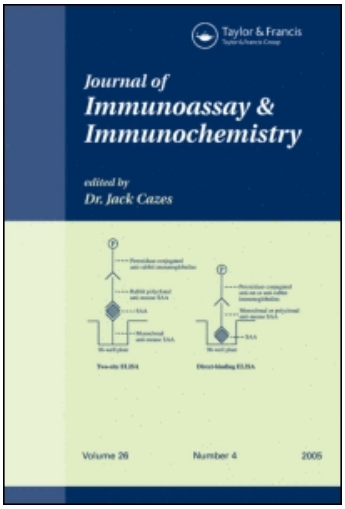

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Development of an Immunoassay to Measure Progesterone using Printed Biosensors, and its Application to the Assessment of Ovarian Function in the Numbat (*Myrmecobius fasciatus*)

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Abstract: A biosensor system was developed to measure progesterone levels in the urine of female numbats (*Myrmecobius fasciata*) as an index of ovarian function. Screen printed sensors were coated with a monoclonal progesterone antibody, and incubated in a mixture of sample/standard and progesterone-3-CMO-horseradish peroxidase (HRP). The difference in potential between the working and reference electrode was measured, after exposure to an HRP substrate. EIA and biosensor standard curves showed parallelism, and the biosensor gave values similar ($r = 0.83$) to the conventional EIA. Progesterone concentrations at different stages of the oestrus cycle were not significantly different to those obtained by EIA.

Keywords: Biosensor, Immunoassay, Numbat, Oestrus, Progesterone, Urine

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INTRODUCTION

The measurement of hormones in urine or faeces gives the opportunity to study non-invasively reproductive cycles in wild animals without stress to the animal or danger to the investigator.[1] Captive breeding programmes are an important component in the conservation of an increasing range of species throughout the world[2] and a thorough knowledge of reproductive cycles is imperative to the successful implementation of such programmes. The steroidogenic switch by the ovarian follicle at luteinisation, where it changes from producing predominantly oestrogens to progestogens, makes the analysis of steroids an effective way of monitoring the appearance and demise of the corpus luteum. Because the range of metabolites can vary widely across species, the use of antibodies that cross-react with a number of related compounds has proven valuable in working with wildlife. A typical example is the monoclonal antibody CL425 to progesterone that cross-reacts with a number of reduced pregnanes,[3] making it useful in a range of species from toads[4] to the Asian elephant.[5]

Enzymeimmunoassay (EIA) methods to measure steroids have proven particularly popular because of the significant advantage of using non-isotopic labels. However, screen printed electrochemical sensors are now becoming available that can allow such non-isotopic competitive immunoassays to be performed for a wide range of analytes rapidly, cheaply and reliably.[6] This technology has already been applied to monitoring the reproductive status of dairy cattle through the measurement of progesterone in milk[7,8] and there is no reason why it should not extend to wildlife endocrinology.

The present study describes the development of a biosensor system for the measurement of progesterone in urine, and its application to the study of ovarian function in the threatened Western Australian marsupial the numbat (*Myrmecobius fasciatus*).

EXPERIMENTAL

Enzymeimmunoassay

EIA was performed using a monoclonal antibody to progesterone (CL425)[3], and a label of progesterone-3-CMO-horseradish peroxidase (prog/HRP) (both supplied by Coralie Munro, University of California at Davis, USA). Standards were made with authentic progesterone (Sigma P0130) over the range 0.0156 ng/mL to 4 ng/mL. Sample volumes of 50 µl were analysed directly. The assay gave a sensitivity of 0.013 ng/mL, and intra- and inter-assay coefficients of variation of $<12\%$. Linearity was demonstrated by serially diluting four neat samples to 1:8 with a mean recovery of 91% (range 86 to 101%).

Biosensor Measurements

Reagents

The antibody, label, and authentic progesterone were the same as used in the EIA as described above.

Sensors

Screen printed sensors (Universal Sensors, Cambridge, UK) were obtained in strips of 40, and stored dehydrated in air-tight bags at 4°C until use. Sensors were coated by placing directly onto each working electrode 3 μL of a dilution of the CL425 monoclonal antibody in phosphate buffered saline (pH 7.2). The electrodes were placed under a gentle stream of air until just dry. Immediately after drying, 3 μL of post-coating buffer (Tropbio, Townsville, #050317; pH 7.7) was dispensed onto the working electrode and dried at 37°C for 15 minutes.

Use of the Integrated Biosensor Platform

Coated sensors were cut from the strip and washed twice by immersing in PBS +0.05% w/v Tween 20 for 3 minutes, followed by 1 minute. The sensors were then immersed in the sample or standard for the required incubation time, and washed twice again. Individual sensors were placed in sequence into the reader (Universal Sensors, Cambridge, UK), and immersed into 400 μL of basic buffer (0.05 M Citrate buffer with 0.1 mg/mL O-phenylenediamine) in wells of a 96-well polypropylene low binding plate (Greiner Bio-one, # 6150), to establish the baseline signal from each sensor. The sensors were then immersed in 400 μL of basic buffer supplemented with 0.03% w/v sodium perborate as a substrate for the prog/HRP. Sensor outputs were read continuously throughout these steps using the proprietary software (Universal Sensors, Cambridge, UK). The initial read was for 400s, with no initial enforced 0 mV step. Subsequent reads in the enhanced buffer alone were for 90s with an enforced 10s 0 mV step at the start of the read. Multiple readings were taken to explore the importance of the delay prior to taking a reading.

Optimisation Studies

Labelled Progesterone

Initial experiments to determine the level of binding of prog/HRP to the sensors were performed with sensors coated as above, either with a 1/500

dilution of the antibody stock supplied, or with PBS alone. Two-fold dilutions were made of a 1/200 working stock of prog/HrP in assay buffer, from 1/2 down to 1/512, and 160 µl of each dilution placed in sequential wells of a low binding polypropylene 96 well plate.

Monoclonal Antibody Concentration

The optimum concentration of monoclonal antibody used to coat the sensors was determined empirically by coating sensors with 3 µL of serial dilutions of the antibody from 1/500 of working stock, to 1/512,000 as above. All the sensors were immersed into 1/250 dilution of the stock prog/HRP in assay buffer for 1 h, prior to washing and reading as described above.

Standard Curve, Samples, and Parallelism

Final standard curves and analysis of samples was done using a 1/500 dilution of the monoclonal antibody and a 1/200 dilution of the prog/HRP diluted in assay buffer (0.1 M phosphate buffer containing 0.148 M NaCl and 1 mg/ml BSA). Authentic progesterone standards of 4, 2, 1, 0.5, and 0.25 ng/mL in assay buffer were prepared from a stock concentration of progesterone at 16 µg/mL in ethanol, and 160 µL of each concentration was dispensed into wells of a low-binding polypropylene 96 well plate. A polynomial standard curve of the form: $y = a+bx+cx^2$ was fitted to a Ln/Ln plot of the sensor output against the standard progesterone concentrations. Numbat urine samples were spiked with prog/HRP stock to give a final concentration of 1/200 of the working stock, and 160 µL pipetted into the wells of a low binding plate and assayed as described above. The progesterone concentrations in the unknown samples were calculated from their sensor outputs using standard curves generated for each experiment. Parallelism was confirmed by serially diluting a sample of urine in assay buffer to give progesterone concentrations closely related to these in the standard curve.

Biological Samples

Animals

The Numbats were part of the Native Species Breeding Programme (NSBP) at Perth Zoo, Western Australia, which breeds animals for release to the wild, in accordance with the Department of Environment and Conservation recovery plan for this threatened species.[9] They were fed a diet of termites and formulated custard, given water *ad libitum*, and housed individually throughout the year until the breeding season.[10]

Urine Collection

Females were caught, examined and then placed until urination occurred into a wire cage trap (57 cm x 20 cm x 20 cm) which rests on a tray lined with a fly wire filter tray sloped down to a collecting specimen jar. Urine was separated from faeces via the fly wire inner tray, the urine centrifuged and the supernatant stored at −20°C for hormonal analysis. The stage of the oestrous cycle at which the sample was collected was determined by examination of cloacal swabs for the presence of cornified epithelial cells and leucocytes.

RESULTS

Prog/HRP Dilution

The effect of serially diluting the prog/HRP label whilst using a fixed concentration of monoclonal antibody is shown in Fig 1. The greatest resolution between the levels of binding of prog/HrP to sensors coated with 1/500 antibody and non-specifically to sensors with no antibody at all was at a concentration of 1/256 of the working stock of prog/HRP. HRP. At concentrations above this the antibody-coated sensors were saturated with prog/HRP, and non-specific binding increased in a dose-related manner to reduce the difference between the signal from

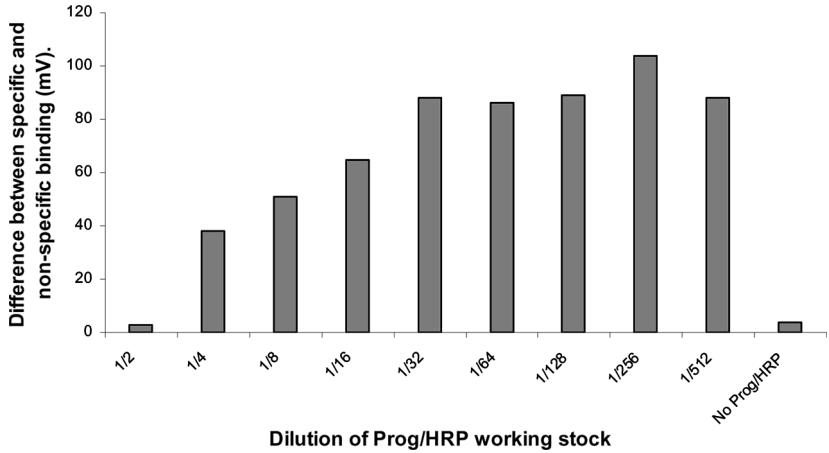

Figure 1. The effect upon the assay of varying labelled progesterone concentration. The polyclonal antibody concentration used was 1:500 of the supplied stock solution.

specific and non-specific binding. A working dilution of prog/HRP at 1/200 was therefore chosen for future assays as the level where the sensor was just saturated, and any displacement with unlabelled competing sample would be detectable.

Antibody Dilution

The effect of varying the antibody dilution in the presence of a fixed prog/HRP concentration (1/250 of the working stock) is shown in Fig 2. The antibody concentration was optimal at 1/500; any greater dilution decreased the signal as less prog/HRP was bound specifically. Prog/HRP was at a concentration to give minimal background due to non-specific binding. A working dilution of 1/500 was therefore used in future work.

Incubation Times

The duration of incubation of the sensors in substrate, prior to reading was not critical as shown in Fig 3, with the difference between signals for progesterone standards of 0.5 ng/mL and 4 ng/ml being maintained over an extended period. An increase in the variance of the signal was apparent at the higher concentration after extended incubation, and the signals from the sensors after incubation of 20 minutes prior to reading

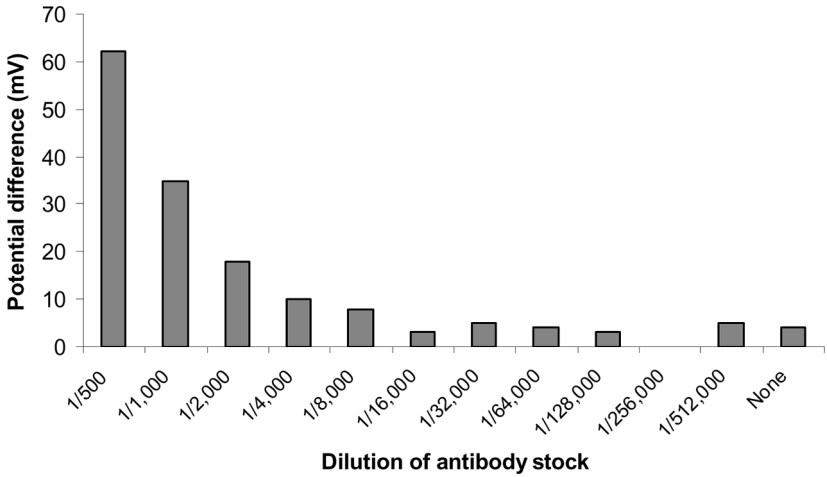

Figure 2. Effect upon sensor output of varying antibody concentration whilst the Prog/HRP is at a constant dilution of the working stock of 1:250.

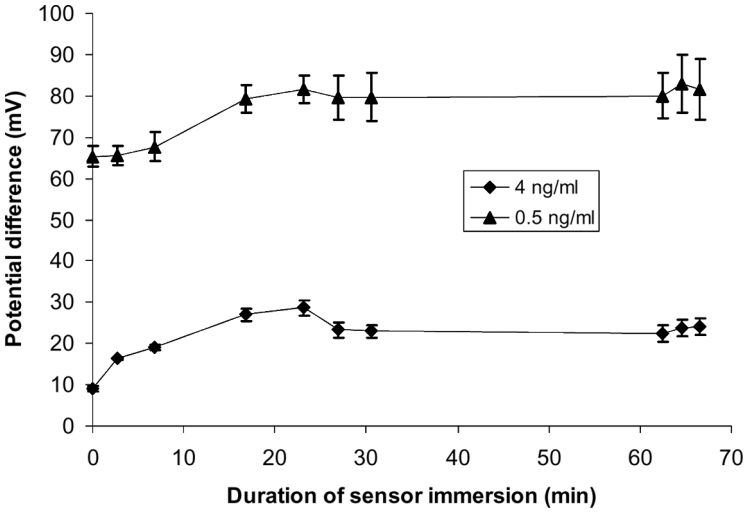

Figure 3. Sensor output from progesterone standards read at increasing time post exposure to substrate.

(to allow the potential build-up on the sensor surface) were utilised to calculate the concentration of progesterone in the unknown samples.

Standard Curve

The polynomial fitted to the standards typically gave R^2 values above 0.98. A typical standard curve (prior to transformation) obtained with an antibody dilution of 1/500 and a prog/HRP dilution of 1/200 is shown in Fig 4. This is compared to a typical curve obtained with the conventional EIA.

Urine Samples

The serial dilution of a urine sample taken at the diestrus stage showed parallelism with the serially diluted standard as shown in Fig 5. The progesterone concentrations in urine collected from numbats at the various stages of the oestrous cycle, as measured both by EIA and with the sensors, are shown in Table 1. Both assay systems confirmed a significant rise in progesterone after ovulation at the diestrus stage of the cycle, and there were no significant differences between the sensor and EIA results at any stage (all $p > 0.1$).

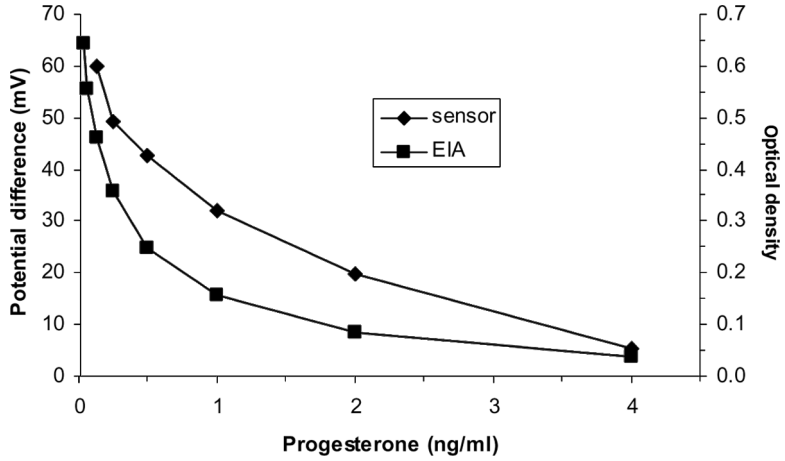

Figure 4. Standard curves prior to transformation of data.

DISCUSSION

The route of excretion of steroid hormones varies considerably between species, with different proportions being excreted in the urine and the faeces, as well as in the range of metabolites.[1] Consequently,

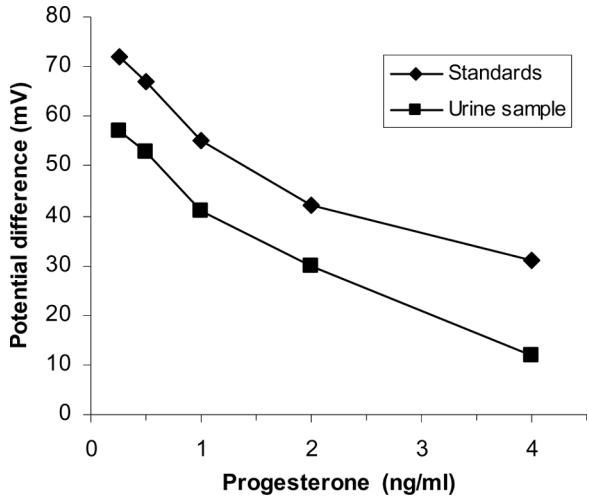

Figure 5. Assay of serial dilutions of numbat urine, showing parallelism with dilutions of authentic progesterone in assay buffer.

Table 1. Progesterone concentrations (mean ± sem) in urine for 6 animals measured at different stages of the oestrous cycle using either the sensors or enzymeimmunoassay

	Progesterone (ng/mL)	
Stage of oestrous cycle	Enzymeimmunoassay	Sensor
Early proestrus	1.36 ± 0.22	1.62 ± 0.23
Late proestrus	0.91 ± 0.18	1.10 ± 0.36
Oestrus	0.98 ± 0.43	1.93 ± 0.97
Diestrus	3.72 ± 0.11	4.40 ± 0.64

the measurement of hormones, usually progestogens, for monitoring ovarian function in wildlife in either urine[11] or faeces[12] depends on the availability of the samples and the pattern of excretion. The potential value of biosensors for monitoring wildlife reproduction has already been demonstrated by measuring progestogen in faecal extracts of the Field Vole[13] with a system designed to measure progesterone in bovine milk, and comparing the results with a conventional ELISA.

The present study was able to monitor the female numbats by virtue of the ability of the antibody to cross-react with the main metabolites excreted in urine, though the usefulness of this assay system for other species would require validation. Interestingly, the use of common reagents (e.g., the antibody, labelled progesterone and standard) in both the standard EIA and the biosensor system allowed a direct comparison of the two assays and confirmed the biosensor system to function well compared with the EIA in terms of the hormone results obtained. Hormone concentrations in urine, like any other urinary compound, are usually indexed against creatinine concentration or specific gravity[14] in order to correct for differences in urine output. Indexing is not easy to do when urinary compounds are measured with hand-held monitors, urine test sticks or similar systems where this adds another measurement and calculation. Although it is not possible to index samples using the biosensor in the present study without introducing a second analysis procedure, it is thought that this should not significantly affect results as the hormonal profiles seen in individual women when the reproductive hormones are indexed or not are remarkably similar.[15]

The biosensor was seen to have a number of advantages over the EIA. Firstly, results could be obtained more quickly. A sample incubation time of 60 minutes with the sensors compares with 2 hours for the EIA, and this incubation step could be reduced to as low as

5 minutes with some loss of sensitivity (results not shown). Also, as shown in Fig 3, the time taken to read the sensors was short, with a difference in potential almost immediately evident, between high and low standards on immersion into substrate. Secondly, the results obtained with the biosensors require very simple robust equipment and would prove easier to undertake out in the wild, a virtue that has already proved attractive to field biologists monitoring the reproductive performance of field voles as a marker of environmental disruption.[13] The development of systems to measure other hormones would seem a natural extension of this work. A future application that would seem ideally suited to this technology would be the identification of hormonal thresholds that are useful in the management of the breeding of animals; the semi-quantitative measurement of hormones would only require a single reference standard and could be done close to the animals with a rapid turnaround as indicated above. One example that springs to mind is in the captive breeding of elephants[16,17] where thresholds are used to determine (i) the end of the preceding luteal phase by observing progesterone to have dropped to baseline levels, (ii) the onset of the first anovulatory LH surge and (iii) the second ovulatory LH surge.

In summary, the present study has shown that printed biosensors can be used to monitor ovarian function in the numbat, and the technology gives similar results to conventional EIA, but quicker and in a format more suited to work in the field.

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