

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

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7 **Abstract**

8 Pregnancy status in harbour seals can be estimated from concentrations of progesterone in blubber
9 as well as in blood samples, which are significantly higher in pregnant than non-pregnant animals.
10 This study investigated the accuracy of estimating pregnancy rates using samples from live-captured
11 and released harbour seals from three regions around Scotland, coupled with observed pregnancy
12 outcomes. Concentrations of progesterone in blood (plasma) and blubber were obtained during the
13 capture of animals early in the year (February to May). Individual animals were identified from the
14 unique markings on their pelage, with a proportion (n=51) of females re-sighted during the
15 subsequent breeding season and the reproductive outcomes determined (pregnant or possibly non-
16 pregnant) during observations from long-term photo-identification studies. Generalised linear
17 models with a binomial link function were fitted to training (60% of the data) and test datasets (40%
18 of the data) to estimate pregnancy status from progesterone concentrations in blubber, plasma or
19 both, and a received operating curves (ROC) approach was used to evaluate the performance of
20 each classifier. The accuracy for the plasma concentrations was 85% with a high classification
21 performance (as estimated from an area under the curve (AUC) of 0.82). The Youden method to
22 determine the cut-point (threshold) and bootstrapping the training dataset resulted in a cut-point of
23 58 ng ml⁻¹ (95th percentiles, 25 – 102 ng ml⁻¹). For blubber, the accuracy was 77% (AUC=0.86) with an
24 optimal cut-point of 56 ng g⁻¹ (95th percentiles, 26 – 223 ng g⁻¹). In the combined analysis (both
25 blubber and plasma), the accuracy was 87.5% (AUC 0.81) with the cut-points of 72 ng ml⁻¹ (95th
26 percentiles, 25 – 103 ng ml⁻¹) in plasma and 56 ng g⁻¹ (95th percentiles, 26 – 223 ng g⁻¹) in blubber.
27 These thresholds were then used to estimate the pregnancy proportions among adult females at the
28 three study sites, including those that were not included in the photo-id studies. Proportions were
29 high at all sites, (63% - 100%) regardless of which matrices were used and were not statistically
30 significantly different from each other but suggested that analysing concentrations in both sample
31 matrices would minimise the uncertainty.

32

33 **Introduction**

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

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

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

65 Circulating concentrations of progesterone can be used to indicate pregnancy as significantly higher
66 concentrations are seen in pregnant compared to the non-pregnant females (Gardiner et al. (1996).
67 The gestation period in harbour seals is approximately 11 months, which includes a 2-3 month
68 period of embryonic diapause. Reijnders (1990) described the longitudinal endocrine patterns in
69 the monoestrus harbour seal throughout the year in pregnant compared to non-pregnant and
70 immature animals. A peak in oestradiol, indicating ovulation, was seen in July, with implantation
71 probably occurring approximately three months later. This was followed by elevated concentrations
72 of progesterone in pregnant females which rose significantly in the last three to four months of
73 gestation, around late February to late May. In non-pregnant females, progesterone declined
74 significantly during the same period and in immature animals remained low throughout the year.
75 Circulating concentrations of progesterone are therefore influenced by stage of gestation and the
76 threshold at which pregnancy is diagnosed may not be consistent between assays and studies. For
77 example, Gardiner et al. (1996) suggest that a minimum circulating concentration of 18 ng ml⁻¹ is
78 necessary to sustain pregnancy. However, Greig (2002) reported that female harbour seals from
79 Monterey Bay in California with plasma concentrations >8 ng ml⁻¹ had a 95% probability of being
80 pregnant and those with concentrations < 7 ng ml⁻¹ had a 95% probability of not being pregnant. In
81 addition to relying on these circulating levels, it is possible to extract steroid hormones from blubber
82 tissue to determine the concentration of these lipophilic hormones in biopsy samples. This
83 approach has now been used successfully on many cetacean species (Perez et al., 2011; Trego et al.,
84 2013) and California sea lions (*Zalophus californianus*) (Beaulieu-McCoy et al., 2017) in which
85 concentrations of steroid hormones have been reliably determined in blubber but to our knowledge,
86 no investigations in phocid seals have been reported.

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

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

93 **Materials and Methods**

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

106 Table 1. Female harbour seals sampled for pregnancy status determination by Seal Management
107 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

108

109 Seal captures were attempted at sites where photo-id were collected during the breeding season to
110 increase the likelihood of future observations and hence determine reproductive outcome for some
111 of the mature females sampled. Where this was not possible because the haulout sites were only
112 used during the breeding season, seals were captured at adjacent sites. Animals were captured
113 using seine nets and were sedated with Zoletil 100 (Virbac, Carros, France) at a dose rate of
114 0.1mg/kg body mass. Animals were weighed, measured (nose-tail standard length and axillary girth)
115 and blood samples were collected from the extradural vein using the Vacutainer system (Becton
116 Dickinson, Franklin Lakes, USA) and the plasma was collected following centrifugation (n=103).
117 Blubber biopsy samples were also taken from some individuals at the pelvic region using sterile 4mm
118 biopsy punches (Acuderm inc., Ft Lauderdale, USA) and all samples were stored at -20°C until
119 analysis (n=79). Thus 78 paired blood and blubber samples were available, and one individual
120 provided a blubber but not a blood sample.

121

122 *Progesterone concentrations*

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

127 Steroid hormones were extracted from the blubber samples following the method of Kellar et al.
128 (2006) and as applied to harbour seal samples by Kershaw and Hall (2016). Briefly, 0.1 g blubber
129 tissue from each animal was homogenised using an Omni tissue homogeniser fitted with a
130 disposable soft tissue homogenizing tip (Omni International, Kennesaw, Georgia, USA) in 1 ml
131 ethanol for 3 mins. Samples were vortexed at high speed for 2 mins and centrifuged at 3,000 *rcf* for
132 10 mins. The supernatant was evaporated under a stream of dry nitrogen while incubating at 25°C.
133 Ethanol:acetone (2ml at a ratio of 4:1) was added to the residue. Diethyl ether (1ml) was added
134 after further evaporation under dry nitrogen, followed by extraction and evaporation with
135 acetonitrile (1 ml) and hexane (1 ml) with 3 min vortexing and 20 min centrifugation at at 3,000 *rcf*
136 between extractions. The solvents formed two immiscible layers with hexane on the top. The
137 hexane layer was removed, and the acetonitrile layer re-extracted with a further 1 ml hexane. The
138 final acetonitrile layer was aspirated and evaporated as before and the final residue was dissolved in
139 phosphate buffered saline containing 1% bovine gamma globulin and assayed using the ELISA kit.
140 The *inter*- and *intra*-assay coefficients of variation were 11.7% and 6.9% respectively. Reliability was
141 assessed from parallelism with the standard curve (see Supplementary material).

142 *Photo-identification*

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

156 *Determining pregnancy thresholds from plasma and blubber*

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

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

166 matrices was the explanatory variable. In order to account for any bias attributable to inter-assay
 167 variability, the ELISA plate number was included in the model as an independent variable. In
 168 addition, because samples were collected at different stages prior to parturition, the sample
 169 collection date was also included in the model. Other predictor variables included were the mass,
 170 length and girth of the individuals. The best predictive model was then determined using Akaike's An
 171 Information Criterion (AIC) and the *step* function in R in which the most parsimonious model was
 172 chosen from the minimal AIC value among the models. A Received Operating Curves (ROC)
 173 approach was used to evaluate the sensitivity (the proportion of correctly classified pregnant
 174 females) and specificity (the proportion of correctly classified non-pregnant females) of the classifier
 175 (Zweig and Campbell, 1993). The method also provides with an Area Under the Curve (AUC), a
 176 measurement of the performance of the classifier, with AUC = 0.5 for a classifier with no predictive
 177 power and AUC = 1 for a perfect classifier. Pregnancy determination thresholds for plasma and
 178 blubber progesterone concentrations were evaluated separately and then jointly. Analyses were
 179 carried out using the packages *caret* (Kuhn, 2008), *pROC* (Robin et al., 2011) and *cutpointr* (Thiele,
 180 2019) in the statistical program R (R Development Core Team, 2019). The specificity and sensitivity
 181 estimates are also reported as the number of false positives and false negatives within the dataset
 182 for each of the matrices alone and for when both agreed. Success rates using the ROC curves applied
 183 to the unknown status dataset were evaluated from the optimised sensitivity and specificity.

184 *Estimating the proportion of pregnant females in different study sites*

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

191 **Results**

192 *Relationship between plasma and blubber progesterone*

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

201

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

	Mean	Median	Min	Max	²⁰⁴ n
Mass (kg)	78.2	82.6	21.8	110.7	203 203
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	206 79

207

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 57 ng ml^{-1} (95th percentiles, 25 - 103 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} (95th 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} (95th percentiles, 25 - 103 ng ml^{-1}). For the
243 blubber progesterone the median in the joint model was 56 ng g^{-1} (95th percentiles, 26 - 223 ng g^{-1}).

244

245

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.

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

251

252

253 Table 3. Number and percentage (with 95% binomial confidence intervals) of mature females
 254 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

255

256 The proportion of animals categorised as pregnant was high in all regions (>60% depending on the
 257 matrix). Proportions were highest in the mature females from the Isle of Skye using either plasma or
 258 blubber diagnostic approaches and including the paired samples ‘agreed’ results. However, there
 259 was no statistically significant difference in the proportions among areas due to the small sample
 260 sizes and wide confidence intervals. Thus, the use of either plasma or blubber gave very similar
 261 results although the paired ‘agreed’ samples resulted in a slightly higher proportion of females being
 262 categorised as pregnant. The largest discrepancy was seen in the proportions estimated using
 263 blubber in the Moray Firth where almost twice the proportionate number of females were
 264 diagnosed as non-pregnant from blubber (9/24, 38%) than plasma (8/38, 21%). However, one of the
 265 9 females had a blubber concentration of progesterone just below the threshold (55 ng g⁻¹). Such
 266 large differences are also a consequence of small sample sizes.

267 **Discussion**

268 This study is the first to estimate pregnancy proportions in phocid seals using progesterone
 269 concentrations in both blubber and plasma. The utility of using progesterone concentrations in
 270 plasma samples for estimating reproductive status in seals has been well established (Gardiner et al.,
 271 1996) and concentrations in blubber from remote biopsy samples in cetaceans to determine
 272 reproductive status is now being widely used (Pallin et al., 2018a; Trego et al., 2013). However, very
 273 few studies have compared the results from plasma, for which the analytical methods were initially
 274 developed and verified (Elder et al., 1987), with those from blubber samples. A recent study by
 275 Beaulieu-McCoy et al. (2017) evaluated progesterone in the blubber of an otariid, the California sea
 276 lion (*Zalophus californianus*), as a means of determining pregnancy status, but concentrations in
 277 paired blood samples were not available. However, Mingramm et al. (2019) found progesterone
 278 concentrations in captive bottlenose dolphins (*Tursiops truncatus*) where patterns, in relation to
 279 reproductive status, were similar between serum and blubber and were significantly elevated in
 280 pregnant females. Interestingly, the blubber progesterone concentrations were lower than the
 281 serum progesterone concentrations (recognising that these concentrations are not directly
 282 comparable due to the differences between the matrices) which is the opposite to our findings,

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

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

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

321 When the reproductive outcome accuracies from the confusion matrices were compared, results
322 obtained from both plasma and blubber resulted in a slightly higher accuracy of 87% (compared with
323 85% for the plasma and 77% for the blubber alone). All the samples were collected between
324 February and May, which is between approximately 2 - 5 months before the breeding season.
325 Samples obtained during captures in February were less accurate as the animals were not observed
326 until late June to early July (within the breeding season), during which time they may have aborted
327 their pup before the observation period.

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

341 Blubber progesterone concentrations have not been routinely measured in pinnipeds, with only one
342 study in otariids (Beaulieu-McCoy et al., 2017). However, there have been several studies
343 investigating the use of blubber progesterone to estimate pregnancy rates in cetaceans (Kellar et al.,
344 2006; Mansour et al., 2002; Perez et al., 2011, Galligan et al., 2020). The results presented here
345 indicate that concentrations in the blubber of $> 56 \text{ ng g}^{-1}$ are indicative of pregnancy. This is similar
346 to the concentrations found in three delphinid species, in which the maximum concentrations in the
347 non-pregnant and immature animals was $\sim 50 \text{ ng g}^{-1}$ (Kellar et al., 2006). In minke whales
348 (*Balaenoptera acutorostrata*) it was only $\sim 3.5 \text{ ng g}^{-1}$ (Mansour et al., 2002) and in the study by Perez
349 et al. (2011) that included six cetacean species, the maximum in the non-pregnant mature animals
350 was also around 35 ng g^{-1} . However, none of these studies investigated the relationship between
351 blood and blubber progesterone and although Mingramm et al. (2019) reported progesterone in
352 serum and blubber from eleven captive bottlenose dolphins, the relationship between paired
353 samples was not shown.

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

361 The utility of estimating pregnancy status in female harbour seals is observed when the rates are
362 compared among the different regions and Seal Management Areas. All areas had high proportions
363 of pregnant females although significant differences among proportions could not be established
364 due to the small sample sizes. Gardiner et al. (1996) reported that 79% of the mature females
365 caught in the Moray Firth between February and May, 1988-1992, were pregnant and 21% were not
366 pregnant. This is the same as the proportion reported here (79% were also pregnant based on
367 plasma samples). Fecundity rates for the population in Loch Fleet are currently estimated at 0.89
368 (0.75-0.95) for multiparous and 0.69 (0.64-0.74) for ≥ 3 y olds (Cordes and Thompson, 2014). The
369 most reliable estimate of pregnancy indicates fecundity rate of 0.82, perhaps suggesting a low rate
370 of pup loss between the sample collection and the breeding season. The highest pregnancy rate was
371 seen at the Isle of Skye, a region where the population appears to have been stable for a number of
372 years (Thompson et al., 2019) and this was similar to the high proportion reported by Greig (2002) in
373 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.

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392

393 **References**

394 Arso Civil, M., Smout, S., Duck, C., Morris, C., Onoufriou, J., Thompson, D., Brownlow, A., Davison, N.,
395 Cummings, C., Pomeroy, P., McConnell, B., Hall, A.J., 2016. Harbour seal decline - vital rates and
396 drivers. Sea Mammal Research Unit, St Andrews.

397

398 Beaulieu-McCoy, N.E., Sherman, K.K., Trego, M.L., Crocker, D.E., Kellar, N.M., 2017. Initial validation
399 of blubber cortisol and progesterone as indicators of stress response and maturity in an otariid; the
400 California sea lion (*Zalophus californianus*). *General and Comparative Endocrinology* 252, 1-11.

401

402 Bradshaw, C.J.A., McMahon, C.R., 2008. Fecundity, in: S.E. Jorgensen, B.D. Fath (Eds.), *Encyclopedia*
403 *of Ecology*. Elsevier, Copenhagen, Denmark, 3120.

404

405 Burgess, E.A., Lanyon, J.M., Brown, J.L., Blyde, D., Keeley, T., 2012. Diagnosing pregnancy in free-
406 ranging dugongs using fecal progesterone metabolite concentrations and body morphometrics: A
407 population application. *General and Comparative Endocrinology* 177, 82-92.

408

409 Cordes, L.S., Thompson, P.M., 2014. Mark-recapture modeling accounting for state uncertainty
410 provides concurrent estimates of survival and fecundity in a protected harbor seal population.
411 *Marine Mammal Science*. 30, 691-705.

412

413 Cordes, L.S., Thompson, P.M., 2015. Mark-resight estimates of seasonal variation in harbor seal
414 abundance and site fidelity. *Population Ecology* 57, 467-472.

415

416 Elder, P.A., Yeo, K.H.J., Lewis, J.G., Clifford, J.K., 1987. An enzyme-linked-immunosorbent-assay
417 (ELISA) for plasma progesterone – immobilized antigen approach. *Clinica Chimica Acta* 162, 199-206.

418

419 Freeman, E.D., Larsen, R.T., Peterson, M.E., Anderson, C.R., Hersey, K.R., McMillan, B.R., 2014.
420 Effects of Male-Biased Harvest on Mule Deer: Implications for Rates of Pregnancy, Synchrony, and
421 Timing of Parturition. *Wildlife Society Bulletin* 38, 806-811.

422

423 Galligan, T.M., Boggs, A.S.P., Balmer, B.C., Rowles, T., Smith, C.R., Townsend, F., Wells, R.S., Kellar,
424 N.M., Zolman, E.S., Schwacke, L.H., 2020. Blubber steroid hormone profiles as indicators of
425 physiological state in free-ranging common bottlenose dolphins (*Tursiops truncatus*). *Comparative*
426 *Biochemistry and Physiology A- Molecular and Integrative Physiology*, 239, Article Number 110583.

427

428 Gardiner, K.J., Boyd, I.L., Racey, P.A., Reijnders, P.J.H., Thompson, P.M., 1996. Plasma progesterone
429 concentrations measured using an enzyme-linked immunosorbent assay useful for diagnosing
430 pregnancy in harbor seals (*Phoca vitulina*). *Marine Mammal Science* 12, 265-273.

431

432 Greig, D.J., 2002. Pregnancy and parturition rates of harbor seals in Monterey Bay, CA, Department
433 of Biological Sciences. San Jose State University, San Jose State University, 69.
434

435 Hall, A.J., Mackey, B., Kershaw, J.L., Thompson, P., 2019. Age-length relationships in UK harbour seals
436 during a period of population decline. *Aquatic Conservation* 29 (Suppl. 1), 61-70.
437

438 Hamudikuwanda, H., Gallo, G., Block, E., Downey, B.R., 1996. Adipose tissue progesterone
439 concentrations in dairy cows during late pregnancy and early lactation. *Animal Reproduction Science*
440 43, 15-23.
441

442 Kellar, N.M., Kelihier, J., Trego, M.L., Catelani, K.N., Hanns, C., George, J.C., Rosa, C., 2013. Variation
443 of bowhead whale progesterone concentrations across demographic groups and sample matrices.
444 *Endangered Species Research* 22, 61-72.
445

446 Kellar, N.M., Trego, M.L., Marks, C.I., Dizon, A.E., 2006. Determining pregnancy from blubber in three
447 species of delphinids. *Marine Mammal Science* 22, 1-16.
448

449 Kershaw, J.L., Hall, A.J., 2016. Seasonal variation in harbour seal (*Phoca vitulina*) blubber cortisol - A
450 novel indicator of physiological state? *Scientific Reports* 6, 21889.
451

452 Kuhn, M., 2008. Building Predictive Models in R Using the caret Package. *J Stat Softw* 28, 1-26.
453 Mackey, B.L., Durban, J.W., Middlemas, S.J., Thompson, P.M., 2008. A Bayesian estimate of harbour
454 seal survival using sparse photo-identification data. *Journal of Zoology* 274, 18-27.
455

456 Mansour, A.A.H., McKay, D.W., Lien, J., Orr, J.C., Banoub, J.H., Oien, N., Stenson, G., 2002.
457 Determination of pregnancy status from blubber samples in minke whales (*Balaenoptera*
458 *acutorostrata*). *Marine Mammal Science* 18, 112-120.
459

460 McHuron, E.A., Schwarz, L.K., Costa, D.P., Mangel, M., 2018. A state-dependent model for assessing
461 the population consequences of disturbance on income-breeding mammals. *Ecological Modelling*
462 385, 133-144.
463

464 Mingramm, F.M.J., Dunlop, R.A., Blyde, D., Whitworth, D.J., Keeley, T., 2019. Evaluation of
465 respiratory vapour and blubber samples for use in endocrine assessments of bottlenose dolphins
466 (*Tursiops spp.*). *General and Comparative Endocrinology* 274, 37-49.
467

468 Pallin, L., Robbins, J., Kellar, N., Bérubé, M., Friedlaender, A., 2018a. Validation of a blubber-based
469 endocrine pregnancy test for humpback whales. *Conservation Physiology* 6 (1), coy031.
470

471 Pallin, L.J., Baker, C.S., Steel, D., Kellar, N.M., Robbins, J., Johnston, D.W., Nowacek, D.P., Read, A.J.,
472 Friedlaender, A.S., 2018b. High pregnancy rates in humpback whales (*Megaptera novaeangliae*)
473 around the Western Antarctic Peninsula, evidence of a rapidly growing population. *Roy Soc Open Sci*
474 5(5), 180017.
475

476 Perez, S., Garcia-Lopez, A., De Stephanis, R., Gimenez, J., Garcia-Tiscar, S., Verborgh, P., Mancera,
477 J.M., Martinez-Rodriguez, G., 2011. Use of blubber levels of progesterone to determine pregnancy in
478 free-ranging live cetaceans. *Marine Biology* 158, 1677-1680.
479

480 Rahman, S.A., Grant, L.K., Gooley, J.J., Rajaratnam, S.M.W., Czeisler, C.A., Lockley, S.W., 2019.
481 Endogenous Circadian Regulation of Female Reproductive Hormones. *Journal of Clinical*
482 *Endocrinology & Metabolism* 104, 6049-6059.

483 Reijnders, P.J.H., 1990. Progesterone and Estradiol-17-Beta Concentration Profiles Throughout the
484 Reproductive-Cycle in Harbor Seals (*Phoca-Vitulina*). Journal of Reproduction and Fertility 90, 403-
485 409.

486

487 R Development Core Team, 2019. R: A Language and Environment for Statistical Computing. R
488 Foundation for Statistical Computing, Vienna, Austria.

489

490 Robin, X., Turck, N., Hainard, A., Tiberti, N., Lisacek, F., Sanchez, J.C., Muller, M., 2011. pROC: an
491 open-source package for R and S plus to analyze and compare ROC curves. BMC Bioinformatics 12,
492 77.

493

494 Saether, B.E., Engen, S., Moller, A.P., Weimerskirch, H., Visser, M.E., Fiedler, W., Matthysen, E.,
495 Lambrechts, M.M., Badyaev, A., Becker, P.H., Brommer, J.E., Bukacinski, D., Bukacinska, M.,
496 Christensen, H., Dickinson, J., du Feu, C., Gehlbach, F.R., Heg, D., Hotker, H., Merila, J., Nielsen, J.T.,
497 Rendell, W., Robertson, R.J., Thomson, D.L., Torok, J., Van Hecke, P., 2004. Life-history variation
498 predicts the effects of demographic stochasticity on avian population dynamics. American Naturalist
499 164, 793-802.

500

501 Schaub, M., Abadi, F., 2011. Integrated population models: a novel analysis framework for deeper
502 insights into population dynamics. Journal of Ornithology 152, 227-237.

503

504 SCOS, 2019. Special Committee on Seals: Scientific Advice on Matters Related to the Management of
505 Seal Populations 2018. Sea Mammal Research Unit, University of St Andrews, Sea Mammal Research
506 Unit, University of St Andrews, St Andrews, 155pp.

507 Sharples, R.J., Moss, S.E., Patterson, T.A., Hammond, P.S., 2012. Spatial Variation in Foraging
508 Behaviour of a Marine Top Predator (*Phoca vitulina*) Determined by a Large-Scale Satellite Tagging
509 Program. PLoS ONE 7, 0037216.

510

511 Shero, M.R., Bergfelt, D.R., Testa, J.W., Adams, G.P., 2018. Pairing ultrasonography with
512 endocrinology to elucidate underlying mechanisms of successful pregnancy in the northern fur seal
513 (*Callorhinus ursinus*). General and Comparative Endocrinology 255, 78-89.

514

515 Šimundić, A.-M. (2009). "Measures of Diagnostic Accuracy: Basic Definitions." The Journal of the
516 International Federation of Clinical Chemistry and Laboratory Medicine 19, 203-211.

517

518 Thompson, D., Duck, C.D., Morris, C.D., Russell, D.J.F., 2019. The status of harbour seals (*Phoca*
519 *vitulina*) in the UK. Aquatic Conservation 29 (Suppl. 1), 40-60.

520

521 Thompson, P.M., Wheeler, H., 2008. Photo-ID-based estimates of reproductive patterns in female
522 harbor seals. Marine Mammal Science 24, 138-146.

523

524 Trego, M.L., Kellar, N.M., Danil, K., 2013. Validation of Blubber Progesterone Concentrations for
525 Pregnancy Determination in Three Dolphin Species and a Porpoise. Plos One 8, 0069709.

526

527 Zweig, M.H., Campbell, G., 1993. Receiver-Operating Characteristic (Roc) Plots - a Fundamental
528 Evaluation Tool in Clinical Medicine. Clinical Chemistry 39, 561-577.

529

530