PLASMA PROGESTERONE CONCENTRATIONS MEASURED USING AN ENZYME-LINKED IMMUNOSORBENT ASSAY USEFUL FOR DIAGNOSING PREGNANCY IN HARBOR SEALS (PHOCA VITULINA)

K. J. Gardiner1,2,3
I. L. Boyd2
P. A. Racey1
P. J. H. Reijnders3
P. M. Thompson1

1Dept of Zoology, University of Aberdeen, Tillydrone Avenue, Aberdeen, AB9 2TN, Scotland.
2British Antarctic Survey, Natural Environment Research Council, High Cross, Madingley Road, Cambridge, CB3 0ET, U.K.
3Institute for Forestry and Nature Research, Zuiderhaaks 17, 't Horntje, P. O. Box 167, NL-1790 AD Denburg, Texel, Netherlands.

ABSTRACT

Wild-caught female harbor seals (Phoca vitulina) were classified as sexually mature or immature on the basis of standard body length (< 125 cm immature, > 125 cm mature) and plasma progesterone concentrations measured using an enzyme-linked immunosorbent assay (ELISA), a technique usable in the field. Sexually mature females were classified as pregnant or non-pregnant on the basis of their plasma progesterone concentrations. Of 28 wild mature female harbor seals caught in the Moray Firth, N.E. Scotland, between the end of February and the end of May, 79% had plasma progesterone concentrations greater than 60 nmol liter⁻¹, the lowest plasma progesterone concentration measured in one of eight females later observed with a pup, and were diagnosed as pregnant. A linear discriminant function, calculated to provide a method of distinguishing pregnant and non-pregnant females, predicted 100% of non-pregnant females and 95.8% of pregnant females using plasma progesterone concentration, standard length, and month of capture as parameters. Plasma progesterone concentrations were less than 30 nmol liter⁻¹ in all mature and immature males and immature females. In mature females plasma progesterone concentrations ranged from 0–318 nmol liter⁻¹.

Keywords: ELISA, progesterone, pregnancy, harbor seal, Phoca vitulina.
Previously, the determination of pregnancy rate, an important parameter in studies of population demography, was made from culled samples of a population. Behavioral and tracking studies had to rely on visual signs of the reproductive status of a marked individual. Progesterone is the hormone associated with the luteal phase of the estrous cycle and pregnancy in mammals and may be a useful indicator of pregnancy in pinnipeds. Among pinnipeds, which are characterized by two to five months delayed implantation during gestation (Daniel 1981), plasma progesterone concentrations vary in relation to the stage of gestation. This has been demonstrated in the phocids, grey seals (Halichoerus grypus), and harbor seals (Phoca vitulina) (Raeside and Ronald 1981; Boyd 1984, 1985; Reijnders 1990) and the otariids, northern and antarctic fur seals (Callorhinus ursinus and Arctocephalus gazella, respectively) (Daniel 1981, Boyd 1991). The pupping season of the harbor seal in the Moray Firth, N.E. Scotland, where part of this study was carried out, occurs in June and July. Lactation lasts approximately four weeks, at the end of which copulation occurs. Implantation occurs in October or November and is followed by an eight-month period of active gestation before the next pupping season.

Plasma progesterone is elevated towards the end of the period of active gestation (i.e., postimplantation) in phocids, probably as the result of placental synthesis of the steroid (Hobson and Boyd 1984). The duration of this increase towards the end of gestation has been given as six to seven weeks in harbor seals (Raeside and Ronald 1981), the final three to four months of gestation in harbor seals (Reijnders 1990), and the final month of gestation in grey seals (Boyd 1984).

The aims of this study were to investigate the use of plasma progesterone concentrations to diagnose pregnancy in individual harbor seals and to provide an estimate of the pregnancy rate for harbor seals in the sampled population.

Methods

Study Animals

Captive seals—Two captive harbor seals, both sexually mature females, were kept at the Institute for Forestry and Nature Research (I.B.N.-D.L.O.), Texel, Netherlands from October 1991 to December 1992. These seals which were on loan from Ecomare, a museum of natural history, Texel, were held in tanks (30 × 6 × 1.8 m) from which they were able to haul out onto a concrete platform (30 × 3 m). The tanks were supplied with running sea water (approximately 50 m³ h⁻¹).

Wild seals—Wild male and female, mature and immature harbor seals were caught in the Moray Firth area on haul-out sites at low tide either by approaching haul-out groups in an inflatable boat or a four-wheel-drive vehicle and catching animals individually, using hoop nets (Thompson et al. 1992); or by setting seine nets from the back of boats to catch seals as they entered the water (Norgaard et al. 1991).
Collection of Blood Samples

Captive seals—Single blood samples were collected from the two captive females at irregular intervals from January 1992 until September 1992. Animals were restrained on a V-shaped bench (Reijnders 1990). Blood samples (4–5 ml) were collected from the extradural vein or hind flipper into heparinized syringes, centrifuged for 15 min, and the plasma decanted and subsequently frozen at −20°C.

Wild seals—All wild seals (n = 168) were transferred from the nets to a flat restraining board and, in some cases, sedated lightly with tiletamine hydrochloride and zolazepam ("Zoletil", Reading, Z.A.C. France) (Thompson et al. 1992). The standard length of each individual was measured and the individual later classified as either mature (> 125 cm) or immature (< 125 cm). A blood sample was taken from the extradural vein of each seal using heparinized vacuum tubes (Vacutainers, Becton Dickinson, U.K. Ltd.) (Thompson et al. 1992) and treated in the same way as those taken from the captive animals. Plasma progesterone concentrations were determined for samples collected from wild harbor seals caught over four years (1988–1992), during two different periods of their reproductive cycle: months four to seven of active gestation (February–May) and the period of lactation/delayed implantation (June/August and September). A representative set of samples from wild immature females (n = 25), immature males (n = 15), and mature males (n = 17) caught between the end of February and the end of May were analyzed to determine baseline concentrations of plasma progesterone.

Hormone Assay

Samples were analyzed in duplicate against a progesterone standard curve using an enzyme-linked immunosorbent assay (ELISA) kit originally designed for use with human plasma (Progesterone Serozyme, Sereno Diagnostics Ltd, U.K.). The volumes of the substrates and samples were one-quarter of those normally prescribed in the instructions, to allow the use of a microtitre plate. However, the reagents were used in the concentrations at which they were supplied.

Progesterone standard curves were prepared by serial dilution in charcoal-stripped seal plasma and run in duplicate on each plate. The seal plasma was stripped by adding charcoal (Norit SX1G, Norit N.V., Netherlands) at 75 mg ml⁻¹ plasma. The solution was then mixed on a magnetic stirrer overnight at 4°C. The plasma was spun at 5,000 rpm for one hour and filtered twice through a 0.2-μm filter before being divided into aliquots and frozen. Distilled water was used as the blank in the assay in order to provide a value below the limits of detection. Charcoal-stripped seal plasma also gave a value below the limits of detection. Addition of a known amount of progesterone to a plasma sample which gave a low value in the assay (2.92 nmol liter⁻¹) gave a mean recovery of 104 ± 16.97%. A plasma sample which gave a high (318 nmol liter⁻¹) value in the assay was diluted in charcoal-stripped seal plasma at 1:1, 1:2, 1:4,
1:8, 1:16, 1:32, 1:64 and this gave a dilution curve which was parallel to the standard curve. The interassay coefficient of variation, used to determine the variation in results between assay plates, was 25.5% for quality controls of 8.5 ± 2.8 nmol liter⁻¹ and 37.6 ± 6.7 nmol liter⁻¹ run in duplicate at the start and end of each individual plate. The quality controls were plasma samples with progesterone concentrations at either end of the standard curve. The intra-assay coefficient of variation was 5.7%.

Seal plasma samples were defrosted and assayed directly. Plasma, 12.5 μl, was incubated at 37 ± 1°C for 45 min with 50 μl fluorescein-labelled steroid and 50 μl of enzyme-labelled monoclonal antibody. Subsequently, 50 μl anti-fluorescein coupled to a magnetic solid phase was added and incubated at 37 ± 1°C for 20 min. After magnetic separation, the supernatant was discarded and the sediment washed with 125 μl sodium phosphate buffer. The separation was repeated and the sediment incubated at 37 ± 1°C for 30 min with 75 μl enzyme substrate solution. The reaction was then stopped by adding 200 μl weak sodium hydroxide solution. Results were obtained by measuring the light absorbency of the supernatant at 550 nm using a Titercek photometer (Flow laboratories Ltd, Scotland). The unknown hormone concentrations were inversely proportional to the intensity of the color formed by the enzyme reaction and were determined from the standard curve, 0–318 nmol liter⁻¹.

Statistical Analysis

A linear discriminant function was calculated to provide an objective method of distinguishing pregnant and non-pregnant females, based on standard length, plasma progesterone concentration, and month of sampling.

RESULTS

Captive seals—One of the two captive seals produced a pup, which was born on 9 July 1992. Plasma progesterone concentrations in the non-pregnant female showed a decline throughout January, remained low in March, and by June had decreased to an undetectable level (Fig. 1). In contrast, the pregnant female showed an increase in plasma progesterone concentrations throughout January, varying through March, but remaining high until 17 June, the last sample taken before the pup was born. Progesterone was undetectable during lactation, 15 July–4 August. However, a sample taken one week after weaning on 12 August had a progesterone concentration of 60 nmol liter⁻¹, suggesting ovulation had already occurred.

Wild seals—Plasma progesterone concentrations of all female harbor seals caught between February and May were plotted against their standard length (Fig. 2). Based on the clear break in the progesterone concentrations, individuals < 125 cm were classified as sexually immature and individuals > 125 cm were classified as sexually mature. Of the 28 mature females, the lowest plasma
Figure 1. Plasma progesterone concentrations measured in single blood samples taken over nine months between January and September 1992 from two mature captive female harbor seals. The pregnant female is shown by • and the non-pregnant female is shown by ○.

Progesterone concentration measured from a sample of eight of them which were subsequently known to have pupped was 60 nmol liter\(^{-1}\). This progesterone concentration was therefore taken as the minimum required to sustain pregnancy. On this assumption 79% (\(n = 22\)) of mature females caught between February and May were classified as pregnant and 21% (\(n = 6\)) as non-pregnant. All immature females, immature males, and mature males had plasma progesterone concentrations less than 30 nmol liter\(^{-1}\) between February to May and June, August, and September (Fig. 3).
Figure 2. Scatterplot of plasma progesterone level on nose to tail length of all wild female harbor seals for which plasma progesterone concentrations were measured. Females which were subsequently observed with a pup are shown by □.

Discussion

Plasma progesterone concentrations in the latter stages of gestation in this study were in the range reported in previous studies. Reijnders (1990) found the mean progesterone concentration in eight pregnant captive harbor seals to be approximately 150 nmol liter$^{-1}$ in early June, i.e., approximately three weeks before the peak of pupping. Raeside and Ronald (1981) also found similar plasma progesterone concentrations (127–190 nmol liter$^{-1}$) in the last six to seven weeks of pregnancy in a captive harbor seal.

The minimum progesterone concentration of any of the sampled females subsequently observed with a pup determined a value for the lowest progesterone concentration necessary to sustain pregnancy in the final four months of gestation for the females in this study. Because the distribution of plasma progesterone concentrations required to maintain pregnancy is unknown, a proportion of females which were pregnant may have been classified incorrectly using this technique if they had a plasma progesterone concentration below 60 nmol liter$^{-1}$ and were not subsequently observed with a pup. However, evidence from the single non-pregnant captive female, together with the comparable low plasma progesterone concentrations measured in immature and male harbor seals, suggests that during the third trimester of pregnancy, non-pregnant females will have progesterone concentrations well below 60 nmol liter$^{-1}$. Progesterone concentrations increase towards the end of gestation in grey seals, probably as a result of placental progesterone (Hobson and Boyd 1984). If this is also the case in harbor seals, then the closer to parturition the test is carried out, the more accurate the pregnancy diagnosis is likely to be.

There was no independent confirmation of pregnancy for the females not
Figure 3. Plasma progesterone concentrations during two different time periods corresponding to the second half of gestation (February–May), and lactation/delayed implantation (June, August and September) in wild mature □ and immature □ male and mature ■ and immature ■ female harbor seals caught in the Moray Firth, N.E. Scotland.

observed with a pup but, based on the classification of females as pregnant or non-pregnant using progesterone concentrations as described above, a linear discriminant function was calculated to assist future researchers, once they have attained a plasma progesterone concentration, in deciding whether their females are pregnant or not. The linear discriminant function predicted 100% of non-pregnant females and 95.8% of pregnant females (Table 1). However, more
Table 1. Parameters for a linear discriminant function, defining to which of the two classes, pregnant or non-pregnant, each harbor seal belongs.

<table>
<thead>
<tr>
<th>Non-pregnant</th>
<th>Pregnant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant (K)</td>
<td>-41.47</td>
</tr>
<tr>
<td>X1</td>
<td>0.62</td>
</tr>
<tr>
<td>X2</td>
<td>-0.02</td>
</tr>
<tr>
<td>X3</td>
<td>3.85</td>
</tr>
</tbody>
</table>

Parameters calculated for a linear discriminant function such that \( D = K + X1 \) Lengths + \( X2 \) Progesterone + \( X3 \) Month. When applied to data for a particular individual whichever is the greater value of \( D \), non-pregnant or pregnant, defines the class to which the seal belongs. Months are numbered sequentially from the beginning of the calendar year.

Independent observations would be needed to determine the accuracy of this function for other populations and other subspecies of harbor seals.

A pregnancy rate of 0.79 is similar to that calculated for harbor seals in the Dutch Waddensea (Rejinders 1978) but lower than previously reported for Northeast Pacific harbor seals (0.88) (Bigg 1969) and for most other pinniped species except Weddell seals (Testa 1987) and antarctic fur seals (Lunn et al. 1994). In the Weddell seal and antarctic fur seal, pregnancy was determined using long-term observations of individuals over consecutive reproductive cycles. Other studies have relied mainly on pregnancy rates from culled samples of the population. It is often impossible to select randomly from a population during culling and, therefore, estimates of pregnancy rate based on such samples may be biased. It is possible that the method used to capture seals in the present study is less selective of pregnant females than culling.

Maintenance of elevated plasma progesterone (Fig. 1) is essential for the continuation of pregnancy in most mammals (Amoroso and Perry 1977), and this probably includes all seals (Daniel 1981, Raeside and Ronald 1981). However, before using this assay to diagnose pregnancy in other seal species, reference endocrine data for each species must be determined. For example, Boyd (1984) measured much lower plasma progesterone concentrations (32 nmol liter\(^{-1}\)) during the final month of gestation in a captive grey seal.

The applications of pregnancy determination by this method are numerous. The assay technique described here provides a method by which plasma progesterone concentrations can be determined in the field for up to 38 individual samples on one microtitre plate. Results can be obtained within three hours of the samples being collected, provided standards and quality controls are prepared in advance, and a centrifuge, incubator, and photometer (all of which are available as portable, battery-powered units) are available in the field. The method could be used for predicting the timing of ovulation and implantation in pinnipeds, once annual hormone profiles and the magnitude of change in plasma progesterone concentrations during ovulation and implantation have been described. Pregnancy determination could also provide an accurate means of determining pregnancy rates in population studies or as a means of establishing the reproductive status of marked individuals in behavioral or tracking studies.
ACKNOWLEDGMENTS

Karen Gardiner was funded by the Science and Engineering Research Council as part of a three-year research studentship. The field sampling was supported by a contract from the Scottish Office Agriculture and Fisheries Department. Thanks go to the staff of I.B.N., Texel, especially Pete Wim van Leeuwen without whose help the captive seal work could not have happened. Thanks also go to everyone who helped with the capture and handling of the wild seals in the Moray Firth.

LITERATURE CITED


Received: 27 October 1993
Accepted: 31 July 1993