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1 **Exposure of harbour seals (*Phoca vitulina*) to *Brucella* in declining populations across**
2 **Scotland**

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21 **ABSTRACT**

22 Since 2000 there have been major declines in the abundance of Scottish harbour seals (*Phoca*
23 *vitulina*). The causes of the declines remain uncertain. The aim of this study was to establish
24 the extent to which the seals in the regions of greatest decline have been exposed to *Brucella*,
25 a bacterial pathogen that causes reproductive failure in terrestrial mammalian hosts. Tissues
26 from dead seals collected between 1992 and 2013 were cultured for *Brucella* (n=150). Serum
27 samples collected from live capture-released seals (n=343) between 1997 and 2012 were
28 tested for *Brucella* antibodies using the Rose Bengal plate agglutination test (RBT) and a
29 competitive Enzyme-Linked Immunosorbent Assay (ELISA). 16% of seals cultured had
30 *Brucella* isolated from one or more tissues but there were no pathological signs of infection.
31 The cELISA results were more sensitive than the RBT results showing that overall, 25.4% of
32 seals were seropositive with the highest seroprevalence in juveniles. As there was no
33 evidence of either a higher seroprevalence, or higher circulating antibody levels in
34 seropositive animals in the areas with the greatest declines, it was concluded that *Brucella*
35 infection is likely not a major contributing factor to recent declines. However, the
36 consistently high proportion of seals exposed to *Brucella* indicates possible endemicity in
37 these populations, likely due to *Brucella pinnipedialis*, which has demonstrated a preference
38 for pinniped hosts. Importantly, given the close proximity between seals, humans and
39 livestock in many areas, there is the potential for cross-species infections.

40 **KEY WORDS**

41 Pinnipeds, *Brucella*, disease, cultures, seroprevalence, antibodies, ELISA, Rose Bengal plate
42 agglutination test

43

44 **INTRODUCTION**

45 Aerial surveys have been carried out by the Sea Mammal Research Unit to monitor harbour
46 seal (*Phoca vitulina*) populations around Scotland since 1985, and declines in a number of
47 these populations have been seen since 2000 (Lonergan et al., 2007). Major declines of 68%
48 in Orkney, 50% in Shetland, and 90% in the Firth of Tay have been documented in particular
49 (SCOS, 2012). However, the pattern of the declines is not universal, as some areas remain
50 more stable while the populations in other areas continue to decrease in size. The population
51 in the Eden and Firth of Tay Special Area of Conservation, for example, has experienced the
52 most dramatic and sustained declines of over 90% in the last 15 years with a most recent
53 estimate of just 29 individuals left in 2014 (Hanson *et al.* 2015).

54 Many potential causes of the decline have been suggested, but the contributing factors remain
55 uncertain. Some of these include predation by killer whales (Bolt et al., 2009), competition
56 for food with other marine top predators (SCOS, 2012), exposure to biotoxins from harmful
57 algal blooms (Hall and Frame, 2010), deliberate shooting (Thompson et al., 2007), accidental
58 mortalities as a result of interactions with shipping vessels (Thompson et al., 2010), and
59 predation by grey seals (*Halichoerus grypus*) (Brownlow *et al.* 2016). A further potential
60 contributing factor to these declines is infectious disease, but there has been a lack of reports
61 of sick animals by the Scottish Marine Animals Strandings Scheme (SMASS) or the Scottish
62 Society for the Prevention of Cruelty to Animals (SSPCA). This suggests that if infectious
63 disease was present in these populations, and was contributing to the observed declines, it
64 could either be affecting the reproductive success of the animals, or, causing them to die very
65 quickly once infected, or both. A particular infectious agent of interest in this respect is
66 *Brucella* as it is known to cause reproductive failure in other mammalian hosts. The aim of
67 this study was to establish the extent to which harbour seals in Scotland have been exposed to

Scottish harbour seals' exposure to *Brucella*

68 *Brucella* over time, both before and during the observed population declines, and whether
69 this could be a potential contributing factor to the major declines in some areas.

70 Members of the genus *Brucella* are Gram-negative, rod-shaped bacteria that cause chronic
71 disease most commonly associated with abortions and infertility in domestic livestock
72 (Seleem et al., 2010). Since the first reports of *Brucella* in a marine mammal in 1994 (Ross et
73 al., 1994), infections have been recognised in a range of pinniped and cetacean species
74 worldwide (Thakur et al., 2012). Strains isolated from marine mammals have been shown to
75 be phenotypically and genetically distinct from those isolated from terrestrial mammals, and
76 two species have been described that have pinnipeds and cetaceans as their preferred hosts,
77 *Brucella pinnipedialis* and *Brucella ceti* respectively (Foster et al., 2007). *Brucella ceti*
78 infections in cetaceans have been associated with various pathologies which include
79 abortions and neonatal mortality (Miller et al., 1999), epididymitis in males (Dagleish et al.,
80 2008), meningoencephalitis (González et al., 2002; Jauniaux et al., 2010; Alba et al., 2013;
81 Garofolo et al., 2014), abscesses (Foster et al., 1996; Foster et al., 2002), endocarditis
82 (González-Barrientos et al., 2010), mastitis, pneumonia, peritonitis, osteomyelitis and spinal
83 discospondylitis (Foster et al., 2002). In contrast, pathology associated with *Brucella*
84 *pinnipedialis* in seals is lacking despite several reports of its isolation (Foster et al., 2002;
85 Nymo et al., 2011; Siebert et al., 2017; Tryland et al., 2005). With respect to harbour seals
86 specifically, *Brucella* has previously been isolated from wild animals (Foster et al., 2002:
87 Garner et al., 1997; Prenger-Berninghoff et al., 2008; Ross et al., 1994), and in several areas
88 they have also been found to be seropositive (Gaydos et al., 2005; Hueffer et al., 2013;
89 Maratea et al., 2003; Ross et al., 1996). However, the extent to which harbour seals in
90 Scotland, or the United Kingdom in general, are currently exposed to this pathogen since the
91 onset of the declines is unknown. Here, tissue samples from dead harbour seals collected by
92 the SMASS over 20 years were cultured to test for *Brucella* isolates. In addition, harbour seal

93 serum samples from live-capture release studies by the Sea Mammal Research Unit over a 14
94 year period were tested for *Brucella* antibodies. Tempo-spatial patterns in seroprevalence
95 were examined in order to investigate the potential role of *Brucella* as a contributing factor to
96 the Scottish harbour seal declines.

97 **MATERIALS & METHODS**

98 ***Brucella* Cultures**

99 Tissue samples from 150 dead harbour seals from across Scotland, which had received post
100 mortem examination were collected by the SMASS between 1992 and 2013 as part of
101 systematic surveillance studies. Microbiological culture was performed including specific
102 methods for *Brucella* isolation. The selected tissues varied between animals but typically
103 included lung, liver, kidney, spleen, and small intestine, but also brain, pancreas, reproductive
104 tissue, various lymph nodes and any abscesses apparent at post mortem. These were collected
105 from approximately equal numbers of males (n = 61) and females (n = 41) and unsexed
106 animals (n = 48). The majority of cases were adult animals although some juveniles were also
107 sampled. Tissues were processed using a standardised method (Foster et al., 2002) and
108 cultured on Columbia sheep blood agar (CSBA) (Oxoid, Basingstoke, UK) and Farrell's
109 medium (Farrell, 1974) incubated at 37°C in air with 5% added CO₂. Isolates with colonial
110 appearance typical of *Brucella* on either medium were identified as *Brucella* using
111 phenotypic tests as previously described (Foster et al., 2002). A multi-locus variable number
112 of tandem repeats analysis (MLVA-16) was used to confirm species designation (Macquart et
113 al., 2009).

114 **Serum Sampling Procedure**

115 Blood samples were collected from 343 live-captured harbour seal adults, juveniles (<50kg
116 and/or 120cm) and suckling pups from multiple haul out sites around five areas of Scotland;
117 the South East (the Eden Estuary, the Firth of Tay and the Firth of Forth), the North East (the
118 Moray Firth, Dornoch Firth, Loch Fleet and the Pentland Firth), the North West (the Isle of
119 Skye, Loch Shildaig, and the Loch Nan Uamh Islands), the South West (the Sound of Jura
120 and south east Islay), and Orkney (Fig.1). Samples were collected between 1997 and 2012 at
121 varying times of the year. Due to the opportunistic nature of the analysis of stored samples,
122 sample sizes varied regionally, across years and between age classes although the male to
123 female ratio was approximately equal with 182 males and 160 females (Table 1). The seals
124 were captured in nets on haul outs or in the water, and were sedated with Zoletil 100 (Virbac,
125 France) at a dose rate of 0.5 ml/100 kg body weight intravenously. Blood samples were taken
126 from the extradural vein immediately after the immobilisation of the animal. The whole blood
127 samples were spun, sera was collected and aliquots were frozen at -20°C for later analysis.
128 Samples were collected under the Animal (Scientific Procedures) Act, 1986, Home Office
129 Project and Personal Licences issued to the Sea Mammal Research Unit.

130 **Serological Methods**

131 Rose Bengal plate agglutination test: In a preliminary trial, the Rose Bengal plate
132 agglutination test (RBT) was used to test stored serum samples for the presence of *Brucella*
133 antibodies. Serum samples were tested with the Micropath Rose Bengal kit against the *B.*
134 *abortus* antigen (Omega Diagnostics, Alva, United Kingdom) following the kit instructions.
135 Samples were either classed as positive or negative based on visually discernible
136 agglutination of antigens. A positive and a negative control supplied by the kit were used for
137 each set of four serum samples tested simultaneously.

138 Following the completion of the Rose Bengal trials with the successful detection of
139 antibodies, the variation in seroprevalence, i.e. the proportion of animals with antibody levels
140 higher than a background threshold level set for terrestrial mammals, as well as the absolute
141 antibody levels in the samples, were investigated using a competitive ELISA.

142 Competitive ELISA (cELISA) - Polystyrene microtitre plates were coated with *Brucella*
143 *melitensis* lipopolysaccharide (LPS) antigen. Both positive (positive serum from an
144 experimentally infected goat with *B. melitensis*) and negative controls (no sera) were tested.
145 Serum and a peroxidase labelled monoclonal antibody (BM40 from a locally held hybridoma)
146 were added to the plates and incubated for 30 min at room temperature. The plate was then
147 washed, and chromogen and substrate were added and incubated for a further 15 minutes at
148 room temperature, shaking at 160rpm. The plates were read at an optical density (OD) of
149 450nm. The mean OD of duplicate wells was expressed as a percentage of antibodies binding
150 to the plate. Test samples with an OD of less than 60% of the conjugate only control (no sera
151 added) were recorded as positive. This cut-off threshold was established based on serology
152 results from terrestrial mammals (Perrett et al., 2010). As such, a weak reaction, indicative of
153 low antibody levels, was considered to be between 30-60% antibody binding, while a strong
154 reaction, indicative of high circulating antibody levels, was considered to be <30%.

155 **Statistical Analysis of Serological Data**

156 All statistical analyses were performed using the statistical package, R, version 3.1.2 (R Core
157 Development Team, 2014). Statistical significance was taken at $p = 0.05$. Two different
158 statistical approaches were taken to investigate firstly, variation in the seroprevalence data,
159 and secondly, variation in the antibody levels in the seropositive individuals.

160 Seroprevalence data: Generalised linear models (glms) with a binomial distribution were
161 fitted to the seroprevalence data with individuals classed as seropositive (1) and seronegative

162 (0) for the two tests separately. The 14 years of data was split into four time periods: 1997-
163 2000 (n = 63) represents the years before the start of the decline, and the years between 2001
164 and 2012 were split into 3 periods with approximately equal numbers of samples in each to
165 give the maximum statistical power for the analysis. These were 2001-2005 (n = 87), 2006-
166 2008 (n = 94), 2009-2012 (n = 99). A global model including all explanatory variables of
167 interest (region, sex, age-class, time period and an interaction between region and time
168 period) was generated, and backwards variable selection using the 'step' function in the 'car'
169 library in R (version 2.11.1) was performed to identify the combination of variables that best
170 explained the variation in the data by producing the model with the lowest AIC.

171 Antibody levels data: In addition, variation in the levels of circulating *Brucella* antibodies
172 were investigated in the seropositive individuals identified using the cELISA data. Antibody
173 binding results of only the seropositive seals were modelled using a glm with a gamma
174 distribution and a log-link function to model the non-normal distribution of the antibody
175 binding data as most individuals had low circulating antibody levels while few were very
176 high. Again, a global model with region, sex, age-class, time and an interaction between
177 region and time was generated and backwards variable selection using the 'step' function was
178 used to identify the combination of variables that best explained the variation in the data.

179 **RESULTS**

180 ***Brucella* Cultures**

181 Of the 150 animals examined bacteriologically between 1992 and 2013, *Brucella* was
182 isolated from the tissues of 24 individuals (16 %). Details for 11 of these animals have been
183 reported previously (Foster et al., 2002). None of the culture positive animals showed any
184 signs of pathological lesions associated with infection and the cause of death was always
185 associated with starvation, trauma or some other viral or bacterial infection, but not *Brucella*.

186 Of the tissues cultured, the two that were the most commonly culture positive were lung
187 (45.8%) and spleen (41.7%) although not all tissues were sampled consistently across the
188 individuals (Table 2). MLVA-16 analysis identified isolates as *B. pinnipedialis* belonging to
189 one of two sequence types: ST 24 or ST 25. Serum was sampled from 12 of these culture
190 positive seals, two of which were seronegative and were sampled from healthy animals that
191 had been shot (Table 2).

192 **Serology**

193 **Test Performance**

194 The RBT trials were able to detect antibodies in the archived serum samples, and results
195 showed that across all study sites over the whole sampling period, the prevalence of *Brucella*
196 antibodies was 15.9%. However, the cELISA results showed a higher overall seroprevalence
197 of 25.4%. The prevalence across all age and sex classes, as well as across regions and over
198 time was lower for the RBT results compared to the cELISA results (Table 3).

199 All of the samples that were positive using the RBT were also classed as positive using the
200 cELISA. These samples had the lowest antibody binding indicating the highest circulating
201 *Brucella* antibodies. Specifically, the mean antibody binding of the samples classed as
202 positive by the RBT was $45.0 \pm 0.06\%$, while those classed as negative had a mean antibody
203 binding of $74.0 \pm 0.02\%$ (Two sample t-test; $t = 8.79$, $df = 51.54$, $p < 0.0001$). Therefore, it
204 seems that the RBT is only able to detect high antibody levels in the serum samples, and is
205 the least sensitive of the two serological methods tested. As a result, the RBT results may
206 have underestimated the prevalence of *Brucella* antibodies in these harbour seals (Table 3).

207 **Seroprevalence**

208 Using the cELISA seroprevalence data where individuals were classed as either seropositive
209 or seronegative, the best binomial glm after backwards variable selection included only age
210 class as an important explanatory variable. Juveniles had a significantly higher
211 seroprevalence than adults and pups (p values < 0.025), while adults and pups were not
212 significantly different to each other ($p = 0.11$). There were no significant changes in
213 prevalence over time or between regions, and there were equal numbers of seropositive males
214 and females.

215 **Antibody Levels**

216 Variation in the levels of circulating *Brucella* antibodies were investigated in the seropositive
217 individuals. Backwards variable selection of the glm using the cELISA antibody binding
218 results of only the seropositive seals revealed that age class and an interaction between region
219 and time period were retained in the final model with significant effects. Pups had near-
220 significant higher antibody binding than both adults and juveniles (p values both < 0.07),
221 indicating the lowest circulating antibodies in these seropositive individuals (Fig. 2). There
222 was no difference between the circulating antibody levels in juveniles and adults (Fig. 2). The
223 interaction between region and time revealed that there were different patterns in circulating
224 antibody levels in the seropositive seals between regions over the 14 year sampling period.
225 The highest average circulating *Brucella* antibody levels (shown as the lowest % antibody
226 binding in Fig. 3) were measured in the 2001-2005 time period in the South West and these
227 then decreased over the following years ($p = 0.035$). All areas showed a decrease followed by
228 an increase again over the whole time frame with the exception of Orkney that showed a
229 sustained decrease in circulating antibodies between 2001 and 2012 (Fig. 3), and the North
230 West where seropositive individuals were only recorded in the final time period, but this is
231 likely a reflection of very limited sampling before 2009 (Table 1) rather than a recent
232 introduction of the bacteria to the area. Males and females had similar antibody levels.

233 **DISCUSSION**

234 *Brucella* species were isolated from 16% of the dead stranded animals tested, but there were
235 no signs of *Brucella*-specific pathological lesions associated with infection in these 24
236 animals. They all appear to have died of other causes, although it is possible that *Brucella*
237 acted as a secondary infection in these cases. The results presented here therefore suggest that
238 harbour seals can be infected by *Brucella*, likely *Brucella pinnipedialis*, without evidence of
239 associated disease. *Brucella pinnipedialis* has also been cultured from apparently healthy
240 tissues of a number of other pinniped species including grey seals (*Halichoerus grypus*)
241 (Foster et al., 2002; Prenger-Berninghoff et al., 2008), hooded seals (*Cystophora cristata*)
242 (Foster et al., 2002; Tryland et al., 2005), ringed seals (*Pusa hispida*) (Forbes et al., 2000)
243 and harp seals (*Pagophilus groenlandicus*) (Forbes et al., 2000). At present, there is only
244 limited evidence of *Brucella* infection causing disease in any species of phocid seal (Jauniaux
245 et al., 2013), although *Brucella* isolation was suggested as a possible cause of abortion in an
246 otariid species, the California sea lion (*Zalophus californianus*), with recovery of *Brucella*
247 from the placenta and stomach contents of an aborted foetus (Goldstein et al., 2009).
248 Extensive typing of a large number of marine mammal *Brucella* strains in a recent study,
249 however, found the Californian Sea lion isolates to be similar to *B. ceti* recovered from
250 bottlenose dolphins in the USA, and that *B. pinnipedialis* isolates from harbour seals in the
251 USA were found to be closely related to Scottish strains (Whatmore et al., submitted for
252 publication).

253 Animal experimentation in cattle with a *Brucella* isolate recovered from a Pacific harbour
254 seal (*Phoca vitulina richardsii*) resulted in seroconversion and abortion in 2 of 3 pregnant
255 animals, suggesting that some strains of *B. pinnipedialis* may have abortifacient potential
256 (Rhyan et al., 2001). However, there has been no evidence in pinnipeds of disease due to *B.*
257 *pinnipedialis* as has been seen with *B. ceti* infection in dolphins and porpoises where chronic

258 disease with significant clinical and pathological signs including male infertility,
259 neurobrucellosis, cardiopathies, bone and skin lesions, and live strandings have been
260 documented (Guzmán-Verri et al., 2012). While no evidence of disease has been reported in
261 pinnipeds, detecting abortions in wild populations is very difficult, especially if the occurrence
262 remains constant over time and there is limited data on pupping success, as is the case for the
263 populations sampled here. The ability to detect abortions and monitor pupping success in
264 different populations is therefore needed in order to determine that *B. pinnipedialis* does not
265 cause disease in these seals.

266 Similar to this study, *Brucella* antibodies have been detected in sera from a number of marine
267 mammal species using Rose Bengal tests (Hernández-Mora et al., 2008; Jensen et al., 2013;
268 Retamal et al., 2000; Tryland et al., 1999; Tryland et al., 2005), and using both indirect
269 ELISA (iELISA) and competitive ELISAs primarily designed for ruminants (Jensen et al.,
270 2013; Lynch et al., 2011; Nielsen et al., 2001; Nymo et al., 2013a; Roe et al., 2010;
271 Tachibana et al., 2006; Tryland et al., 1999; Van Bresseem et al., 2001). Here, differences in
272 the prevalence estimates obtained from the RBT and the cELISA results highlight the need to
273 consider test performance when conducting serological studies. It seems that the RBT is only
274 able to detect antibodies when at higher levels, and as such, seropositive samples with low
275 levels of antibody are not recognised.

276 Our results suggest that the cELISA appears to be a more sensitive test than RBT and is thus
277 able to distinguish between seronegative samples and samples with low antibody levels. The
278 cELISA results were therefore chosen for further analysis over the RBT results as this is
279 thought to be a more robust and objective test. However, when detecting *Brucella* antibodies
280 using serological methods, serological cross-reactions and false positives are potentially a
281 major problem, and may contribute to the higher seroprevalence seen in the cELISA results.
282 It is thought that in cattle, most problems caused by cross-reactivity are the result of

283 antibodies produced through the immune response of the animal to other microorganisms
284 sharing similar structural characteristics with the O-polysaccharide of *Brucella* species
285 (Corbel, 1985). We cannot rule out the possibility that other cross-reacting bacteria could
286 affect these results. In cattle, it is thought that the cELISA is a more appropriate serological
287 test than the iELISA as it is better able to distinguish between antibodies to *Brucella* species
288 and antibodies to other cross-reacting Gram-negative bacteria (Nielsen, 1990; Samartino et
289 al., 1999). In two studies on Australian fur seals (Lynch et al., 2011) and Hawaiian monk
290 seals (*Monachus schauinslandi*) (Nielsen et al., 2005), it was concluded that the iELISA was
291 an unreliable test for the identification of seropositive individuals. Thus, based on this
292 previous evidence, the cELISA was chosen here as an appropriate assay as it is more
293 conservative than an iELISA with a reduced chance of false positives.

294 The cELISA results indicate that approximately 25% of the seals sampled had antibodies to
295 *Brucella*. This is within the range of previous studies on harbour seal populations in the North
296 Atlantic where prevalence ranged between 3.1% (n=96) in the St Lawrence Estuary, 14% (n
297 = 21) (Maratea et al. 2003) and 50% (n=8) off the Atlantic coast of the United States (Nielsen
298 et al., 2001). Serology testing of 300 Scottish harbour seals prior to 2002 found 147 (49 %) to
299 be positive (Foster et al. 2002). As there appears to have been no change in antibody
300 prevalence over this 14 year sampling period, or between regions, these data suggest that
301 *Brucella* may be endemic in Scottish harbour seals and exposure to the bacteria seems to
302 have remained constant over the study period. For endemicity to occur, a high and regular
303 rate of transmission of the bacteria is required within a population.

304 The transmission of *Brucella* in marine mammals is poorly understood as there is little
305 evidence to support any particular route of infection in these species. It is likely that the
306 routes of transmission are similar to those of terrestrial mammals, whereby transmission
307 occurs through exposure to infected placenta, birth fluids and vaginal secretions as well as by

308 venereal spread (Young, 2006). As *Brucella* has been isolated from the reproductive organs
309 of several cetacean species (Foster et al., 2002; González-Barrientos et al., 2010; Miller et al.,
310 1999), and from an aborted foetus of a captive bottlenose dolphin (Ewalt et al., 1994), the
311 most likely mode of transmission of *B. ceti* appears to be through sexual intercourse, vertical
312 transmission from mother to foetus, maternal feeding and contact with aborted fetuses and
313 placental tissues (Guzmán-Verri et al., 2012). The transmission between pinnipeds is even
314 less well understood, but it could be similar to cetaceans. However, transmission may also
315 occur through contact with infected individuals in gregarious species that haul out together in
316 large groups. *Brucella* was cultured from the faeces of a seropositive juvenile harbour seal in
317 captivity (Gaydos et al., 2005), suggesting that some *Brucella*-positive seals are actively
318 shedding the bacteria. In addition, *B. pinnipedialis* was cultured or detected by PCR in
319 harbour seal salivary gland secretions, lungs, urinary bladder, and faeces (Lambourn et al.
320 2013), suggesting that seals could be exposed to the bacterium via exposure to oral
321 secretions, urine, or faeces on haul-outs. *Brucella* has also been isolated from subcutaneous
322 lesions in cetaceans (Foster et al., 1996; Foster et al., 2002), so the potential for direct contact
323 with similarly infected skin lesions, should they occur, in pinnipeds that haul out together
324 may present another mode of transfer of the bacteria, although such lesions have not been
325 reported to date. Together, this could make harbour seals more at risk of bacterial transfer at
326 particular times during their life cycle when they haul-out in larger numbers during the
327 breeding season and during the moult. Thus, the requirements of a high and regular rate of
328 transmission of the bacteria for endemicity to occur could be met for harbour seals.

329 There was no regional variation in seroprevalence across Scotland, indicating that seals in the
330 declining populations in Orkney and along the East coast have similar proportions of
331 seropositive seals to the stable populations along the West coast. In addition there has been
332 no change in the prevalence over time, even in declining populations, and none of the seals

333 sampled in this study showed any overt signs of ill health. These results further support the
334 hypothesis that they may be infected by a strain of the bacteria that appears to be having little
335 effect on their health. Other pinniped species have also been shown to be seropositive and yet
336 remain apparently healthy and asymptomatic (Nielsen et al., 2005; Nielsen et al., 1996;
337 Nymo et al., 2011; Retamal et al., 2000). Together, these results indicate that the bacteria
338 may only cause a mild and transient infection, and *B. pinnipedialis* is most likely not a major
339 cause of the harbour seal decline in Scotland. Other potential causes of the declines should
340 therefore continue to be investigated.

341 Juveniles showed the highest overall prevalence of the three age classes. It has been reported
342 that the higher incidence in juveniles may be as a result of recent exposure to the pathogen
343 due to a change to a prey-based diet after they are weaned (Lynch et al., 2011; Nymo et al.,
344 2013b). Lungworms carrying *Brucella* in fish prey species may be a means by which marine
345 mammals become infected with the bacterium as was suggested when *Brucella* was isolated
346 from the lungworms in a harbour porpoise (*Phocoena phocoena*) (Dawson et al., 2008).
347 *Brucella* species have also been demonstrated by immunohistochemical staining in
348 the uterus and the intestinal lumen of female *Parafilaroides* lungworms from a Pacific
349 harbour seal (Garner et al., 1997) and it was postulated that, based on the life cycle of the
350 parasite, the larvae migrate through the respiratory tract and are then swallowed. From there,
351 they pass through the digestive tract and out into the environment in the faeces where they are
352 taken up by fish and ultimately by the seal. The parasitic larvae are released into the
353 gastrointestinal tract of the animal, and when they mature into adults, they migrate to the
354 lungs and continue the life cycle (Howard et al., 1983). It may also be significant that lung
355 was the body tissue with the highest isolation rate from the 24 harbour seals that were
356 positive by *Brucella* culture. As such, the high levels of antibodies in seropositive juveniles
357 may suggest a more recent exposure to *Brucella* as they first start to eat fish containing the

358 infected parasites, but it is not necessarily indicative of an active infection. The seropositive
359 adults have high antibody levels which may be indicative of both previous and regular
360 exposure to the bacteria.

361 The finding that there were lower levels of *Brucella* antibodies in the seropositive pups
362 compared to both seropositive adults and juveniles is surprising as it would be expected that
363 passively transferred maternal antibodies would be present in pups, as they are found in the
364 offspring of antibody-positive mothers in terrestrial species (Ray et al., 1988; Rhyan et al.,
365 2009; Thakur et al., 2002). However, a lower seroprevalence was seen in Australian fur seal
366 (Lynch et al., 2011), Hawaiian monk seal (Aguirre et al., 2007), hooded seal (Nymo et al.,
367 2013b) and Alaskan harbour seal pups (Zarnke et al., 2006) compared to both adults and
368 juveniles. In these studies, it was concluded that pups may have had maternal antibodies at
369 titres lower than the threshold of detection used in their serological tests. These data support
370 this theory that pups likely have low levels of maternal antibodies, and that they may not be
371 exposed to infection until a later stage postweaning (Lynch et al., 2011). These findings
372 further highlight the need for investigations into the timing of first exposure to *Brucella* and
373 seroconversion as well as the development of specific thresholds of detection for antibodies
374 to marine mammal strains of *Brucella* in various serological tests.

375 While the overall proportion of positive seals did not change across the different sampling
376 regions over time, there were varying patterns of high and low antibody levels measured in
377 the seropositive seals. Higher antibody levels were not recorded in the declining populations
378 however, and there were no populations with consistently higher or lower antibody levels.
379 The presence of antibodies does not necessarily suggest that the animals had a current or
380 active infection at the time of sampling. The variation over time seen here between
381 populations likely reflects cycles of infection followed by clearance in infected individuals
382 that do not show any clinical signs of the disease. While the apparently high exposure rates of

383 Scottish harbour seals to *Brucella* appear not to be having a negative impact on their
384 populations, such levels may have important implications for cross-species infections
385 between humans and domestic livestock where infections may lead to disease. Currently a
386 total of 53 marine mammal species worldwide have been shown to be seropositive for
387 *Brucella* antibodies, and 20 of these species have been positive for *B. ceti* or *B. pinnipedialis*
388 by culture or PCR assays (Foster et al., 2015; Hernández-Mora et al., 2013). The high
389 seroprevalence seen here in all populations across Scotland suggests that wildlife
390 professionals working with live seals could be exposed to the bacterium, and care should be
391 taken when handling the animals and working with samples. To date, there have been four
392 documented cases of humans infected with *Brucella ceti* (Brew et al., 1999; McDonald et al.,
393 2006; Sohn et al., 2003), demonstrating the zoonotic potential of that species, but human
394 infections with *B. pinnipedialis* have not been documented.

395 In conclusion, over a quarter of Scottish harbour seals have detectable levels of antibodies to
396 *Brucella* which may indicate endemicity in these populations, possibly to a strain of the
397 pathogen that has little effect on the health of individuals. These prevalence rates do not
398 appear to explain the declines in Orkney and along the East coast as the prevalence in these
399 areas is the same as in populations along the west coast that remain stable. The causes of the
400 decline are likely to vary between regions and are probably due to a combination of factors,
401 but *Brucella* infection does not appear to be one of them, based on our findings and
402 comparison with seroprevalence rates for Scottish harbour seals before 2002 (Foster et al.,
403 2002). Despite the routine use of the serological tests used here in many assessments of
404 exposure to *Brucella* species, further validation of the tests for marine mammals is needed,
405 and the discrepancies between the two test types here highlight the need for careful
406 interpretation of the results.

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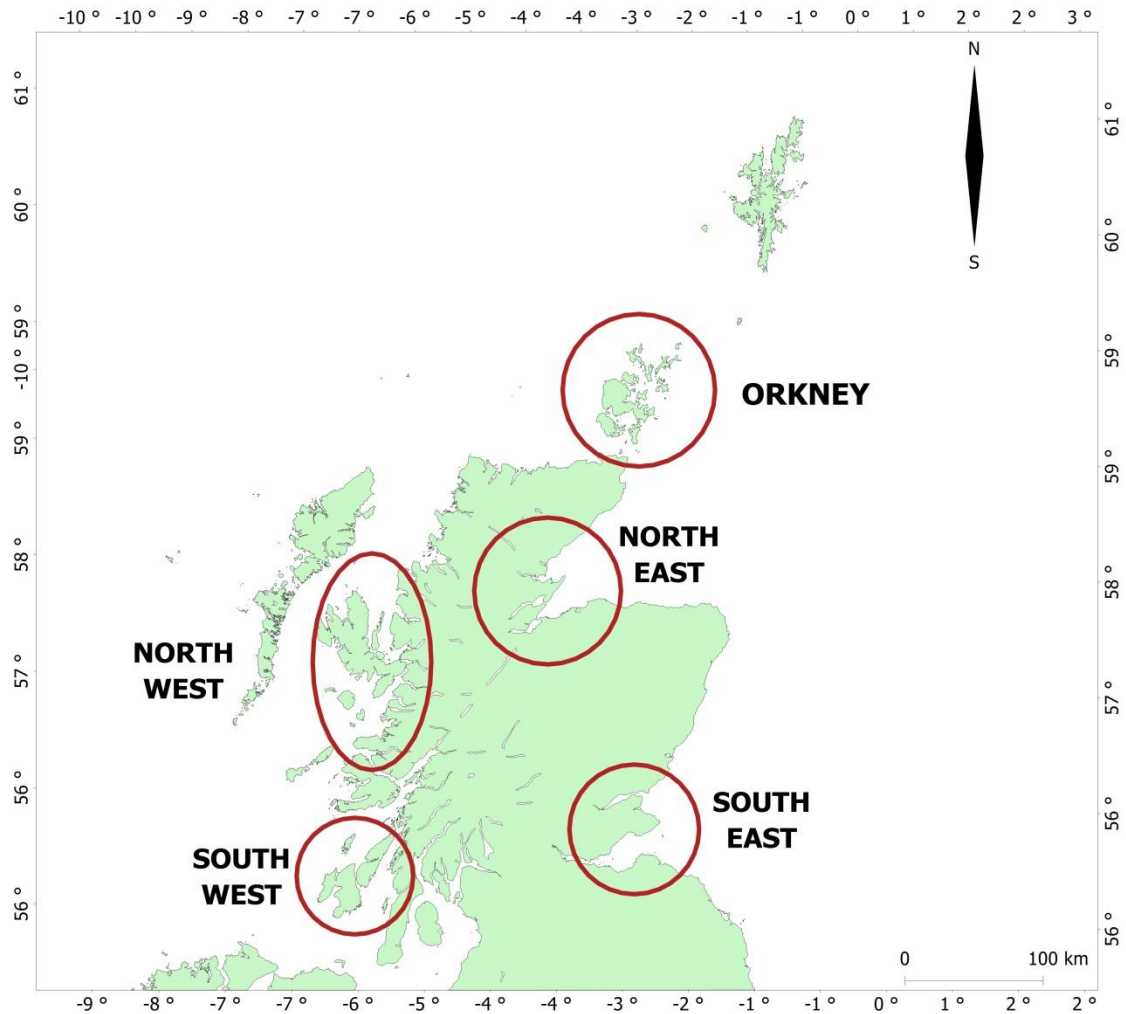
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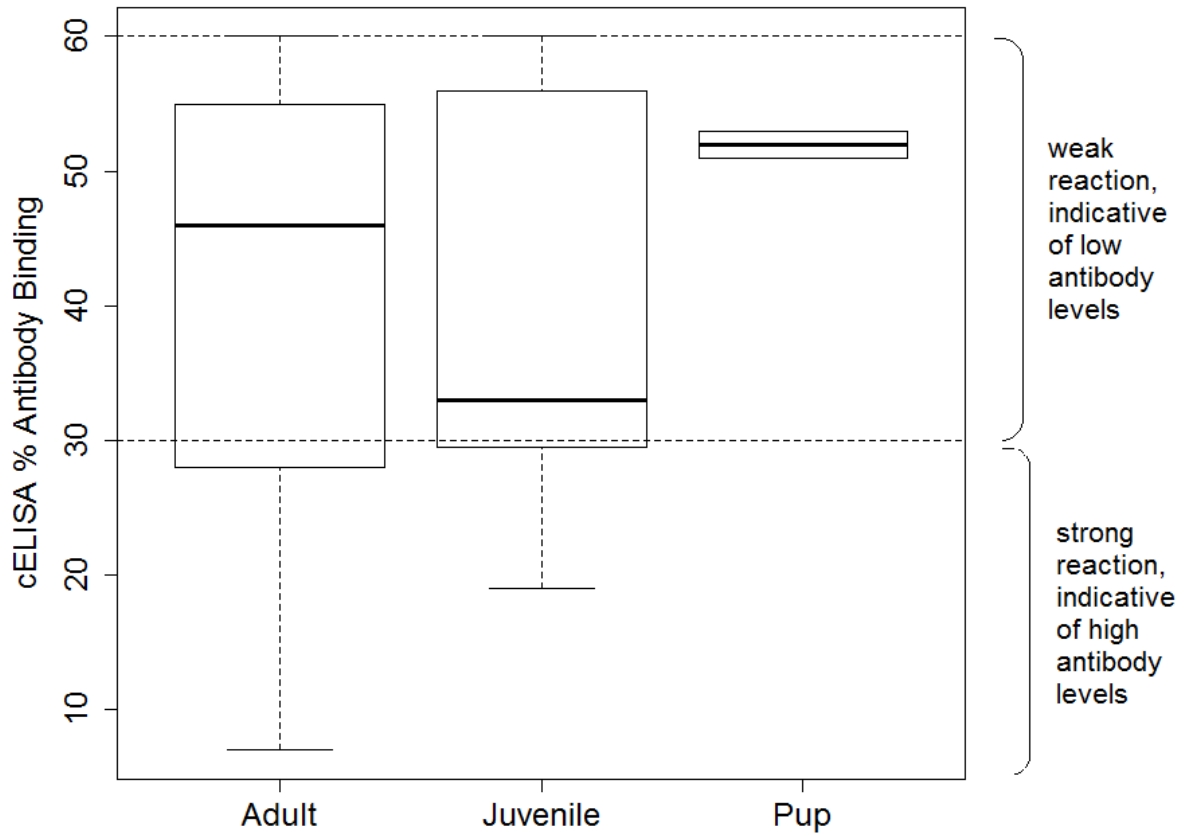


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628 **Fig 1. Sampling regions of live-captured harbour seals across Scotland between 1997 and**
629 **2012 by the Sea Mammal Research Unit. Serum samples were grouped into 5 main regions**
630 **across Scotland as indicated by the circles on the map. Over this time period, the populations**
631 **along the west coast, marked as 'North West' and 'South West' were stable while the**
632 **populations in 'Orkney', the 'North East' and the 'South East' underwent precipitous**
633 **declines (SCOS, 2012).**

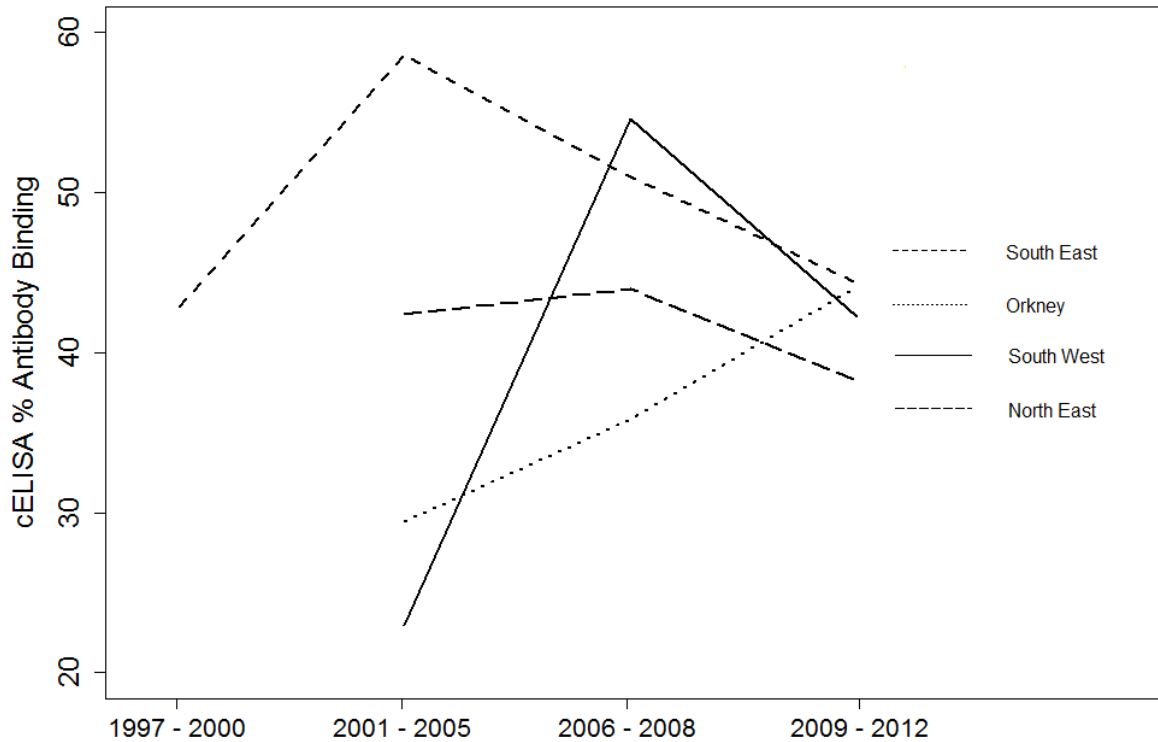
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637 **Fig. 2. cELISA antibody binding of the seropositive animals by age class. Low antibody**
638 **binding indicates high levels of circulating *Brucella* antibodies in the seals. The pups had**
639 **lower circulating antibody levels than both adults and juveniles.**



640

641 **Fig. 3. Interaction plot of the cELISA % antibody binding over time for each sampling**
642 **region showing variation in the seropositive individuals. Low antibody binding indicates**
643 **high levels of circulating *Brucella* antibodies in the seals. The North West sampling region is**
644 **not included here as seropositive animals were only identified between 2009 – 2012. With the**
645 **exception of Orkney, the other sampling regions showed a decrease in the circulating levels**
646 **of *Brucella* antibodies, followed by an increased again over this time period.**

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652 **Table 1** – Serological samples collected from harbour seals across Scotland across the five
 653 sampling regions and over 12 years broken down into 4 time periods. Samples are grouped by
 654 sex and age class. A total of 306 adults, 15 juveniles and 22 pups were sampled.

Sampling Period	Sex	Age Class	Serological Samples Collected by Region				
			North East	North West	Orkney	South East	South West
1997 - 2000	Males	Adults	-	-	-	37	-
		Juveniles	-	-	-	1	-
		Pups	-	-	-	3	-
	Females	Adults	-	-	-	21	-
		Juveniles	-	-	-	1	-
		Pups	-	-	-	-	-
2001 - 2005	Males	Adults	5	-	11	15	14
		Juveniles	-	-	-	1	-
		Pups	-	-	-	-	-
	Females	Adults	10	-	12	9	4
		Juveniles	1	-	-	2	2
		Pups	-	-	-	-	1
2006 - 2008	Males	Adults	3	1	10	16	8
		Juveniles	-	-	-	-	1
		Pups	-	-	-	-	5
	Females	Adults	7	1	15	6	7
		Juveniles	1	-	-	-	-
		Pups	2	-	11	-	-
2009 - 2012	Males	Adults	1	14	21	10	2
		Juveniles	1	-	1	-	1
		Pups	-	-	-	-	-
	Females	Adults	6	11	20	1	8
		Juveniles	1	-	-	-	1
		Pups	-	-	-	-	-

655 **Table 2** - Details of 24 *Brucella* culture positive harbour seals. The last two digits of the
 656 reference number indicate the year of stranding. * Indicates individuals that were culture
 657 positive but seronegative. MLN, mesenteric lymph node; IILN, internal iliac lymph node;
 658 EILN, external iliac lymph node; GLN, gastric lymph node, ManLN, mandibular lymph
 659 node; HLN, hepatic lymph node; TLN, thoracic lymph node and CLRN, colorectal lymph
 660 node; SI, small intestine.

Reference Number	Positive Cultures	Negative Cultures
M2357/93	Spleen	lung, liver
M2466/93	Spleen	lung, MLN
M2533/93	Spleen	MLN, SI
M292/94*	Spleen	testes, MLN, SI
M336/94*	IILN	spleen, MLN
M339/94	GLN	spleen, IILN
M972/94	EILN, manLN	spleen, MLN
M490/95	EILN, HLN, IILN, TLN	lung, spleen, brain, CRLN, GLN, manLN, blood, SI
M514/96	Lung	
M445/99	Lung	liver, spleen, kidney, brain, MLN, blood, SI
M13/01	Lung	liver, spleen, kidney, blood
M250/02	lung, liver, spleen, kidney, MLN, blood	brain, SI
M305/02	Spleen	lung, liver, kidney, brain, MLN, cellulitis, SI
M342/02	MLN	lung, liver, spleen, kidney, brain, blood
M374/02	lung, liver, spleen, kidney	brain, MLN
M449/02	Lung	
M599/02	MLN	lung, liver, spleen, kidney
M43/09	lung, liver, spleen, kidney, MLN, SI	
M91/10	lung, brain	liver, spleen, kidney, MLN
M228/10	pancreas	lung, liver, spleen, kidney, brain,

Scottish harbour seals' exposure to *Brucella*

		MLN, SI
M244/10	lung, liver, spleen, brain, MLN, SI	Kidney
M273/10	lung, MLN	liver, spleen, kidney, brain, pre- scapular LN, SI
M341/11	lung, spleen, MLN, SI	liver, kidney, brain, abscess
M337/13	kidney, brain	lung, liver, spleen

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676 **Table 3** - Comparison of the prevalence (% of seropositive animals) using the Rose Bengal
 677 plate agglutination test (RBT) and the competitive ELISA (cELISA). The ELISA results
 678 indicate a higher overall prevalence of *Brucella* antibodies in harbour seals than the Rose
 679 Bengal results.

	% of Seropositive Animals	
	RBT	cELISA
Scotland (n = 343)	15.9	25.4
North East (n = 38)	28.6	28.9
North West (n = 27)	14.8	33.3
Orkney (n = 101)	12.9	28.7
South East (n = 123)	19.8	20.3
South West (n = 54)	3.9	24.1
Males (n = 183)	14.5	25.7
Females (n = 160)	17.4	24.4
Adults (n = 306)	14.5	25.2
Juveniles (n = 15)	60.0	53.3
Pups (n = 22)	4.5	9.09
1997-2000 (n = 63)	12.3	22.2
2001-2005 (n = 87)	24.7	26.4
2006-2008 (n = 94)	12.0	18.1
2009-2012 (n = 99)	13.7	33.3

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