enzyme immunoassay (Rettenbacher et al. 2004). The antibody, biotinylated steroid and standard were obtained from R. Palme (University of Veterinary Medicine, Vienna; LabCode 32a). The sensitivity of the total method was 260 ng/g droppings. The assay was biochemically validated in our lab by demonstrating parallelism between a serially diluted plasma pool and the standard curve. Assay procedures were similar to those previously described (Rettenbacher et al. 2004). All of an individual's samples were run on a single plate. The intra-assay coefficient of variation (CV) was <15%.

***Data analysis***

Data were analysed using the R package hormLong (Fanson and Fanson 2015). For each individual, baseline FGM values were calculated using an iterative process excluding all points greater than the mean +2.5 standard deviations (Wielebnowski et al. 2002). Peaks were defined as points exceeding this threshold.

**Table 1.** Change in faecal glucocorticoid metabolites in little penguins following capture, handling and being moved to a new enclosure.

| ID | Sex | Baseline (ng/g) | Peak (ng/g) | Magnitude (fold increase) | Excretion lag (h) |
|---|---|---|---|---|---|
| Claus | Male | 1360 | 5778 | 4.25 | 7 |
| Katie | Female | 2232 | 23612 | 10.58 | 5 |
| Penny | Female | 768 | 4873 | 6.34 | 7 |
| Toby | Male | 792 | 2872 | 3.63 | 24 |

## Results

All penguins exhibited a peak in FGM concentrations within 24 h of the start of the health check (mean ± SD = 10.8 ± 8.9 h; median = 7.0 h; Table 1, Figure 1). In three birds, the peak occurred between 5 and 7 h. In the fourth bird, the peak occurred at 24 h. On average, FGM concentrations increased 6.2 ± 3.1 times above an individual's mean baseline value. For most penguins, the peak was only detected in a single sample. The exception was Katie, who exhibited a second, smaller peak the morning after the health check (approximately the same time as Toby's peak).

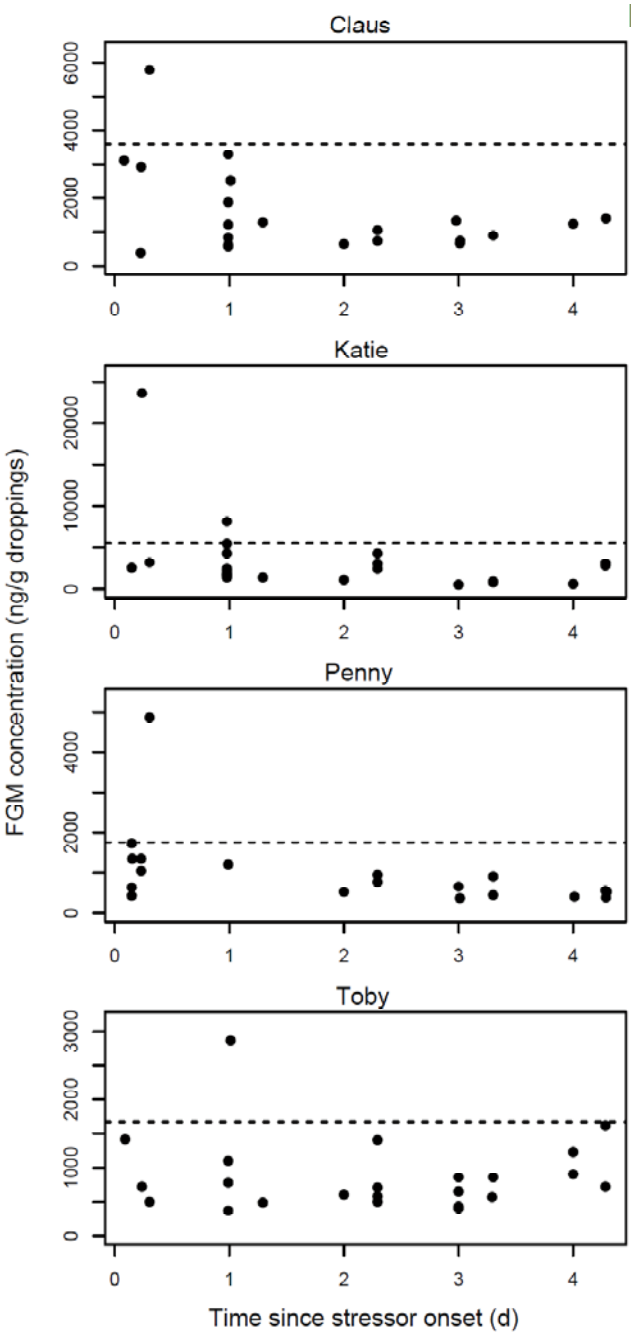


**Figure 1.** Profiles of faecal glucocorticoid metabolites (FGM) of little penguins following capture, handling and being moved to a new enclosure over the four study days. The dashed line represents the baseline cut-off value (mean + 2.5 SD) and points above this line are considered peaks.

## Discussion

We were able to detect an increase in FGM for little penguins exposed to a series of stressors (capture, handling, new enclosure and change in social environment). We selected an assay that targets glucocorticoid metabolites because unmetabolised corticosterone is generally not present in faeces (Touma and Palme 2005). This particular assay, which targets FGM with a 3,11-dioxo structure, has been shown to work well for monitoring FGM in other bird species (e.g. Rettenbacher et al. 2004; Thiel et al. 2005). Another group-specific assay that targets FGM with a 5β-3α,11β-diol structure and is made in the same laboratory has been validated for Adelie penguins (*Pygoscelis adeliae*; Nakagawa et al. 2003; Ninnes et al. 2010).

The average excretion lag time was 10.8 h (median = 7.0 h), which is similar to another study on African penguins that reported the FGM peak to be between 7 and 10 h after the stressful event (Anfossi et al. 2014). However there was also some individual variability in our results, with lag times ranging from 5 to 24 h. This is common in FGM studies and may reflect individual variation in hepatic clearance or excretion times (Touma and Palme 2005). Alternatively, it may reflect individual variation in perception of the events. For example, the individuals with earlier peak times may have experienced a strong physiological response to the initial capture, whereas the individual with later peak time (Toby) may have responded more to being isolated overnight. It is also possible that we missed the initial peak for Toby, despite efforts to collect all droppings.

In three of the four birds, the FGM peak was only detected in a single sample, despite the fact that there was potential for the adrenal gland to be activated longer as a result of the move to a new environment, which some animals can take a day or two to acclimatise to. This finding suggests that the validation study did not have a sustained impact on adrenal function in these individuals. The fact that the birds maintained auditory and olfactory contact with familiar conspecifics (even when they were spatially isolated) may have helped mitigate the potential stress of the new environment. Because the peak was so transient and the timing variable among individuals, researchers studying acute stressors in this species should adopt a fairly intensive sampling regime.

The validation of this assay for monitoring adrenal activity in little penguins is a valuable technique that can now be used to develop a better understanding of the impact of the environment on the welfare of little penguins both in zoos and in the wild. Indeed, FGM monitoring in wild individuals is likely to be useful in informing the management of populations, particularly as previous studies have shown that tourist visits can have negative impacts on populations. For example, little penguins in Victoria and New South Wales, Australia, showed avoidance of nesting areas exposed to high levels of human visitation (Giling et al. 2008). However, FGM concentration has never been assessed in these situations so it is unclear whether impacts on the population are a result of stress or other factors associated with tourists.

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