Blubber Cortisol as an Indicator of Physiological State in Phocid Seals: The Result of Passive Accumulation or Local Production?

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Joanna L. Kershaw and Ailsa J. Hall

Introduction

In terrestrial mammals, the glucocorticoids (cortisol and corticosterone), as well as playing a major role in the stress response, have been proposed as long-term regulators of both energy intake and storage (Strack et al., 1995). Glucocorticoids increase in circulation in response to energetic needs, and their levels are generally interpreted as indicators of allostatic load (Bonier et al., 2009). Cortisol is of particular interest in the regulation of whole body energy stores as it is involved in maintaining the balance between fat storage where triglycerides are deposited, and fat depletion where they are catabolised and released into circulation (McMahon et al., 1988; Peckett et al., 2011). Overall, cortisol is known to increase lipolysis, stimulate gluconeogenesis, mobilise amino acids, and increase circulating concentrations of plasma proteins (Bergendahl et al., 1996; Exton et al., 1972). Plasma cortisol concentrations could therefore also be used as indicators of overall physiological state and provide vital information on the health and resilience of a population as a whole. For these reasons, conservation biologists are increasingly using glucocorticoid hormone assessments to monitor both stress and health to better inform management efforts (Walker et al., 2005; Wikelski and Cooke, 2006).

However, studying underlying variability in glucocorticoid hormone concentrations in wild populations is inherently very difficult as the temporary capture and restraint necessary for sampling may alter the physiological parameters of interest. Animals are typically manually restrained or sedated for varying lengths of time, and it is well recognised that these sampling procedures are potentially highly stressful for the study animals (Morton et al., 1995). It is therefore very important to appreciate the magnitude and duration of the response to the capture event as it may compromise the specific aims of different studies by masking any underlying additional variation in these hormones levels.

In pinniped research, animal handling cannot be avoided for the collection of physiological samples, and several methods of physical restraint including nets and nooses have been used in the field and in captivity. Increases in circulating cortisol concentrations are characteristic of their stress response to such handling and physical restraint procedures (Champagne et al., 2012; Engelhard et al., 2002; Harcourt et al., 2010). Natural cycles due to reproductive and physiological condition may therefore be masked by the effect of handling on cortisol secretion. Sedation has been shown to reduce the stress response in phocids, such that hormone concentrations under these conditions are more likely to reflect basal values (Harcourt et al., 2010; Champagne et al., 2012). However, there is a time delay between capture and sedation which is then reflected in the lag in circulating cortisol concentrations post-sedation (Champagne et al., 2012).

Sampling other fluids, tissues and excreta instead of the plasma may therefore provide more representative baseline information on cortisol concentrations that are likely to be less affected

by the sampling procedure. Previous work has shown that cortisol can be extracted and quantified from blubber biopsy samples of harbour seals (*Phoca vitulina*), and that hormone concentrations in this tissue are unaffected by handling stress, likely because of the slower turnover of cortisol in the blubber compared to the blood (Kershaw and Hall, 2016). These concentrations are therefore more likely to represent baseline cortisol values for these individuals, and any variation is as a result of within-population variability and does not reflect individual variation in the response to the handling procedure. For example, seasonal differences have been observed in blubber cortisol concentrations, for example, concentrations are, on average, an order of magnitude higher during the moult than over the rest of the year (Kershaw and Hall, 2016).

While we can measure these changes and link them to an increased demand on energy stores when individuals fast during the moult, it is not known whether blubber *passively accumulates* or *actively produces* and secretes glucocorticoid hormones as is the case in other mammalian adipose tissues including humans (Lee et al. 2014). If it does produce cortisol (from the local conversion of stored inert cortisone to cortisol through what is known as the cortisol-cortisone shuttle), it is likely that the blubber's importance in energy regulation extends to the secretion of hormones that are involved in the control of lipid metabolism, fasting energetics and the regulation of physiological processes that take place during periods of high energy demand. The blubber could therefore be acting as an endocrine organ in its own right, comparable to white adipose tissue in terrestrial mammals, including humans (Kershaw and Flier, 2004).

The main objective of this study was to therefore measure the rate of exchange of cortisol between the plasma and the blubber in order to investigate whether harbour seal blubber is passively accumulating cortisol from the blood, or actively producing cortisol from cortisone, through the activity of the 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) enzyme in the adipocytes (Stimson et al. 2009). Prednisolone, a synthetic steroid hormone both physically and metabolically similar to cortisol and used as a standard steroid treatment, was administered as a proxy for cortisol in order to track concentrations of the hormone over time in both the blood and the blubber. A harbour seal housed at the SMRU Home Office Licenced captive facility was restrained, prednisolone was administered intravenously before the animal was then anesthetised and blood and blubber biopsy samples were taken over an 80 minute period. Measuring the concentrations of the hormone in both matrices over time will establish whether cortisol in the blubber reflect changes in the blood, thus demonstrating to what extent, and at what rate the blubber is passively sequestering cortisol. Determining the dynamics of cortisol in this species at its site of action, in the adipocytes, would be a key step towards understanding the endocrine control of energy regulation in pinnipeds, and establishing whether glucocorticoid concentrations in this matrix can indeed be used as biomarkers to better understand health and condition in wild populations.

Methods

1. Sample Collection

A juvenile male harbour seal housed at the SMRU captive seal facility was anesthetised with Zoletil 100 (Virbac, France) at a dose rate of 0.5ml/100kg body weight intravenously at Time 0 minutes (Fig. 1). The first plasma sample was taken simultaneously from the extradural vein into a heparinized Becton Dickinson Vacutainer (Oxford, UK) as the 'background' plasma sample, and 1ml of 0.25mg/ml methylprednisolone (Solu-Medrone^{TM}, Zoetis, 8R3154) in isotonic saline solution was then administered intravenously. The dose was established at a level well below the threshold that would produce a physiological response but high enough to be traced in the tissues. A further seven plasma samples were taken over the next 80 minutes (15 min, 25 min, 35 min, 45 min, 55 min, 70 min and 80 min) while the animal remained anesthetised (Fig. 1). The blood samples were centrifuged at 1,500*rcf* for 10 minutes and aliquots of the plasma collected and stored at -20^{0}C before analysis. Four blubber biopsies were also taken during this time period from the left and right flank areas following local anaesthesia using subcutaneous injections of Lignol (Dechra, UK) administered 3 minutes before each sample was taken (Fig. 1). A small incision was made through the skin, and a core biopsy punch was used to obtain a full depth blubber sample. The blubber samples were wrapped in aluminum foil, placed in individual plastic vials, and stored at -20^{0}C. All procedures were carried out under the SMRU Home Office project licence.

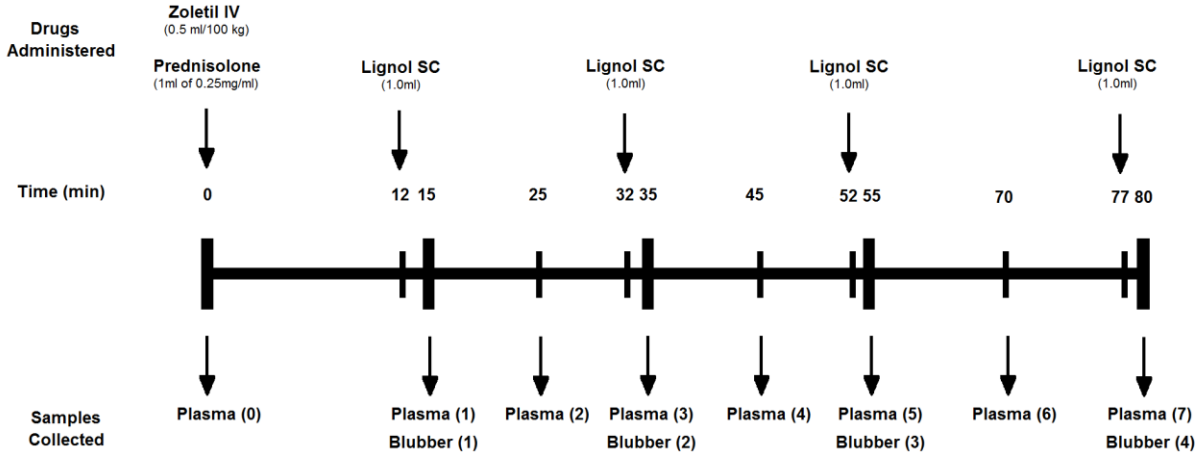

Fig. 1. Plasma and blubber sampling timeline for the anesthetised harbour seal. The animal was anesthetised with an intravenous (IV) dose of Zoletil, and the prednisolone dose was administered at Time 0, at the beginning of the trial. Subcutaneous (SC) doses of Lignol were administered at the biopsy sites before each sample was taken over the 80 minute time period.

2. ELISA Verifications

To date, attempts to measure prednisolone concentrations in a subset of the plasma samples have been made, using a commercially available Enzyme Linked Immunosorbent Assay (ELISA) kit for the quantification of prednisolone in human cell culture supernatants, plasma, serum and tissue homogenates (LifeSpan BioSciences Inc., Human Prednisolone ELISA, LS-F27799). The concentrations were measured according to the ELISA kit instructions with a standard curve ranging between 0 and 100ng/ml. The hormone concentrations in the samples were determined using a 4 parameter log-logistic model based on the standard curve of known concentrations.

2.1 Parallelism Assays

Parallelism of the standard curve of known concentrations with the dilution curve of the extracts to be analysed supports the assumption that the binding characteristics allow the reliable determination of hormone levels in the samples (Grotjan and Keel, 1996). To assess parallelism, firstly, the plasma sample with the highest expected prednisolone concentration (Plasma (1)) was serially diluted (1, ½, ¼, $^1/_8$, $^1/_{16}$) with phosphate buffered saline (PBS). Secondly, the background plasma sample was spiked with 250ng/ml of prednisolone and then also serially diluted. Parallelism was assessed between these two series of dilutions and the standard curves of known concentrations. Statistical comparisons among the standard curves and the plasma dilution curves were carried out by modelling the dilutions using the 'drm' function in the 'epicalc' library in the statistical package R, version 3.1.2 (R Development Team, 2014). Parameter estimates for each model were then compared to assess if the curves were parallel to each other and to the standard curve.

2.2 Matrix Effect Tests

Successful immunoassays require an optimal pH and ionic strength to promote the specific antibody–antigen complexes needed, while reducing the nonspecific binding of other proteins in the samples that increase interference. As the ELISA kit used here was designed for use with human samples, the compatibility of the kit with harbour seal plasma and blubber tissue extracts resuspended in PBS was assessed. The expected and the measured concentrations in the spiked samples should show a positive linear relationship with a slope of approximately 1.0 (Hunt et al., 2014). Harbour seal plasma was assessed as a suitable matrix by comparing the measured and the expected concentrations in the serial dilutions of the sample spiked with 250ng/ml of prednisolone (see above). Harbour seal blubber extracts were assessed as a suitable matrix by spiking four extracted blubber samples (samples A, B, C and D in Table 1) used in previous work (Kershaw and Hall, 2016) with 125ng/ml of prednisolone. The original blubber extracts as well as the spiked extracts were all assayed in tandem for comparison.

Results and Conclusions

1. ELISA Verifications

1.1 Parallelism Assays

Visual inspection of the dilution curves shows very clearly that they are not parallel (Fig. 2) and the absorbance values in the seal plasma largely remained constant over the dilution series. There were indeed significant differences between the parameter estimates of each dilution curve with the standard curves (p values < 0.1), indicating that this ELISA kit is unfortunately not suitable for measuring the concentrations of prednisolone in the seal samples.

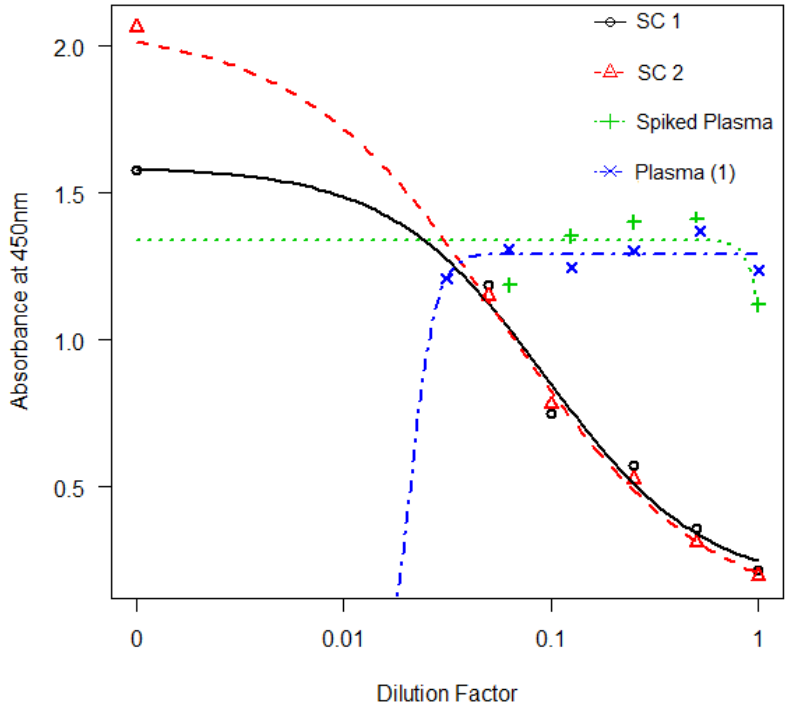

Fig. 2. Dilution curves for the standard curves and the plasma dilutions do not show parallelism. The spiked and unspiked plasma samples showed no parallelism with the two standard curves (SC), thus showing that this ELISA kit is not reliably measuring prednisolone in these samples.

1.2 Matrix Effect Tests

Similarly to the parallelism assay, the matrix effect tests with the spiked blubber and plasma samples confirmed that this ELISA cannot be used to measure prednisolone in these samples. All samples showed very similar measured concentrations and there is no relationship between the expected and the measured concentrations (Table 1).

Table 1. Measured and expected concentrations in plasma and blubber extracts spiked with varying concentrations of prednisolone.

Samples	Spiked Prednisolone Concentration (ng/ml)	Measured Prednisolone Concentration (ng/ml)	Expected Prednisolone Concentration (ng/ml)
Plasma (0)	0	6.33	0
Spiked Plasma (0)	500	4.34	250
Spiked Plasma (0)	250	3.26	125
Spiked Plasma (0)	125	3.79	62.5
Spiked Plasma (0)	62.5	4.24	31.25
Spiked Plasma (0)	31.25	3.75	15.63
Spiked Plasma (0)	15.63	4.57	7.81
Pv[1] Blubber A	0	10.85	0
Spiked Pv Blubber A	125	7.49	62.5
Pv Blubber B	0	10.77	0
Spiked Pv Blubber B	125	8.05	62.5
Pv Blubber C	0	10.36	0
Spiked Pv Blubber C	125	9.92	62.5
Pv Blubber D	0	11.21	0
Spiked Pv Blubber D	125	9.94	62.5

Conclusions and Next Steps

It is likely, based on the similar concentrations measured in all of the diluted samples as well as the spiked and un-spiked blubber samples that there is a metabolite within the samples interfering strongly with the assay, thus preventing the prednisolone in the samples from binding to the plate. There may also be a problem with the cross-reactivity between the form of prednisolone (*methyl*prednisolone was administered here as this is most commonly used in veterinary medicine for intravenous treatment of dogs and cats) administered to the seal, and the human anti-rat prednisolone antibodies in the immunoassay.

However, additional aliquots of all plasma samples collected are available for further analysis, our next step is to use a different analytical approach. We will collaborate with colleagues at the Marine Scotland Science, Marine Laboratory in Aberdeen and use high-performance liquid chromatography (as established by Majid et al., 2011) to measure the prednisolone and endogenous cortisol in the eight plasma samples. Once this is confirmed, we will extract the steroids from the blubber biopsy samples using a previously published method (Kershaw and Hall, 2016), and these will also be analysed for prednisolone.

[1] Pv = harbour seal

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