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

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ABSTRACT

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

KEY WORDS

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

INTRODUCTION

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

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

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

Members of the genus *Brucella* are Gram-negative, rod-shaped bacteria that cause chronic disease most commonly associated with abortions and infertility in domestic livestock (Seleem et al., 2010). Since the first reports of *Brucella* in a marine mammal in 1994 (Ross et al., 1994), infections have been recognised in a range of pinniped and cetacean species worldwide (Thakur et al., 2012). Strains isolated from marine mammals have been shown to be phenotypically and genetically distinct from those isolated from terrestrial mammals, and two species have been described that have pinnipeds and cetaceans as their preferred hosts, *Brucella pinnipedialis* and *Brucella ceti* respectively (Foster et al., 2007). *Brucella ceti* infections in cetaceans have been associated with various pathologies which include abortions and neonatal mortality (Miller et al., 1999), epididymitis in males (Dagleish et al., 2008), meningoencephalitis (González et al., 2002; Jauniaux et al., 2010; Alba et al., 2013; Garofolo et al., 2014), abscesses (Foster et al., 1996; Foster et al., 2002), endocarditis (González-Barrientos et al., 2010), mastitis, pneumonia, peritonitis, osteomyelitis and spinal discospondylitis (Foster et al., 2002). In contrast, pathology associated with *Brucella pinnipedialis* in seals is lacking despite several reports of its isolation (Foster et al., 2002; Nymo et al., 2011; Siebert et al., 2017; Tryland et al., 2005).With respect to harbour seals specifically, *Brucella* has previously been isolated from wild animals (Foster et al., 2002: Garner et al., 1997; Prenger-Berninghoff et al., 2008; Ross et al., 1994), and in several areas they have also been found to be seropositive (Gaydos et al., 2005; Hueffer et al., 2013; Maratea et al., 2003; Ross et al., 1996). However, the extent to which harbour seals in Scotland, or the United Kingdom in general, are currently exposed to this pathogen since the onset of the declines is unknown. Here, tissue samples from dead harbour seals collected by 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 seroprevelance
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 CO_2. 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**

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

Serological Methods

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

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

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

Statistical Analysis of Serological Data

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

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

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

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

RESULTS

Brucella Cultures

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

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

Serology

Test Performance

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

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

Seroprevalence

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

Antibody Levels

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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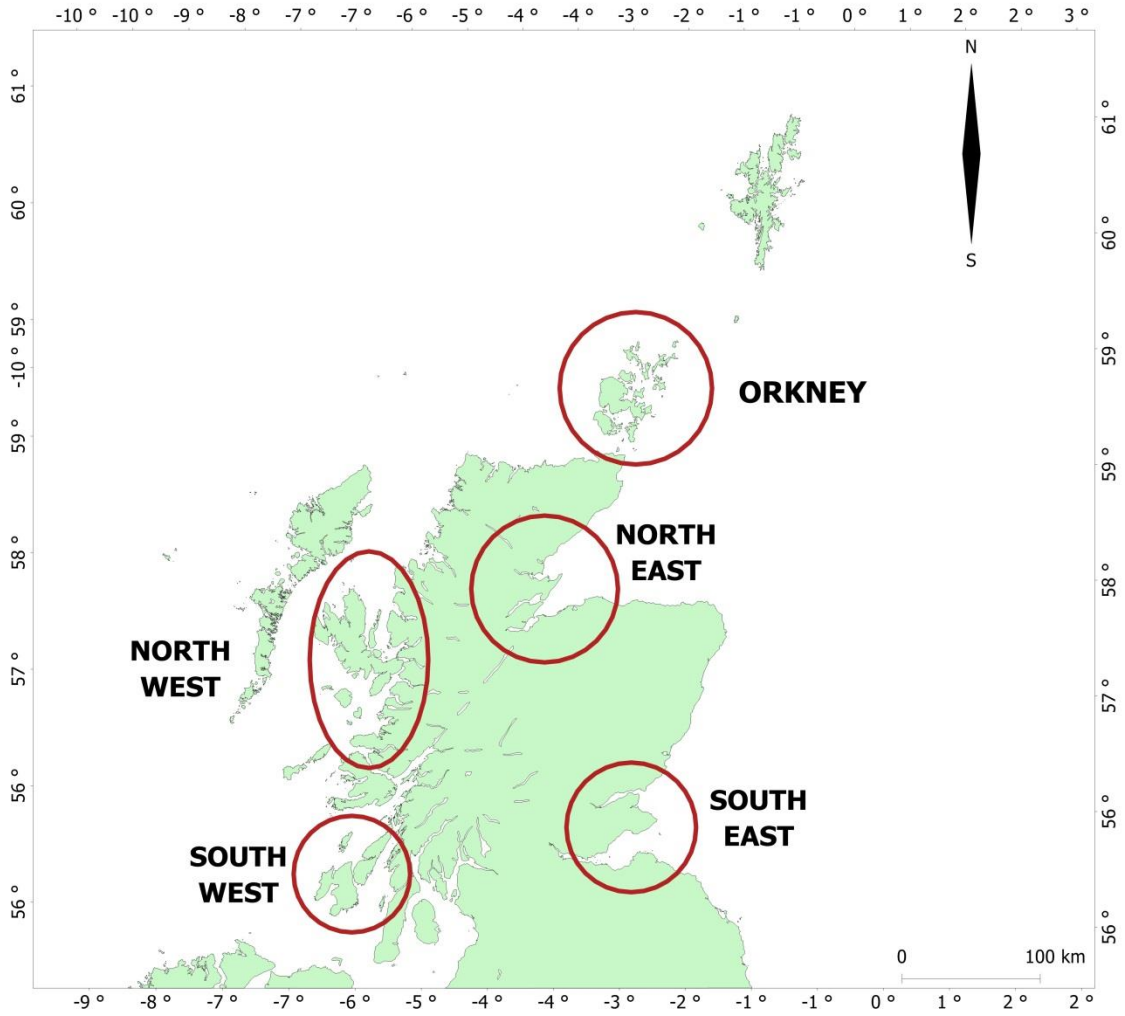

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628 ***Fig 1. Sampling regions of live-captured harbour seals across Scotland between 1997 and***
629 ***2012 by the Sea Mammal Research Unit.*** *Serum samples were grouped into 5 main regions*
630 *across Scotland as indicated by the circles on the 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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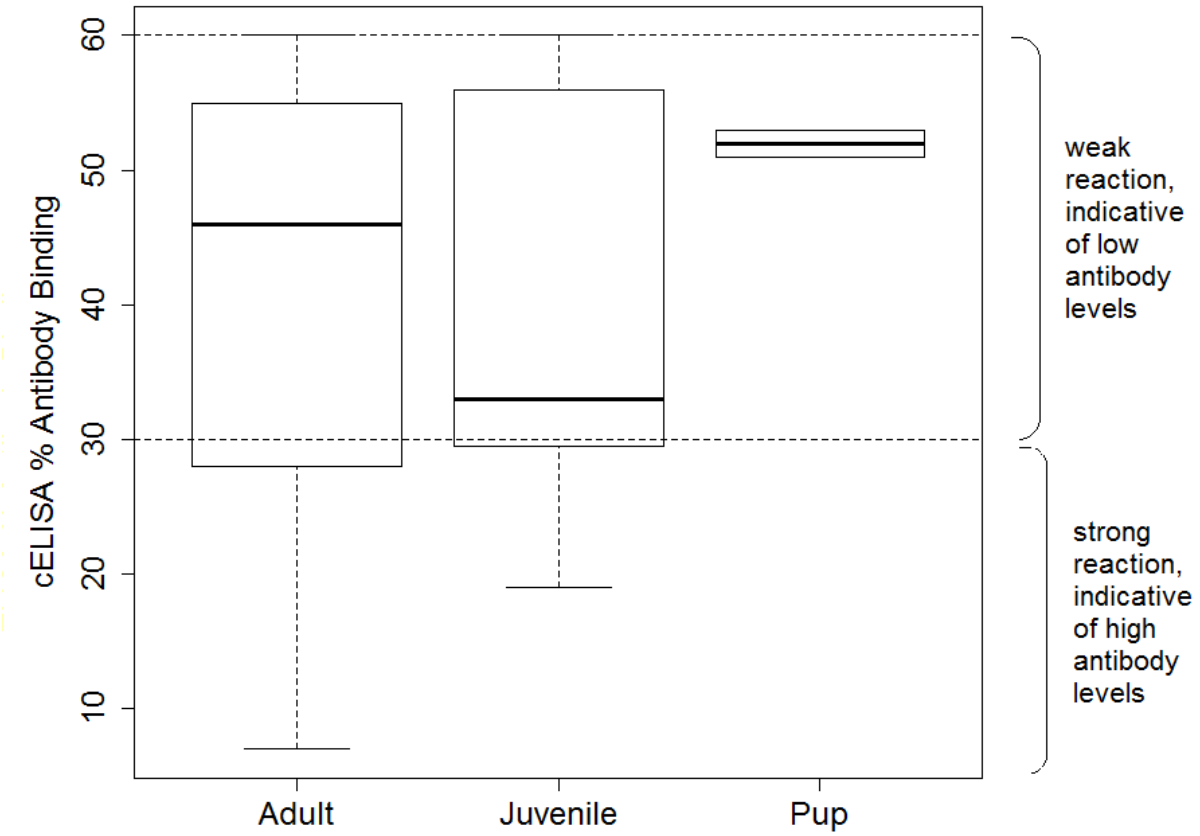
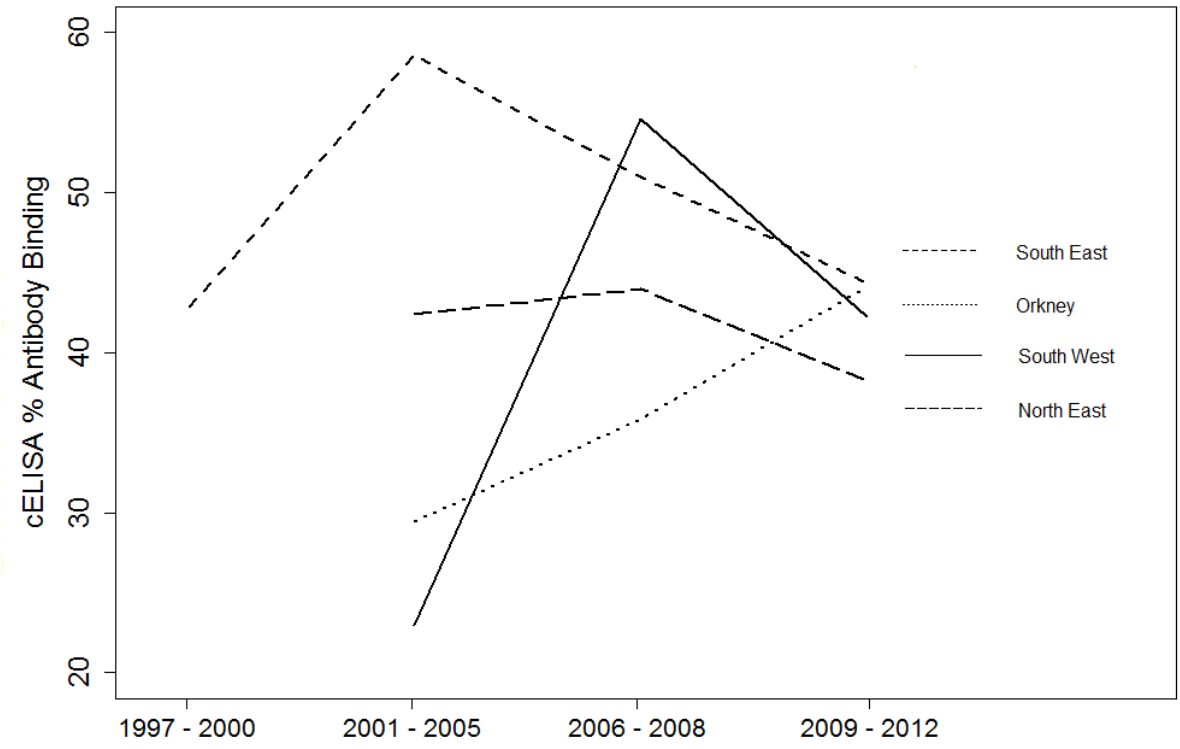


Fig. 2. cELISA antibody binding of the seropositive animals by age class. *Low antibody binding indicates high levels of circulating Brucella antibodies in the seals. The pups had lower circulating antibody levels than both adults and juveniles.*



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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,

		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

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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