

# Evidence for a prolonged postimplantation period in the Australian sea lion (*Neophoca cinerea*)

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Concentrations of circulating progesterone and oestradiol were measured in 96 free-ranging, female Australian sea lions *Neophoca cinerea* from Kangaroo Island, South Australia. There was a marked increase in the concentrations of both hormones (progesterone from approximately 12 ng ml<sup>-1</sup> to approximately 24 ng ml<sup>-1</sup>; oestradiol from approximately 1.5 pg ml<sup>-1</sup> to approximately 14 pg ml<sup>-1</sup>) about 3.5 months after the probable date of mating, reaching peak values in the 5 months after parturition. Progesterone concentrations remained at peak concentrations for about 2 months, decreasing at approximately 8 months to concentrations approximating those of the first 3 months after parturition. Oestradiol concentrations decreased, after reaching a peak, to 3–4 pg ml<sup>-1</sup> at about 8 months after parturition. The timing of the increase in the concentrations of circulating progesterone and oestradiol provides evidence that the blastocyst reactivates and implants between 3.5 and 5 months of pregnancy in Australian sea lions, indicating an embryonic diapause of similar duration to that of other pinnipeds. This would suggest a prolonged postimplantation period of up to 14 months (to fit with the gestation period of 18 months reported for this species) the longest postimplantation period recorded for pregnancy in any pinniped.

## Introduction

The Australian sea lion (*Neophoca cinerea*) is unique amongst pinnipeds in having an aseasonal, 17–18 month breeding cycle with a lack of synchrony between breeding seasons and between the time of breeding at different locations. There is no discernible effect of season on pupping dates (Ling and Walker, 1978; Gales *et al.*, 1992, 1994; Higgins, 1993). Females have a postpartum oestrus approximately 7 days after pupping (range 4–10 days), during which time they are attended by an adult male (Higgins, 1990). Lactation lasts 15–18 months (Higgins and Gass, 1993). Embryonic diapause occurs during pregnancy and is thought to last at least 7 weeks (Tedman, 1991), but otherwise nothing is known of the reproductive physiology of this species (see Gales and Costa, in press, for a review of the unusual life cycle of this species).

Pregnancy in pinnipeds is generally divided into a period of embryonic diapause of variable length followed by a postimplantation period of approximately 8 months (Boyd, 1991). In most species in the wild, birth (and hence lactation) is normally timed to maximize the chance of survival of the offspring and is presumably in response to proximate environmental factors such as climate and the availability of food. It has been postulated that photoperiod is the most influential environ-

mental factor affecting breeding patterns in pinnipeds and acts by cueing the reactivation of the dormant blastocyst at the end of embryonic diapause (Boyd, 1991; Temte and Temte, 1993).

The present study was undertaken to investigate the breeding cycle of female *N. cinerea*, to determine whether the reported aseasonal breeding pattern is reflected by the reproductive endocrinology and whether this endocrinology differs from that of other pinnipeds. Plasma progesterone and oestradiol concentrations were measured throughout pregnancy in an attempt to determine these factors.

## Materials and Methods

The study was conducted at Seal Bay, Kangaroo Island (36°S, 137°20'E) in South Australia, where a detailed study into the reproductive behaviour and maternal investment of Australian sea lions was underway (Higgins, 1990, 1993; Higgins and Gass, 1993). Females that had produced pups had been marked with either a pelage mark (paint or dye) or a flipper tag and the birth date of their pups had been recorded (Higgins, 1993). Pups were also identified with flipper tags or subcutaneous transponder implants (Trovan, AEG, Hellbron).

Females with known birthing dates were identified, captured with a hoop net (Furhman Diversified, Flamingo, TX) and

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physically restrained. A 15–20 ml sample of blood was collected from each animal from the lateral gluteal veins using a 2 inch, 20 gauge needle and a 20 ml syringe. Animals were released within 10 min of capture. Blood samples were stored in lithium heparin vacutainer tubes, which were kept in an insulated container at about 4°C (on ice). Samples were centrifuged at 1200 g for 10 min within 3 h of collection and the plasma was decanted and stored frozen at –20°C until analysis.

Blood samples were collected over three consecutive breeding seasons (Fig. 1). At the time of blood collection, it was known that the female had produced a pup during the previous pupping season, but it was not known whether she was pregnant. Higgins and Gass (1993) documented that approximately 71% of females pupped in consecutive seasons. In the present study, we were unable to confirm pupping after sampling in most females, so it was assumed that a similar proportion of the sampled females were pregnant at the time of sampling.

#### Hormone determinations

Concentrations of progesterone and oestradiol were measured in each plasma sample. Plasma progesterone was measured using a double antibody radioimmunoassay. Two 100 µl replicates of each unknown sample were extracted in 12 mm × 75 mm glass tubes by adding 2 ml distilled hexane and vortexing for 2 min. The solvent layer was removed to clean tubes and dried under a stream of nitrogen. Powdered progesterone (Sigma, Australia) dissolved in ethanol was used to prepare standards ranging from 16 ng ml<sup>-1</sup> to 0.125 ng ml<sup>-1</sup>. Replicates of 0.1 ml standard were dried under nitrogen. The unknown replicates and standards were reconstituted in 0.1 ml of 0.1 mol phosphate-buffered saline l<sup>-1</sup> containing 0.1% (w/v) bovine serum albumin (PBSB). The tracer (0.1 ml, containing 30 000 d.p.m. 1,2,6,7-<sup>3</sup>[H]progesterone) and the antibody (0.1 ml, diluted 1:6000) were added, the tubes mixed and incubated at 4°C for 24 h. Gamma globulin (0.1 ml normal rabbit serum 1:500) and second antibody (0.1 ml diluted 1:20 in PBSB containing 0.05 mol EDTA l<sup>-1</sup>) were added to the tubes which were then mixed and incubated overnight at 4°C, before 1 ml cold PBS was added to all tubes except those used to determine total counts. The tubes were centrifuged at 2000 g for 30 min at 4°C; the supernatant was aspirated and the pellet was redissolved in 0.5 ml 0.05 mol HCl l<sup>-1</sup>. The solution was dispensed into counting vials with 4 ml toluene-based scintillant and counted in a liquid scintillation counter. The antibody (GT1) was raised in a rabbit against progesterone-11α-carboxymethylloxime–human serum albumin and the major crossreactions were with deoxycorticosterone (2% w/v) and 20α-OH progesterone, 17β-OH progesterone, 17α-OH progesterone and allopregnenolone (all <1%). Two pooled samples of sea lion plasma were serially diluted and used to test for parallelism with the standard curve of the assay. The correlation between volume of plasma and mass of hormone gave a coefficient of 0.975. Mean percentage recovery for extraction for the assay was 81% and the sensitivity of the assay was 0.022 ng per tube. All samples were processed in a single assay with an intra-assay coefficient of variation of 11.2%.

Oestradiol was measured in a double antibody radioimmunoassay after an affinity chromatography extraction using an antibody–sepharose 4B gel as described by Webb *et al.* (1985). The antiserum and conjugate for the iodinated tracer were kindly donated by R. Webb (AFRC, Animal Breeding Research Organisation, Dryden Laboratory, Roslin, Midlothian, UK). As the samples to be assayed contained high concentrations of progesterone and testosterone (ng ml<sup>-1</sup>) compared with oestradiol (pg ml<sup>-1</sup>) and the assay antibody R48 cross-reacts 1.2% with testosterone, the affinity chromatography extraction step was tested to check that progesterone and testosterone were removed. Samples of plasma with 4 ng ml<sup>-1</sup> tritiated progesterone and testosterone (*n* = 5) added were incubated at room temperature for 1 h before extraction. Ninety-five per cent of testosterone was removed in the first step. Only 0.11% or 4.4 pg ml<sup>-1</sup>, of which only 1.2% crossreacts, remained in the extract to be assayed. All of the progesterone was removed during the extraction steps.

Major crossreactions of R48 were oestrone (16%), oestriol (3%), testosterone (1.2%) and 5α-dihydrotestosterone, androsterone, androstenedione and progesterone (<1%).

Extracted samples equivalent to 0.5 ml of plasma were assayed in duplicate and the limit of detection was 0.4 pg per tube. The extraction efficiency was 75% and the coefficient of variation in the single oestradiol assay performed was 6.8%. The reported hormone concentrations for both oestradiol and progesterone were corrected for recovery percentage.

#### Analyses of hormone data

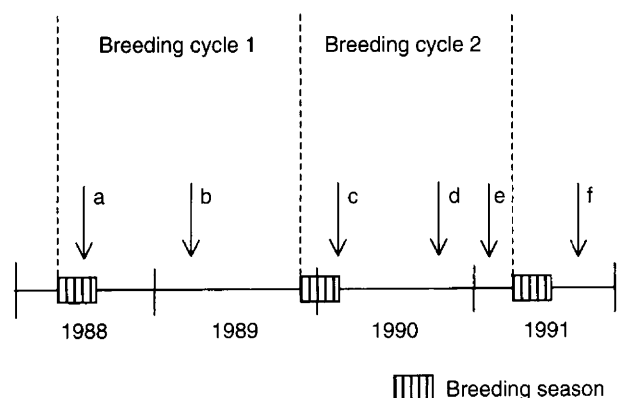
Inspection of distributions of the data indicated that the progesterone concentrations were symmetrically distributed about their mean with constant variation, while the oestradiol values were skewed towards the higher values. This skewness and variance in homogeneity were corrected using a natural log transformation, and the oestradiol values were subsequently analysed on the log scale. In both cases some atypical values (outliers) were detected among the data and these were omitted before analysis to avoid them having undue influence. Analyses were carried out in Minitab (Minitab Inc., 1996).

Values for progesterone and log-oestradiol by months since pupping were plotted (Fig. 2), and a robust, locally weighted regression smoother (Lowess), which indicates the underlying trends in the data (Cleveland, 1979), was applied.

The significances of peaks were tested using one-way analysis of variance with data grouped according to month since pupping. Approximately one-third of the cases had multiple sequential blood samples during the same pregnancy, giving data which were correlated as a result of their association with the same animal, but these were not sufficient in number to adopt repeated measures techniques for analysis. For the analyses these data were reduced to a single observation per birth by taking only the last measurement from multiple sampled animals in each case.

## Results

Blood samples were collected from 96 females during six periods, over three separate breeding cycles (Fig. 1). One-third



**Fig. 1.** Timing of breeding seasons and field trips to collect blood samples from Australian sea lion cows on Kangaroo Island, South Australia. Dates of field trips and number of samples collected on each trip were: (a) 26 July–11 August, 1988,  $n = 10$ ; (b) 14 February–1 March, 1989,  $n = 35$ ; (c) 6–23 February, 1990,  $n = 46$ ; (d) 26 October–1 November, 1990,  $n = 20$ ; (e) 1–16 February, 1991,  $n = 20$ ; (f) 25–30 November, 1991,  $n = 27$ .

of the females were sampled on more than one occasion. Analysis using the last hormone concentration measurement from multiple sampled animals yields the same qualitative results as analysis performed when all the data from multiple bleedings were included and treated as independent, or if only the first measurements of multiple bled animals per birth are taken. Therefore, only the last sample collected from each of these animals was used in the statistical analyses.

The mean progesterone values showed an increase from about  $12 \text{ ng ml}^{-1}$  at 3 months after pupping to a peak of about  $24 \text{ ng ml}^{-1}$  at 5–6 months, subsequently falling to about  $12 \text{ ng ml}^{-1}$  by 8 months after pupping (Fig. 2a). Oestradiol concentrations increased from about  $1.5 \text{ pg ml}^{-1}$  at 3 months after birth to a peak of about  $14 \text{ pg ml}^{-1}$  at 5–6 months. Concentrations then fell to about  $3\text{--}4 \text{ pg ml}^{-1}$  by 8 months, settling at a concentration above the initial values (Fig. 2b).

The mean values for progesterone concentrations by month from birth of pups were determined. The overall test for equality of means was highly significant ( $P < 0.0005$ ). Tukey's pairwise comparison test (Zar, 1984) indicated that there was no significant difference between the means for months 1–4 and 8–16, while the means for months 5–7 were significantly higher ( $P < 0.05$ ), providing strong evidence of a peak in mean values at about 6 months after parturition.

Mean monthly oestradiol concentrations from birth of pups were also determined and were significantly different ( $P < 0.005$ ). Tukey's test indicated that mean concentrations at months 4–7 were significantly higher than at months 1–3 ( $P < 0.05$ ), and oestradiol concentrations at month 6 in particular were higher than at months 8–16. Concentrations at months 8–16 were also significantly higher than at months 1–3 ( $P < 0.05$ ), indicating that the concentrations had not yet returned to pre-peak concentrations 16 months after pupping.

## Discussion

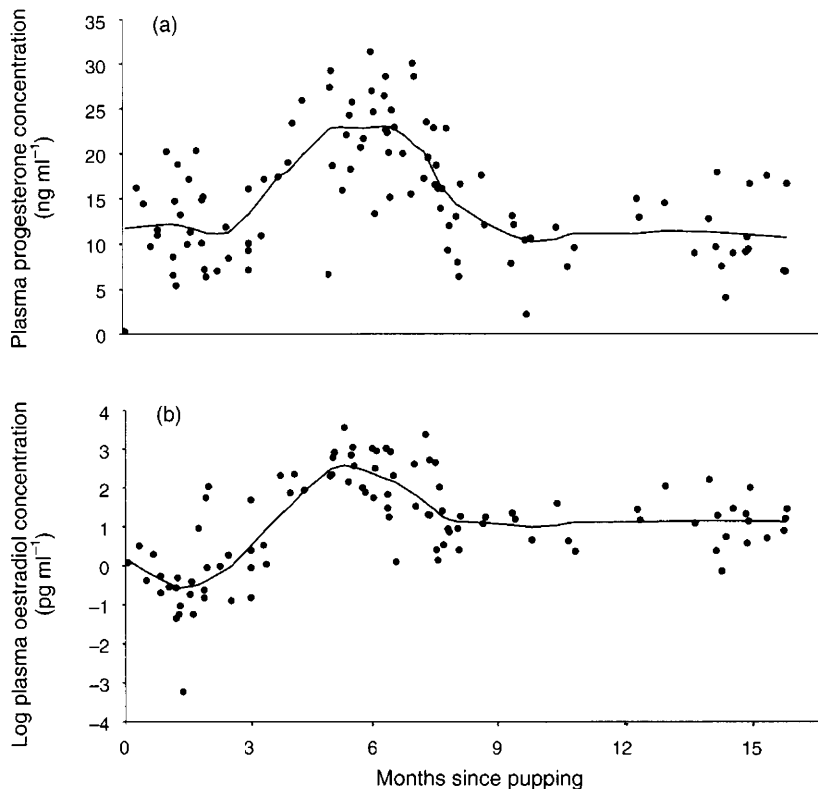
The difficulty in catching, restraining and collecting blood samples from sea lions in their wild habitat limited the data

available for analysis in the present study. Only female seals with a known pupping date were sampled, and sampling depended on identified females being on the beach in accessible parts of the coast. A long day's work often resulted in only four or five animals being sampled. Despite the limited number of samples, some clear hormone patterns were apparent throughout pregnancy.

The concentrations of plasma progesterone and oestradiol during the first 3 months of pregnancy or pseudopregnancy were indicative of a luteal phase following the follicular activity associated with postpartum oestrus. In the only published histological examination of *N. cinerea* ovaries, Tedman (1991) noted that the corpus luteum of pregnancy (at 7 weeks after ovulation) was markedly lobed with a well developed capillary network that extended through most of the cortex and well into the medulla. This histology is consistent with the increased progesterone concentrations measured at a similar stage of pregnancy or pseudopregnancy in the present study. The low concentrations of oestradiol measured in the early months of pregnancy or pseudopregnancy in the present study may reflect the ovarian changes noted by Craig (1964) in her histological studies of the reproductive tracts of mature female Northern fur seals. Craig (1964) postulated that during the first month after ovulation there is a luteal phase with high progesterone concentrations and reduced oestrogen activity.

During this early postpartum phase it is likely that the progesterone profiles of pregnant and nonpregnant sea lions would be similar, as a pseudopregnancy of similar duration to embryonic diapause has been reported in several other species of pinniped (Noonan and Ronald, 1989; Boyd, 1991; Renouf *et al.*, 1994). Progesterone and oestradiol concentrations began to rise significantly at about 3.5 months after parturition. Craig (1964) also reported a luteal phase immediately before implantation, which prepares the uterine mucosa for implantation. Results from the present study indicate that, unlike the luteal phase that follows the postpartum ovulation, there is a concurrent rise in both progesterone and oestradiol concentrations in this preimplantation phase, with both hormones rising to peak concentrations at about 5–6 months after parturition. After the rise to peak values for both hormones, there is a period during which the corpus luteum regresses slowly, allowing for a redevelopment of follicular activity. This slow decline of the functional corpus luteum is reflected in the gently falling concentrations of plasma progesterone and oestradiol.

The marked rise in both oestradiol and progesterone concentrations measured from about 3.5 months after parturition in the present study suggests a pattern of steroid production similar to that measured in other pinnipeds at the time of implantation. Surges of oestradiol (Daniel, 1981) and progesterone (Boyd, 1991) at this stage of pregnancy have been linked to reactivation and implantation of the blastocyst. The marked increase in progesterone concentration at this time is indicative of the activation of the corpus luteum as either a precursor to, or as a result of, reactivation and implantation of the blastocyst (Boyd, 1991). Boyd (1991) argues that, since Daniel (1981) had failed to reactivate the blastocysts of Northern fur seals prematurely by treatment with exogenous progesterone or oestradiol, the change of steroid concentrations at about the time of implantation is a consequence and



**Fig. 2.** Plasma concentrations of (a) progesterone and (b) oestradiol in blood samples collected from sea lion cows plotted against the number of months since pupping, with locally weighted regression smoother (Lowess). Only the final measurements were used for animals with multiple samples.

not the cause of the blastocyst growth phase of pregnancy. The corpus luteum in grey seals increases in size after implantation and throughout the fetal growth phase, but progesterone secretion does not increase (Boyd, 1982). Analysis of the data collected in the present study provides some evidence that reactivation and implantation of the blastocyst in Australian sea lions occurs some time between the initial rise in plasma oestradiol and progesterone concentrations, at about 3.5 months after mating, and the peak hormone concentrations at about 5 months. This evidence, coupled with the reported postpartum mating pattern for this species (Higgins, 1990), indicates that embryonic diapause is of similar duration to that in other pinnipeds. The data also suggest that there is a prolonged postimplantation period in pregnancy of up to 14 months, the longest recorded for any pinniped.

After the surge in progesterone and oestradiol concentrations that signified the termination of embryonic diapause, concentrations of both of these hormones gradually decreased. This pattern is similar to that reported for Antarctic fur seals (Boyd, 1991) and Northern fur seals (Daniels, 1981).

Having established that *N. cinerea* has an extended breeding cycle uncharacteristic of pinnipeds (Higgins, 1993; Gales *et al.*, 1994), and that these animals are aseasonal and asynchronous across their range (Gales *et al.*, 1994), the question arises as to what mechanism could be responsible for cueing implantation. The principal proximate factors that control the timing of birth in pinnipeds are thought to be photoperiod (Boyd, 1991;

Temte and Temte, 1993) and water temperature (Coulson, 1981). Temte and Temte (1993) have demonstrated a relationship between implantation and a photoperiod of 11.48 h day<sup>-1</sup>, which occurred 242 days prepartum in California sea lions. Coulson (1981) has also proposed an intricate model suggesting that grey seals are able to sum daily sea temperatures, implantation occurring at 800 'degree days'. In the case of *N. cinerea*, these models, and any other seasonally based model, are unable to predict implantation timing. We know of no other factors that can be identified as cueing breeding or implantation in *N. cinerea*. Gales and Costa (in press) provide a more detailed discussion of these issues.

Since *N. cinerea* has an extended postimplantation period, it might be expected that *N. cinerea* offspring will be more developed at birth than those of other pinnipeds. Assessment of this in terms of pup birth mass as a percentage of maternal mass suggested that this is not the case. Indeed, *N. cinerea* produce a pup of only 9% maternal body mass compared with  $10.7 \pm 2.6\%$  (mean  $\pm$  SD) for ten other otariids (see summary in Costa, 1991). Therefore, it seems likely that fetal development in *N. cinerea* is slower than in other pinnipeds, except perhaps the walrus which, in common with the Australian sea lion, has an extended postimplantation period (Fay, 1981). Such a reduced growth rate may represent an energetic advantage to female *N. cinerea*, which can spread the energetic cost of gestation over a longer period.

In conclusion, the hormone patterns of pregnancy in the Australian sea lion measured in the present study provide

evidence that the blastocyst reactivates and implants at between 3.5 and 5 months of gestation, suggesting an embryonic diapause of similar duration to that of other pinnipeds. The results also indicate that the species has a prolonged postimplantation period of up to 14 months, the longest postimplantation period of pregnancy recorded in any pinniped.

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