Determining pregnancy status in harbour seals using progesterone concentrations in blood and blubber

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Abstract

Pregnancy status in harbour seals can be estimated from concentrations of progesterone in blubber as well as in blood samples, which are significantly higher in pregnant than non-pregnant animals. This study investigated the accuracy of estimating pregnancy rates using samples from live-captured and released harbour seals from three regions around Scotland, coupled with observed pregnancy outcomes. Concentrations of progesterone in blood (plasma) and blubber were obtained during the capture of animals early in the year (February to May). Individual animals were identified from the unique markings on their pelage, with a proportion (n=51) of females re-sighted during the subsequent breeding season and the reproductive outcomes determined (pregnant or possibly non-pregnant) during observations from long-term photo-identification studies. Generalised linear models with a binomial link function were fitted to training (60% of the data) and test datasets (40% of the data) to estimate pregnancy status from progesterone concentrations in blubber, plasma or both, and a received operating curves (ROC) approach was used to evaluate the performance of each classifier. The accuracy for the plasma concentrations was 85% with a high classification performance (as estimated from an area under the curve (AUC) of 0.82). The Youden method to determine the cut-point (threshold) and bootstrapping the training dataset resulted in a cut-point of 58 ng ml^{-1} (95^{th} percentiles, 25 – 102 ng ml^{-1}). For blubber, the accuracy was 77% (AUC=0.86) with an optimal cut-point of 56 ng g^{-1} (95^{th} percentiles, 26 – 223 ng g^{-1}). In the combined analysis (both blubber and plasma), the accuracy was 87.5% (AUC 0.81) with the cut-points of 72 ng ml^{-1} (95^{th} percentiles, 25 – 103 ng ml^{-1}) in plasma and 56 ng g^{-1} (95^{th} percentiles, 26 – 223 ng g^{-1}) in blubber. These thresholds were then used to estimate the pregnancy proportions among adult females at the three study sites, including those that were not included in the photo-id studies. Proportions were high at all sites, (63% - 100%) regardless of which matrices were used and were not statistically significantly different from each other but suggested that analysing concentrations in both sample matrices would minimise the uncertainty.

Introduction

Estimating the proportion of adult females that are pregnant in a population is a central parameter for understanding and predicting its population dynamics (Schaub and Abadi, 2011), determining the effect of natural and anthropogenic stressors (McHuron et al., 2018; Pallin et al., 2018b) and planning conservation management strategies (Freeman et al., 2014). Ideally, estimates are coupled with studies to determine reproductive outcome and thus fecundity, which, when estimated over a lifetime, is one of the major foundations of population biology (Bradshaw and McMahon, 2008). Whilst pregnancy rates (i.e. the proportion of pregnant females in the population at a given time) are not as informative as fecundity rates (i.e. the proportion of females that give birth to a viable pup), estimates can provide critical information about factors driving a population decline since low

rates may indicate where impacts are occurring and could narrow the field of issues to be investigated.

The abundance of harbour seals (*Phoca vitulina*) in some regions of Scotland has been declining dramatically over the past 20 years or so (Thompson et al., 2019). Whilst the reasons for these changes in population size are not known, the factors driving such changes must be acting on survival, fecundity, causing permanent emigration, or it must be due to a combination of these. It is therefore critical that robust estimates of vital rates are obtained from populations with differing population trajectories (Saether et al., 2004) so that causal hypotheses for the declines can be tested (SCOS, 2019). Estimating such demographic parameters in marine mammals with sufficient accuracy is often logistically difficult and costly. However, for harbour seals this can be achieved through long term individual-based photo-identification (photo-id) studies (Cordes and Thompson, 2015; Mackey et al., 2008) and through tracking individuals using telemetry tags (Sharples et al., 2012). Photo-id data are used in a mark-recapture model framework to estimate survival, fecundity and site fidelity but several years of data are required before reliable rates are available, especially in slow-growing long-lived species such as phocids. The live-capture release of individual animals can therefore assist in providing information on the reproductive status of females sampled from populations with different trajectories. Here the proportion of adult females that were pregnant in three Scottish populations with different dynamics was investigated. The population in Orkney declined by 46% between 2001 and 2006 and since then has continued to decrease at approximately 10% per annum (Thompson et al., 2019). The population on Skye has been stable over the same period as has the population in the wider Moray Firth (Thompson et al., 2019), although the population in Loch Fleet has been increasing (Graham et al., 2017).

Circulating concentrations of progesterone can be used to indicate pregnancy as significantly higher concentrations are seen in pregnant compared to the non-pregnant females (Gardiner et al. (1996). The gestation period in harbour seals is approximately 11 months, which includes a 2-3 month period of embryonic diapause. Reijnders (1990) described the longitudinal endocrine patterns in the monoestrus harbour seal throughout the year in pregnant compared to non-pregnant and immature animals. A peak in oestradiol, indicating ovulation, was seen in July, with implantation probably occurring approximately three months later. This was followed by elevated concentrations of progesterone in pregnant females which rose significantly in the last three to four months of gestation, around late February to late May. In non-pregnant females, progesterone declined significantly during the same period and in immature animals remained low throughout the year. Circulating concentrations of progesterone are therefore influenced by stage of gestation and the threshold at which pregnancy is diagnosed may not be consistent between assays and studies. For example, Gardiner et al. (1996) suggest that a minimum circulating concentration of 18 ng ml^{-1} is necessary to sustain pregnancy. However, Greig (2002) reported that female harbour seals from Monterey Bay in California with plasma concentrations >8 ng ml^{-1} had a 95% probability of being pregnant and those with concentrations < 7 ng ml^{-1} had a 95% probability of not being pregnant. In addition to relying on these circulating levels, it is possible to extract steroid hormones from blubber tissue to determine the concentration of these lipophilic hormones in biopsy samples. This approach has now been used successfully on many cetacean species (Perez et al., 2011; Trego et al., 2013) and California sea lions (*Zalophus californianus*) (Beaulieu-McCoy et al., 2017) in which concentrations of steroid hormones have been reliably determined in blubber but to our knowledge, no investigations in phocid seals have been reported.

The objectives of this study were therefore to (1) establish a threshold concentration of progesterone in harbour seals to determine pregnancy status, using both circulating concentrations

and concentrations in blubber biopsies from the same individuals, in combination with observations of reproductive output during the breeding season and (2) to compare pregnancy rates in mature harbour seals in populations with contrasting trajectories in order to better understand where the drivers for population change may be acting.

Materials and Methods

Harbour seals were captured, sampled and released at haulout sites in three of the Seal Management Areas in Scotland (SCOS, 2019); the Moray Firth (Loch Fleet, Ardersier and the Dornoch Firth), West Scotland (Isle of Skye) and North Coast and Orkney (Orkney and Pentland Firth). Captures were conducted between February and May 2015-2018, before the breeding season (June and July in those areas), and in accordance with the Animal (Scientific Procedures) Act, 1986, Home Office Licence issued to the Sea Mammal Research Unit (Licence No. 192CBD9F) following approval by the University of St Andrews Animal Welfare and Ethics Committee. This period encompasses the mid to late-gestation periods and the third trimester for harbour seals when progesterone concentrations in pregnant females are expected to be at least twice the concentration seen in non-pregnant and immature animals (Reijnders, 1990, Gardiner et al., 1996). The number of animals sampled for this study by region and haulout site location are given in Table 1. This included juvenile as well as adult females (as determined by their mass and length).

Table 1. Female harbour seals sampled for pregnancy status determination by Seal Management Area and location.

Seal Management Area	**Location**	**No animals**
Moray Firth	Loch Fleet	37
	Ardersier	6
	Dornoch Firth	6
North coast and Orkney	Scapa Flow	24
	Pentland Firth	16
West Scotland	Loch Dunvegan, Isle of Skye	15
Total		**104**

Seal captures were attempted at sites where photo-id were collected during the breeding season to increase the likelihood of future observations and hence determine reproductive outcome for some of the mature females sampled. Where this was not possible because the haulout sites were only used during the breeding season, seals were captured at adjacent sites. Animals were captured using seine nets and were sedated with Zoletil 100 (Virbac, Carros, France) at a dose rate of 0.1mg/kg body mass. Animals were weighed, measured (nose-tail standard length and axillary girth) and blood samples were collected from the extradural vein using the Vacutainer system (Becton Dickinson, Franklin Lakes, USA) and the plasma was collected following centrifugation (n=103). Blubber biopsy samples were also taken from some individuals at the pelvic region using sterile 4mm biopsy punches (Acuderm inc., Ft Lauderdale, USA) and all samples were stored at -20^{o}C until analysis (n=79). Thus 78 paired blood and blubber samples were available, and one individual provided a blubber but not a blood sample.

Progesterone concentrations

Blood samples were analysed using a commercially available progesterone ELISA (DRG International Inc, Springfield, USA) and assay reliability was assessed by parallelism of a diluted sample with the standard curve from the kit (see Supplementary material). The *inter*- and *intra*-assay coefficients of variation (CVs) were 16.5% and 5.6% respectively.

Steroid hormones were extracted from the blubber samples following the method of Kellar et al. (2006) and as applied to harbour seal samples by Kershaw and Hall (2016). Briefly, 0.1 g blubber tissue from each animal was homogenised using an Omni tissue homogeniser fitted with a disposable soft tissue homogenizing tip (Omni International, Kennesaw, Georgia, USA) in 1 ml ethanol for 3 mins. Samples were vortexed at high speed for 2 mins and centrifuged at 3,000 *rcf* for 10 mins. The supernatant was evaporated under a stream of dry nitrogen while incubating at 25°C. Ethanol:acetone (2ml at a ratio of 4:1) was added to the residue. Diethyl ether (1ml) was added after further evaporation under dry nitrogen, followed by extraction and evaporation with acetonitrile (1 ml) and hexane (1 ml) with 3 min vortexing and 20 min centrifugation at at 3,000 *rcf* between extractions. The solvents formed two immiscible layers with hexane on the top. The hexane layer was removed, and the acetonitrile layer re-extracted with a further 1 ml hexane. The final acetonitrile layer was aspirated and evaporated as before and the final residue was dissolved in phosphate buffered saline containing 1% bovine gamma globulin and assayed using the ELISA kit. The *inter*- and *intra*-assay coefficients of variation were 11.7% and 6.9% respectively. Reliability was assessed from parallelism with the standard curve (see Supplementary material).

Photo-identification

Photographs of the pelage of the captured seals were taken and incorporated in the catalogue of photographs developed at each site for the long-term individual-based, mark-recapture studies to estimate survival and fecundity rates (Arso Civil et al., 2016; Cordes and Thompson, 2014; Thompson and Wheeler, 2008). Following the method of Thompson and Wheeler (2008), photographs taken during captures were matched with those taken during the subsequent breeding season. The reproductive outcome of sampled females recognised during the breeding season was recorded as 'pregnant' if observed pregnant and/or with a pup (females might be observed pregnant as the major change in body shape is generally noticeable during late gestation, but might subsequently move to a non-monitored site before giving birth), or as possibly 'non-pregnant' if neither a pregnancy nor a pup were observed or their status could not be determined from a photograph. This may have introduced some misclassification, as categorisation was dependent on these observations. If females were not seen again after capture and release, their status was recorded as unknown.

Determining pregnancy thresholds from plasma and blubber

To investigate the relationship between plasma and blubber progesterone concentrations from all the samples, standard major axis (SMA) regression was used after log transforming the data due to negatively skewed distributions of the progesterone concentrations and measurement error.

Training datasets comprising of plasma and/or blubber samples from juvenile and adult females for which the reproductive output was established as 'pregnant' or 'non-pregnant' during the breeding season were then used to determine the progesterone concentrations thresholds that best predict pregnancy in both matrices. Generalised linear models (glm) with a binomial link function were fitted, in which pregnancy status (pregnant or non-pregnant, based on the observed reproductive output) was the response variable and concentration of progesterone in blood, blubber or both

matrices was the explanatory variable. In order to account for any bias attributable to inter-assay variability, the ELISA plate number was included in the model as an independent variable. In addition, because samples were collected at different stages prior to parturition, the sample collection date was also included in the model. Other predictor variables included were the mass, length and girth of the individuals. The best predictive model was then determined using Akaike's An Information Criterion (AIC) and the *step* function in R in which the most parsimonious model was chosen from the minimal AIC value among the models. A Received Operating Curves (ROC) approach was used to evaluate the sensitivity (the proportion of correctly classified pregnant females) and specificity (the proportion of correctly classified non-pregnant females) of the classifier (Zweig and Campbell, 1993). The method also provides with an Area Under the Curve (AUC), a measurement of the performance of the classifier, with AUC = 0.5 for a classifier with no predictive power and AUC = 1 for a perfect classifier. Pregnancy determination thresholds for plasma and blubber progesterone concentrations were evaluated separately and then jointly. Analyses were carried out using the packages *caret* (Kuhn, 2008), *pROC* (Robin et al., 2011) and *cutpointr* (Thiele, 2019) in the statistical program R (R Development Core Team, 2019). The specificity and sensitivity estimates are also reported as the number of false positives and false negatives within the dataset for each of the matrices alone and for when both agreed. Success rates using the ROC curves applied to the unknown status dataset were evaluated from the optimised sensitivity and specificity.

Estimating the proportion of pregnant females in different study sites

The proportion of seals in each study population that were pregnant or not pregnant were calculated using the established thresholds for the plasma and blubber results and for both matrices where the reproductive status agreed. Binomial proportions were calculated with associated 95% confidence intervals and were compared among locations using a Chi Squared test. Only results from adult, mature females, ≥130 cm nose-tail length (Hall et al., 2019) were included in this analysis (n=85, mean length = 141.4, SD ± 5.1 cm, mean mass = 86.2, SD ± 8.9 kg).

Results

Relationship between plasma and blubber progesterone

Plasma and blubber samples were obtained for 103 and 79 out of the 104 captured females, respectively. The concentrations of progesterone in all the female plasma and blubber are summarised in Table 2, together with the mass of the females sampled (adult and juvenile). Progesterone concentrations in both matrices displayed negatively skewed distributions and therefore analysis was carried out following $\log_{10}$ transformation. Standard major axis (SMA) regression was used because both variables contain measurement error and to explore the true relationship between progesterone concentrations in the two matrices collected at the same time (n=78, linear model, p=<0.00001, R^2=0.19, intercept = -0.74, slope =0.985, Fig. 1.).

Table 2. Summary of all harbour seal female mass and progesterone concentrations in blubber and plasma samples.

	Mean	Median	Min	Max	n
Mass (kg)	78.2	82.6	21.8	110.7	103
Plasma progesterone (ng ml^{-1})	76.7	76.4	1.3	281.7	103
Blubber progesterone (ng g^{-1})	285.4	104.6	7.7	3261.7	79

208 **Thresholds for pregnancy estimation**

209 Of the sampled females, the reproductive status of 51 was subsequently observed during the
210 breeding season. They were classed as 'pregnant' (observed pregnant and/or with pup, n=29) or
211 presumed 'non-pregnant' (n=22) at either the Loch Fleet photo-id study site in the Moray Firth
212 (Thompson and Wheeler, 2008) or Scapa Flow, Orkney. Progesterone concentrations in plasma were
213 available for all individuals (n=51). Blubber concentrations were available for 41 animals, for which
214 plasma concentrations were also available. The dataset was then split into training and test sets in
215 which 60% of the data were used for training and 40% for testing.

216 Plasma progesterone concentrations alone were first assessed for their ability to correctly classify
217 animals as pregnant or not pregnant (Fig 2a). The binomial glm (Pregnancy status ~ plasma
218 progesterone + sampling date + plate ID + length + axillary girth + mass) was fitted to a randomly
219 selected training dataset (n=31). Model selection indicated that plasma progesterone was a
220 significant predictor, but that none of the other variables (the sampling date, plate ID, mass, length
221 or girth) were individually significant ($p>0.05$) and none improved the prediction (AIC for full model =
222 40.17). These were therefore removed from the model and the simplest model, pregnancy status ~
223 plasma progesterone was used in the subsequent analyses ($p=0.005$, AIC = 34.30). This model was
224 then compared with how correctly it classified the samples from the remaining test dataset (n=20).
225 The resulting confusion matrix estimated an accuracy of 0.85 (95% CI 0.621, 0.968) with two false
226 positives and one false negative. The sensitivity estimate was 0.9 and specificity 0.727
227 (AUC=0.8227).

228 In order to estimate the uncertainty in the cut-point or threshold values, the data were sampled
229 without replacement into a 60% training dataset 500 times and cut-point estimated. The median
230 optimal cut-point using the Youden method was 58 ng ml^{-1} (95^{th} percentiles, 25 - 102 ng ml^{-1}).

231 A similar model was fitted to the same training dataset for blubber progesterone (Fig 2b). As for the
232 plasma, only blubber progesterone was retained in the model, AIC full model = 34.31 as the best
233 predictor ($p=0.045$, AIC reduced model = 31.61). This resulted in an accuracy of 0.769 (95% CI 0.462,
234 0.949) when compared to the test data, with one false negative and two false positives. The
235 sensitivity was 0.895 and the specificity 0.778 (AUC = 0.860). The median optimal cut-point was 56
236 ng g^{-1} (95^{th} percentiles, 26 – 223 ng g^{-1}).

237 Fitting a model with both concentrations of progesterone (pregnancy status ~ plasma progesterone
238 + blubber progesterone) to the training dataset (n=25 for the paired samples) gave an accuracy of
239 0.875 (95% CI 0.616, 0.985) which when applied to the remaining test dataset (n=16) resulted in two
240 false positives. The sensitivity for the model with two predictors was 0.667 and the specificity 1.0
241 (AUC = 0.81). For the plasma progesterone the median threshold concentration in the joint ROC
242 using both blood and blubber as predictors was 72 ng ml^{-1} (95^{th} percentiles, 25 - 103 ng ml^{-1}). For the
243 blubber progesterone the median in the joint model was 56 ng g^{-1} (95^{th} percentiles, 26 - 223 ng g^{-1}).

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246 **Pregnancy proportions in harbour seals from the Moray Firth, Orkney and the Isle of Skye**

247 The proportion (as a percentage) of mature harbour seal females in each study population that were
248 categorised as pregnant or not pregnant, using the thresholds estimated above, are given in Table 3.

The results using plasma (n=85) or blubber (n=71) individually and both matrices where the results agreed (n=58) are shown.

Table 3. Number and percentage (with 95% binomial confidence intervals) of mature females categorised as not pregnant or pregnant by Seal Management Area.

	Not Pregnant						Pregnant					
	Plasma		Blubber		Both		Plasma		Blubber		Both	
Seal Management Area	No/ Total	%, 95% CI	No/ Total	%, 95% CI	No/ Total	%, 95% CI	No/ Total	%, 95% CI	No/ Total	%, 95% CI	No/ Total	%, 95% CI
Moray Firth	8/38	21, 1-37	9/24	38, 19-59	4/19	21, 6-45	30/38	79, 62-90	15/24	63, 41-81	15/19	82, 57-96
North Coast and Orkney	13/37	35, 20-52	10/37	27, 14-44	6/30	20, 7-39	24/37	65, 47-80	27/37	73, 56-86	24/30	80, 61-92
West Scotland	2/10	10, 2-55	0/10	0, 0-31	0/9	0, 0-33	8/10	79, 62-90	10/10	100, 69-100	9/9	100, 66-100

The proportion of animals categorised as pregnant was high in all regions (>60% depending on the matrix). Proportions were highest in the mature females from the Isle of Skye using either plasma or blubber diagnostic approaches and including the paired samples 'agreed' results. However, there was no statistically significant difference in the proportions among areas due to the small sample sizes and wide confidence intervals. Thus, the use of either plasma or blubber gave very similar results although the paired 'agreed' samples resulted in a slightly higher proportion of females being categorised as pregnant. The largest discrepancy was seen in the proportions estimated using blubber in the Moray Firth where almost twice the proportionate number of females were diagnosed as non-pregnant from blubber (9/24, 38%) than plasma (8/38, 21%). However, one of the 9 females had a blubber concentration of progesterone just below the threshold (55 ng g^{-1}). Such large differences are also a consequence of small sample sizes.

Discussion

This study is the first to estimate pregnancy proportions in phocid seals using progesterone concentrations in both blubber and plasma. The utility of using progesterone concentrations in plasma samples for estimating reproductive status in seals has been well established (Gardiner et al., 1996) and concentrations in blubber from remote biopsy samples in cetaceans to determine reproductive status is now being widely used (Pallin et al., 2018a; Trego et al., 2013). However, very few studies have compared the results from plasma, for which the analytical methods were initially developed and verified (Elder et al., 1987), with those from blubber samples. A recent study by Beaulieu-Mccoy et al. (2017) evaluated progesterone in the blubber of an otariid, the California sea lion (*Zalophus californianus*), as a means of determining pregnancy status, but concentrations in paired blood samples were not available. However, Mingramm et al. (2019) found progesterone concentrations in captive bottlenose dolphins (*Tursiops truncatus*) where patterns, in relation to reproductive status, were similar between serum and blubber and were significantly elevated in pregnant females. Interestingly, the blubber progesterone concentrations were lower than the serum progesterone concentrations (recognising that these concentrations are not directly comparable due to the differences between the matrices) which is the opposite to our findings,

where blubber progesterone concentrations were much higher than those in plasma. The authors suggest this may be because samples were collected during the first trimester or because the extraction efficiency was low due to method differences. Other studies have reported significantly higher concentrations of progesterone in fat tissue (adipose) compared to plasma during pregnancy. For example, concentrations in tailhead adipose in cattle were two orders of magnitude higher than in plasma during pregnancy but were an order of magnitude lower during lactation (Hamudikuwanda et al., 1996). Kellar et al. (2013) reported similar results in bowhead whales (*Balaena mysticetus*) to those reported here and concentrations in blubber were significantly higher at all stages of the reproductive cycle than in serum.

A significant positive linear relationship between progesterone in plasma and blubber was found, also reported by Kellar et al., (2013) for serum and blubber in bowhead whales but only for the pregnant females. The authors found that the relationship was not significant when pregnant animals were excluded. This was not the case in this study of harbour seals. Standardised major axis (SMA) regression was used to explore the true relationship between progesterone levels in two matrices (as compared to using linear regression when the objective is to investigate predictive variables). As expected, when concentrations of progesterone were high in the plasma, they were generally also high in the blubber. However, there was a large degree of scatter around this relationship. This is likely due to the nature of tissues, with blubber sequestering progesterone from the circulation at different rates. In addition, pulsatile secretion of progesterone may account for some of the difference in concentration (Rahman et al., 2019). Nonetheless, either sample matrix was useful for determining reproductive status. Although the highest accuracy was found when both matrices were analysed (0.875, 95% CI 0.616, 0.985), similar accuracy was obtained using plasma concentrations alone (0.85, 95% CI 0.621, 0.968), a difference of 2.5% which, depending on the objectives of the study, may not justify the collection and analysis of both matrices. In addition, a recent study by Galligan et al. (2020) found that other hormones, particularly 17-hydroxyprogesterone and androstenedione, were also significant predictors of pregnancy in bottlenose dolphins.

The progesterone concentrations in the two matrices, plasma and blubber, were combined with visual observations of reproductive outcome. This allowed for a non-lethal training dataset to be used for the evaluation of the predictive power of the concentration thresholds obtained from the plasma and/or blubber results. Other studies have verified pregnancy outcomes against progesterone concentrations in blood or blubber by subsequent observation of the females with offspring (Perez et al., 2011) and or have compared circulating progesterone concentrations with ultrasound assessment of gestational stage (Shero et al., 2018). These approaches all allow the sensitivity and specificity of the concentration thresholds to be determined. The results presented here indicate that both plasma and blubber progesterone can discriminate between pregnant and non-pregnant harbour seal females with levels of sensitivity and specificity that would be acceptable in a clinical diagnostic situation (AUC both > 0.8, Šimundić, 2009).

When the reproductive outcome accuracies from the confusion matrices were compared, results obtained from both plasma and blubber resulted in a slightly higher accuracy of 87% (compared with 85% for the plasma and 77% for the blubber alone). All the samples were collected between February and May, which is between approximately 2 - 5 months before the breeding season. Samples obtained during captures in February were less accurate as the animals were not observed until late June to early July (within the breeding season), during which time they may have aborted their pup before the observation period.

The threshold concentration for plasma using the ROC method was estimated at ~58 ng mL^{-1}. This is higher than the minimum estimate of ~19 ng mL^{-1} in plasma suggested by Gardiner et al (1996). This concentration was suggested as being the level required to maintain the pregnancy during the last trimester. This threshold was similar to that observed by Reijnders (1990) of around 13 ng mL^{-1} as the maximum concentration observed in the non-pregnant and immature animals. Raeside and Ronald (1981) studied circulating concentrations of progesterone in late gestation and found concentrations were >40 ng ml^{-1} around 2 months before parturition. As demonstrated in these studies, progesterone concentrations increase toward the end of pregnancy, so the closer to parturition the samples are collected, the more accurate the diagnosis is likely to be. In addition, it is likely that thresholds will differ depending on the assay kit used. For example, Greig (2002) found that female harbour seals from Monterey Bay in California with progesterone concentrations >7 ng mL^{-1} had a 95% probability of being pregnant. This discrepancy highlights the need, where possible, to determine the threshold for a given species and assay kit combination.

Blubber progesterone concentrations have not been routinely measured in pinnipeds, with only one study in otariids (Beaulieu-Mccoy et al., 2017). However, there have been several studies investigating the use of blubber progesterone to estimate pregnancy rates in cetaceans (Kellar et al., 2006; Mansour et al., 2002; Perez et al., 2011, Galligan et al., 2020). The results presented here indicate that concentrations in the blubber of > 56 ng g^{-1} are indicative of pregnancy. This is similar to the concentrations found in three delphinid species, in which the maximum concentrations in the non-pregnant and immature animals was ~50 ng g^{-1} (Kellar et al., 2006). In minke whales (*Balaenoptera acutorostrata*) it was only ~3.5 ng g^{-1} (Mansour et al., 2002) and in the study by Perez et al. (2011) that included six cetacean species, the maximum in the non-pregnant mature animals was also around 35 ng g^{-1}. However, none of these studies investigated the relationship between blood and blubber progesterone and although Mingramm et al. (2019) reported progesterone in serum and blubber from eleven captive bottlenose dolphins, the relationship between paired samples was not shown.

Other studies have combined the concentrations of hormones in various matrices with morphometric measurements in a discriminant analysis (DFA) to determine their ability to collectively diagnose pregnancy. Burgess et al. (2012) showed that faecal progesterone metabolite data, together with body morphometrics from cross-validated results using ultrasound, the DFA correctly classified 100% of the pregnant and non-pregnant female dugongs. This analysis produced additional morphometric thresholds which could be used in conjunction with the faecal progesterone threshold to diagnose pregnancy in free-ranging dugongs.

The utility of estimating pregnancy status in female harbour seals is observed when the rates are compared among the different regions and Seal Management Areas. All areas had high proportions of pregnant females although significant differences among proportions could not be established due to the small sample sizes. Gardiner et al. (1996) reported that 79% of the mature females caught in the Moray Firth between February and May, 1988-1992, were pregnant and 21% were not pregnant. This is the same as the proportion reported here (79% were also pregnant based on plasma samples). Fecundity rates for the population in Loch Fleet are currently estimated at 0.89 (0.75-0.95) for multiparous and 0.69 (0.64-0.74) for ≥3 y olds (Cordes and Thompson, 2014). The most reliable estimate of pregnancy indicates fecundity rate of 0.82, perhaps suggesting a low rate of pup loss between the sample collection and the breeding season. The highest pregnancy rate was seen at the Isle of Skye, a region where the population appears to have been stable for a number of years (Thompson et al., 2019) and this was similar to the high proportion reported by Greig (2002) in Monterey Bay where 90% (35/39) of the females caught between September and March were

374 pregnant. For the Isle of Skye study, it was 100%, but this was only based on 9 or 10 animals.
375 Further studies are underway investigating the causes of the decline in harbour seal numbers in
376 areas of Scotland, including Orkney and the east coast. These results might suggest that it is unlikely
377 to be driven by changes in pregnancy proportions, however the sample size is too small to make any
378 robust conclusions at this stage.

379 This study verifies the reliability of using progesterone concentrations in blubber or blood to
380 estimate pregnancy status in phocid seals. The results therefore indicate that where it is not
381 possible to collect blood samples, blubber biopsies (from live or freshly dead carcasses) could also be
382 used as an alternative matrix in this species. In addition, the closer the sampled seals are to
383 parturition, the more reliable the results will be as the concentrations of progesterone rise sharply
384 during the last trimester in order to maintain the uterus.

Acknowledgements

The authors would like to thank all the staff and students who helped collect the samples in the field, particularly Simon Moss, Matt Bivins, Paul Thompson, Isla Graham and Tim Cândido Barton. This study was made possible through funding from the UKRI Natural Environment Research Council (grant numbers SMRU10001 and NE/R015007/1), the Scottish Government (grant number MMSS/002/15), Beatrice Offshore Wind Ltd. (BOWL), Moray Offshore Renewables Ltd. (MORL), Marine Scotland Science, The Crown Estate and Highlands and Island Enterprise.

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