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EPIZOOTIOLOGY OF *BRUCELLA* INFECTION IN AUSTRALIAN FUR SEALS

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ABSTRACT: Novel members of the bacterial genus *Brucella* have recently emerged as pathogens of various marine mammal species and as potential zoonotic agents. We investigated the epizootiology of *Brucella* infection in Australian fur seals (*Arctocephalus pusillus doriferus*) by establishing demographic and temporal variations in antibody prevalence, attempting isolation of the causative agent, and determining whether this potential pathogen is involved in frequent abortions observed in this pinniped species. Two competitive enzyme-linked immunosorbent assays (cELISAs), an indirect ELISA, and a fluorescence polarization assay (FPA) were used to test sera for *Brucella* antibodies. The FPA and cELISA proved suitable for use in this species. Significant differences in antibody prevalence were found between age classes of seals sampled between 2007 and 2009 at one colony. Pups sampled at this site ($n=134$) were negative for *Brucella* antibodies by all serologic tests but 17 of 45 (38%) of juveniles were antibody-positive. Antibody prevalence in adult females was significantly higher than in juveniles ($P=0.044$). Antibody prevalence for adult females between 2003 and 2009 varied significantly over time ($P=0.011$), and for individuals sampled between 2003 and 2005, the likelihood of pregnancy was greater in individuals positive for *Brucella* antibodies ($P=0.034$). Inflammatory lesions suggestive of infectious agents were found in 14 of 39 aborted Australian fur seal pups, but pathologic changes were not uniformly consistent for *Brucella* infection. Culture and PCR investigations on fetal tissues were negative for *Brucella*. Culture and PCR on selected fresh or frozen tissues from 36 juvenile and adult animals were also negative. We suspect that the prevalence of active infection with *Brucella* in Australian fur seals is low relative to antibody prevalence.

Key words: Abortion, Australian fur seals, *Brucella*, disease, epizootiology, gestational failure, pinniped, serology.

INTRODUCTION

Brucella infections of terrestrial animals are a well-recognized cause of abortion and infertility and, consequently, of high economic concern to the domestic livestock industry (Van Campen and Rhyan, 2010). Infection of marine mammals with *Brucella* spp. has been recognized since the mid-1990s (Ross et al., 1994). Subsequently, serologic evidence of exposure to bacteria of this genus has been found in many species from various geographic areas (Nielsen et al., 2001; Tryland et al., 2005). Marine strains of *Brucella* are genetically and biochemically distinct from other species in this genus, and isolates from cetaceans and pinnipeds have been proposed recently as two new species, respectively, *Brucella ceti* and *Brucella pinnipedialis* (Foster et al., 2007; Dawson et al., 2008b).

Although serology shows evidence of exposure to *Brucella* spp. in cetaceans and pinnipeds in the Southern Hemisphere, no isolates have been made from marine mammals in this region (Blank et al., 2002; Dawson, 2005; Abalos et al., 2009). However, isolates from humans with severe brucellosis who were resident in South America and New Zealand were characterized as marine mammal types (Sohn et al., 2003; McDonald et al., 2006; Dawson et al., 2008b), further supporting

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the organism's presence in marine mammals of the Southern Hemisphere and highlighting its zoonotic potential.

Brucella has been demonstrated to cause fatal neurobrucellosis in striped dolphins (*Stenella coeruleoalba*) from European and Central American waters and has also been detected in placental and fetal tissues from this species (Hernandez-Mora et al., 2008; Davison et al., 2009; Gonzalez-Barrientos et al., 2010). *Brucella* has also been associated with abortion and placentitis in bottlenose dolphins (*Tursiops truncatus*) (Miller et al., 1999), and epididymitis and orchitis in harbor porpoises (*Phocoena phocoena*; Foster et al., 2002; Dagleish et al., 2008), minke whales (*Balaenoptera acutorostrata*), and one Bryde's whale (*Balaenoptera edeni*; Ohishi et al., 2003). These findings suggest that, as in terrestrial mammals, brucellosis in cetaceans is associated with reproductive failure.

In contrast to cetaceans, *Brucella* spp. infections in pinnipeds are not associated with a clear pattern of pathology and the bacterium is often isolated from apparently healthy animals (Foster et al., 2002; Prenger-Berninghoff et al., 2008). *Brucella* spp. infections in pinnipeds appear to be most commonly associated with bronchopneumonia, potentially only acting as a secondary agent after parasitic infection (Prenger-Berninghoff et al., 2008). However, *Brucella* was recently suggested as a possible cause of abortion in California sea lions (*Zalophus californianus*; Goldstein et al., 2009).

Adult female Australian fur seals (*Arctocephalus pusillus doriferus*) have a high prevalence (57%) of antibodies to an unidentified *Brucella* sp. (Lynch et al., unpubl. data). Additionally, spontaneous abortions are commonly observed in Australian fur seals during the latter half of pregnancy, affecting up to 37% of known pregnant females (Gibbens et al., 2010). Therefore, the aims of this study were to investigate whether *Brucella* is associated with abortion in Australian fur seals and to investigate the epizootiology of this potential pathogen. This includes the demographic, temporal, and spatial variations in antibody prevalence, isolation of the causative agent, routes of infection, and pathologic consequences. Establishing these epizootiologic parameters will enable an assessment of the importance of *Brucella* as a potential population regulatory factor in Australian fur seals.

MATERIALS AND METHODS

Blood sampling

The primary study site was the Australian fur seal colony on Kanowna Island (39°10′S, 146°18′E), northern Bass Strait, Australia. Capture efforts were concentrated at one colony to limit potential intercolony confounding factors that might affect antibody prevalence. Sampling of seals belonging to the juvenile and pup age classes was conducted annually between 2007 and 2009. These age classes were sampled as encountered rather than selected on the basis of sex. Animals were classed as juveniles if they were estimated to be >1 yr of age on the basis of the presence of an erupted permanent canine tooth and <3 yr of age on the basis of body weight (<50 kg for females and <100 kg for males). In females, where age class was uncertain, the detection of pregnancy (by palpation) or positive lactational status was used to distinguish adults from juveniles. The pupping season of Australian fur seals is synchronized and occurs between mid-November and mid-December (Gibbens and Arnould, 2009). Pups (<1 yr old) from the 2006 cohort were sampled between April and October in 2007, meaning they were 4–10 mo old. The 2007 cohort were sampled in January 2008 (<2 mo old) and the 2008 cohort in late November 2008 when <2 wk old.

To investigate possible spatial differences in antibody prevalence, pups from three other Australian fur seal colonies, The Skerries (37°45′S, 149°31′E), Seal Rocks (38°30′S, 145°10′E) and Lady Julia Percy Island (38°42′S, 142°00′E), were sampled between late December 2007 and late January 2008. This sampling was conducted concurrently with a mark-recapture study (Kirkwood et al., 2010).

Because adult females sampled on Kanowna Island between 2007 and 2009 had a high prevalence of *Brucella* antibodies, additional analyses were performed on samples and data collected from adult females at the same site

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between 2003 and 2005. For this data set, information concerning each individual's pregnancy and lactation status, age, and reproductive success was available (Gibbens et al., 2010).

Desired sample sizes were calculated on the assumption that the prevalence in the juvenile and pup age classes might be lower than that established for adult females. WinPepi epidemiologic software, Version 10.5 (Abramson, 2004), was utilized. The estimated size of the Kanowna Island colony is 15,000 individuals, which includes an annual production of approximately 3,400 pups (Kirkwood et al., 2010). A desired sample size of 59 for each age class was required to enable a 95% probability of detecting at least one positive animal if antibody prevalence was 5% or greater.

Animals were selected at random and captured by hand (young pups) or with a large hoop net. Seals were manually restrained and blood samples collected from the interdigital hind flipper veins or from a vein lying in the webbing of the fore flipper. Samples were transferred into untreated tubes (BD Vacutainers, Becton, Dickinson, and Co. North Ryde, Australia) and allowed to clot for at least 1 hr before centrifugation. Serum aliquots were stored at ≤−20 C until analysis.

Antibody assays

Sera were assayed for *Brucella* spp. antibodies using a commercially available, competitive enzyme-linked immunosorbent assay (cELISA) kit (Svanovir®, Svanova Biotech, Uppsala, Sweden). Testing of sera with the use of this kit was performed at the laboratories of the Veterinary Diagnostic Services (Department of Primary Industries, Attwood, Victoria 3049, Australia). Additionally, all juvenile and adult female samples plus sera from 10 pups from each year were tested by a second cELISA, an indirect ELISA (iELISA), and a fluorescence polarization assay (FPA) at the Canadian Food Inspection Laboratories (Ottawa, Canada), so that test performance and agreement could be assessed. Although these tests have been used to investigate *Brucella* antibody status in marine mammals (Nielsen et al., 2005), they have not been validated for this purpose.

The Svanovir cELISA, was performed according to the manufacturer's instructions. The test uses a monoclonal antibody specific for the M-84 epitope common to all *Brucella* species. An inhibition value of ≥30% was considered positive. Sera that fell within 5% of this cutoff were retested, and the mean value was used to determine the final result.

The second cELISA, the iELISA, and the FPA were performed according to the methods described by Nielsen et al. (2005). For the cELISA, an inhibition value of ≥30% was considered a positive result. For the iELISA, inhibition of binding ≥20% was considered positive. On the basis of previous experiments with seal sera (Nielsen et al., 2005), a cutoff millipolarization value of ≥90 mP was used to indicate a positive FPA.

Tissue and swab collection

Aborted Australian fur seal pups were collected between 2007 and 2010 during visits to all field sites. Some fetal tissues had been scavenged by seabirds before collection. If available, tissue samples were taken from placenta, lung, liver, spleen, kidney, axillary lymph node, brain, heart, thymus, stomach, intestine, pharynx, and eye and preserved in 10% buffered formalin for histologic examination. For fetuses processed in the field, placenta, lung, liver, spleen, and stomach contents were frozen in liquid nitrogen and then held at −70 C until microbiologic and molecular analysis for *Brucella*. For a small number of fetuses, it was possible to immediately culture fresh tissues.

Juvenile and adult Australian fur seals found dead and dying along the Victorian coastline between 2007 and 2010 were collected for postmortem examination. In addition to the tissue samples listed for fetuses (excepting placenta and thymus), gonad, uterus, mammary gland, mediastinal lymph node, thyroid, adrenal glands, and urinary bladder were routinely collected into formalin. Samples of these tissues were also frozen at −70 C. For some animals, fresh lung and lymph nodes were also cultured for *Brucella* spp.

Frozen lung from four juveniles observed to be infected with lungworm on histologic examination were dissected to isolate the parasite. Slices of lung were immersed in 0.9% saline and individual worms teased from the tissue using a dissecting microscope. Worms isolated were held in saline overnight before PCR analysis for *Brucella*.

Brucella can be isolated from milk of some terrestrial species infected with this pathogen (Rhyan et al., 2009). Milk samples obtained from Australian fur seals at Kanowna Island between 2003 and 2008 were, therefore, investigated by culture and PCR for the presence of *Brucella*. Milk had been frozen at −20 C and thawed two or three times before analysis.

Brucella has been isolated from the digestive tract of some pinnipeds (Prenger-Berninghoff

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et al., 2008). Buccal and rectal microbiologic swabs were collected from six juveniles captured at Seal Rocks in July 2010 for an ecologic study. Swabs were held at 4 C in Amies transport medium (BD Diagnostics) for up to 3 days before culture.

Tissue and swab analysis

Formalin-fixed tissues were embedded in paraffin, sectioned at 5-µm intervals, and stained with hematoxylin and eosin for histologic examination. Fresh tissues were inoculated onto *Brucella*-selective medium, Farrell's agar (Farrell and Robertson, 1972), and either sheep or horse blood agar (HBA). They were incubated at 37 C in 10% CO_2 for ≥3 days and, in most cases, up to 14 days. Homogenate prepared from frozen tissues was used to inoculate *Brucella*-selective biphasic medium (Corner and Alton, 1987), serum dextrose agar, and HBA. Media were incubated at 37 C for 4 wk and checked every 3 days for colonies resembling *Brucella*. Selected colonies were subcultured onto HBA and gram stained, and oxidase and catalase tests were performed to determine whether further biochemical identification tests were warranted.

For PCR analysis, DNA was extracted from tissue homogenate using QIAamp DNA Mini Kit (Qiagen Pty Ltd, Doncaster, Australia). DNA template was added to a PCR reaction mix containing HotStar TaqMaster Mix (Qiagen) primers targeting the *Brucella*-specific *bp26* gene and downstream IS*711* element (Cloeckaert et al., 2000). In addition to the sample templates, positive and negative controls were included. Positive controls included a marine strain of *Brucella* isolated from a New Zealand patient (McDonald et al., 2006), *Brucella suis* bv. 3, and *Brucella melitensis* bv. 2 WHO reference strains (Australian Animal Health Laboratory, Geelong, Victoria, Australia).

Statistical analysis

Statistical analyses were performed with the software program PASW Statistics 18 (SPSS Inc, Chicago, Illinois, USA). Antibody prevalence estimates were compared between age classes and years by chi-square tests for independence. Agreement between serologic tests was investigated by the Kappa measure of agreement. For the 2003–2005 data from adult females, the likelihoods of being pregnant and, if pregnant, giving birth were modeled by binary logistic regression. The analyses repeated those of Gibbens et al. (2010), with the use of the same data set but adding an additional

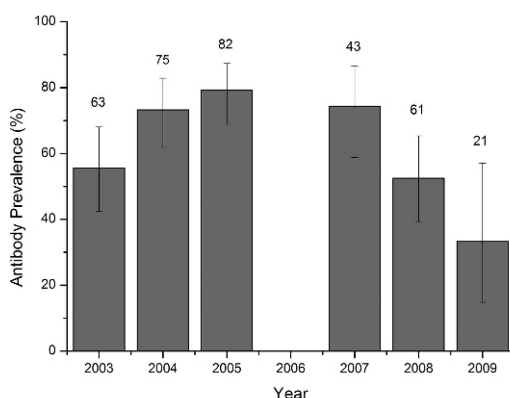

FIGURE 1. Temporal variation in prevalence of antibody to *Brucella* spp. (Svanovir *Brucella* competitive enzyme-linked immunosorbent assay) in adult, female Australian fur seals on Kanowna Island, northern Bass Strait, Australia. Error bars are 95% confidence intervals; samples sizes are shown above bars.

independent variable, *Brucella* antibody status. A backward stepwise elimination method was used, in which the least significant variable ($P>0.1$) was removed after each step.

RESULTS

Using the Svanovir cELISA, 17 of 45 (38%; 95% CI, 23.8–53.5) juvenile seals sampled at Kanowna Island between 2007 and 2009 were positive for *Brucella* antibodies. This was significantly less ($\chi^2=4.06$, $n=170$, $P<0.044$) than the prevalence for adult females sampled in the same period, in which 71 of 125 (57%) were positive. No antibodies to *Brucella* were found in 134 pups sampled at Kanowna Island, and only one antibody-positive individual was identified from 167 pups sampled from Seal Rocks ($n=57$), Lady Julia Percy Island ($n=57$), and The Skerries ($n=53$).

Prevalence of antibody to *Brucella* spp. in adult females sampled between 2003 and 2009 appeared to vary temporally (Fig. 1). Antibody prevalence in 2003 was compared with 2005 and found to have significantly increased ($\chi^2=7.24$, $n=145$, $P<0.007$). Prevalence was then found to have declined significantly compared with years 2007 and 2009 ($\chi^2=8.35$, $n=64$,

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TABLE 1. Numbers of Australian fur seals from Kanowna Island, Australia (by year and age class) tested and found positive for antibody to *Brucella* spp. using two competitive enzyme-linked immunosorbent assays (cELISA), and indirect ELISA (iELISA) and a fluorescence polarization assay (FPA).[a]

		No. positive/tested			
Year	Age class	Svanovir cELISA	cELISA	iELISA	FPA
2007	Pup	0/58	2/10	0/10	0/10
	Juvenile	0/9	2/9	5/9	1/9
	Adult	32/43	24/43	36/43	34/43
2008	Pup	0/57	0/10	0/10	0/10
	Juvenile	16/28	9/28	16/28	2/28
	Adult	32/61	40/61	58/61	24/61
2009	Pup	0/19	0/10	2/10	0/10
	Juvenile	1/8	2/8	1/8	1/8
	Adult	7/21	NT	NT	NT

[a] NT=not tested.

$P<0.004$). One adult female sampled at 6 yr old in 2004 was recaptured in 2010. Her initial sample was positive (inhibition=41.42), but in 2010 she was antibody-negative (inhibition=1.51).

Samples from 30 pups classified as antibody-negative on the Svanovir cELISA were assayed with the additional three serologic tests. Antibody titers exceeding minimum thresholds were found in four individuals: two with the cELISA and two with the iELISA (Table 1). In juveniles, antibody prevalence calculated by the additional tests was 29% (13 of 45) for the cELISA, 49% (22 of 45) for the iELISA, and 11% (4 of 45) for the FPA. Sera from adult females sampled in 2007 and 2008 were retested with the three additional assays. Although their antibody prevalence was 62% with the Svanovir cELISA, prevalences were 62, 90, and 56% with the cELISA, iELISA, and FPA, respectively. Combining all age classes, the agreement between the Svanovir cELISA and each of the additional tests was moderate (Svanovir cELISA*cELISA, K=0.57, $P<0.0005$; Svanovir® cELISA*iELISA, K=0.54, $P<0.005$; Svanovir cELISA*FPA, K=0.63, $P<0.005$).

In females sampled between 2003 and 2005, association between positive antibody status and pregnancy was significant ($\chi^2=4.40$, $n=200$, $P<0.036$). This finding was consistent with logistic regression analysis modeling pregnancy as the dependent variable. The final model contained age (Gibbens et al., 2010) and antibody status ($\chi^2=32.17$, $n=200$, $P<0.025$) as significant independent variables. Although antibody-positive individuals had a greater likelihood of being pregnant, the size of the effect was small (phi correlation coefficient=0.164). Antibody status had no influence on the likelihood of aborting with birth success as the dependent variable in logistic regression analysis. Chi-square tests of serologic status in aged (>10 yr) versus young (≤10 yr) females were not significant.

Thirty-nine fetuses were examined over the course of the study. The placenta was available for sampling from 11 of these. Placentitis, characterized by a diffuse, leukocytic infiltrate was present in one individual (Fig. 2a). The lung was the most common site of pathology. Alveoli and bronchioles in 25 of 29 (86%) lungs examined had mild to marked accumulations of cytokeratin-positive squamous cells, suggesting fetal respiratory distress before abortion. Interstitial pneumonia, characterized by mononuclear (lymphocytic and plasmocytic) infiltrates and interstitial edema was observed in 6 of 29 (21%) of these individuals, and an

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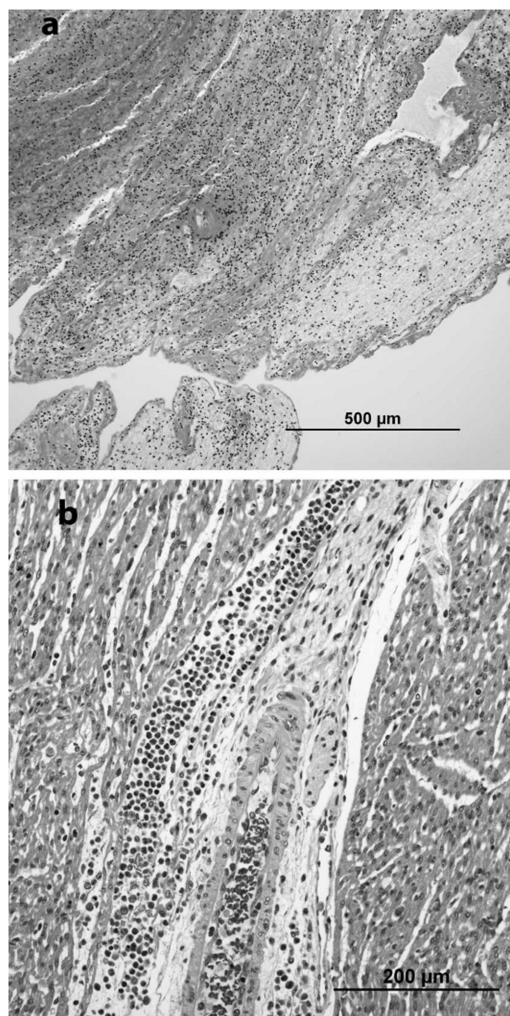

FIGURE 2. Histologic sections demonstrating pathology in tissues of aborted Australian fur seal pups collected on Seal Rocks, northern Bass Strait, Australia (H&E stain). (a) Placentitis characterized by a moderately diffuse infiltration of neutrophils and mononuclear cells from a fetus collected in August 2008. (b) Perivascular infiltrate consisting predominantly of neutrophils and mononuclear cells in the myocardium from a fetus collected in September 2009.

additional two fetuses had foci of mixed inflammatory infiltrates within the lung. One fetus with severe interstitial pneumonia also had lymphoplasmacytic phlebitis of hepatic vessels, a mild diffuse nonsuppurative meningitis, and significant perivascular leukocytic infiltration in the myocardium (Fig. 2b). Cardiac inflammatory infiltrates were noted in 8 of 30 (27%) fetuses. Five of these were of a perivascular nature in the myocardium or epicardium, and three fetuses exhibited pericarditis, characterized by mixed lymphocytic and leukocytic infiltrates.

No *Brucella* spp. were isolated from fresh or frozen tissues collected from fetuses, juveniles, or adults, and no positive *Brucella* PCR results were obtained (Table 2). Two lung worms were isolated from one of four animals examined and were negative on *Brucella* PCR. Culture results on 106 milk samples were negative and 58 of these samples were tested by PCR and were also negative. Swabs of the buccal cavity and rectum collected from six juvenile seals were negative on culture for *Brucella*.

DISCUSSION

The positive titers obtained from multiple assay types and the significant level of agreement between these tests give a high level of confidence that our results are representative of the exposure of Australian fur seals to *Brucella* sp. rather than cross-reacting bacteria (Kittelberger et al., 1995). Differences in prevalence estimates obtained from the four tests highlight the need to consider test performance when presenting results of serologic surveys. The two cELISAs gave similar results, whereas the iELISA gave a higher proportion of positive results in all age classes. In cattle, cELISAs have an advantage over iELISAs in most occasions, in that they can distinguish between antibodies to *Brucella* spp. and those to other cross-reacting gram-negative bacteria (Nielsen, 1990; Samartino et al., 1999). Whether the cELISA behaves similarly in pinnipeds is unknown, but our study is consistent with findings in Hawaiian monk seals (*Monachus schauinslandi*), in which it was concluded that the iELISA was an unreliable test for the identification of *Brucella* antibody–positive individuals (Nielsen et al., 2005). The FPA returned the fewest

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TABLE 2. Number and types of tissue samples investigated for *Brucella* spp. by specific culture (fresh and frozen) and by polymerase chain reaction (PCR) for Australian fur seals collected in Victoria, Australia, 2007–2010.

	No. of samples tested								
	Fetus			Juvenile			Adult		
Sample type	Fresh	Frozen	PCR	Fresh	Frozen	PCR	Fresh	Frozen	PCR
Umbilicus	6								
Placenta		9	2						
Lung	6	25	5	2	18	11	1	8	6
Lymph node[a]	1	2	2	7	16	6	3	8	6
Stomach content	2	14	3						
Spleen	1	23	5	4	17	6		8	6
Kidney	2				2				
Liver		13	5	2	7	4			
Testis/epididymis		3			7	3		2	2
Uterus		1		1	6	2		5	4
Mammary							1	6	4
Brain		2	1		1				
Abscess				3	2			1	1

[a] Numbers represent pooled lymph node samples from individual animals.

number of positive results; this is also consistent with Nielsen et al. (2005), who suggested that the FPA is significantly more specific than the iELISA and marginally more specific than the cELISA.

The finding that no Australian fur seal pups from Kanowna Island had titers of *Brucella* antibodies exceeding threshold values contrasts with the relatively high antibody prevalence in adult females from this location. Pup samples from Kanowna Island included 76 individuals 1–2 mo old, with 19 of these animals less than 2 wk old. It would be expected that passively transferred maternal antibodies would still be present in pups of this age. In addition, only one positive individual was found from 167 pups 1–2 mo of age sampled from three other colonies. The lack of antibodies in these pups contrasts to terrestrial species in which offspring born to antibody-positive dams have detectable passively transferred antibodies on standard serologic tests for 2–5 mo after birth (Ray et al., 1988; Rhyan et al., 2009). The findings suggest that if passive transfer of *Brucella* antibodies to pups occurs in Australian fur seals, antibody titers are well below threshold values used in this study.

The finding that Australian fur seal pups lack detectable *Brucella* antibodies contributes to determining the age that individuals are first exposed to infection. Antibodies to *Brucella* have been detected in many pinniped species, but few studies have included all age classes in serologic surveys. Therefore, understanding of the timing of exposure to *Brucella* and antibody status conversion in pinnipeds is lacking. Serum from 1 of 3 dead pups and 3 of 11 adult female Antarctic fur seals (*Arctocephalus gazella*) were positive for *Brucella* antibodies on cELISA with the use of a threshold inhibition value of 30% (Retamal et al., 2000). The result from the pup, however, was borderline positive and, therefore, possibly doubtful. Fourteen preweaned, live pups sampled from the same population one year later were all negative, but 3 of 61 adult females were antibody-positive (Blank et al., 2001). In harbor seals (*Phoca vitulina*), 2 of 18 pups (undefined weaning status) were positive to *Brucella* on iELISA and cELISA and higher prevalences were found in both juvenile and adult age classes (Zarnke et al., 2006). Increased prevalence with age was also noted in Hawaiian monk seals, in

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which progressively higher proportions of animals with *Brucella* antibodies were found in the weaned pup, juvenile, and adult age classes (Aguirre et al., 2007). We also observed increasing prevalence of *Brucella* antibodies with pup, juvenile, and adult age classes. Although pups between 6 and 10 mo of age were sampled only in 2007, the lack of positive results in this group suggests that antibody status conversion to *Brucella* occurs after 10 mo of age in Australian fur seals.

Routes of infection for *Brucella* spp. include inhalation, ingestion, and venereal transmission (Nicoletti, 1980; Glynn and Lynn, 2008). Timing of antibody status conversion in Australian fur seals might be explained by age at weaning in this species. Australian fur seals normally wean at 10–12 mo of age, with some individuals suckled well into their second year (Warneke and Shaughnessy, 1985; Hume et al., 2001). Our results are, therefore, consistent with a hypothesis that first exposure to *Brucella* spp. in Australian fur seals is associated with the change to a prey-based diet. It has been suggested lungworms carrying *Brucella* are the means by which marine mammals become infected with this bacterium (Garner et al., 1997; Dawson et al., 2008a). Pinnipeds are infected with lungworm by consumption of intermediate host fish species (Daily, 1970), which could be the means that Australian fur seals are first exposed to *Brucella*. Although seal strains of *Brucella* have not yet been isolated from marine fish, *B. melitensis*, an important pathogen of humans and goats, was recently isolated from freshwater fish in Egypt (El-Tras et al., 2010).

Although many pinniped populations have been surveyed for *Brucella* antibodies, no previous studies have stratified and analyzed data temporally. Antibody prevalence to *Brucella* in adult females from Kanowna Island varied significantly between 2003 and 2009. Factors that influence antibody prevalence estimates include the duration of antibody following exposure, the ability of the bacteria to establish chronic infection, and the frequency of repeated exposures to the bacteria. In terrestrial animals, *Brucella* antibody duration in the absence of chronic infection or repeated exposure is 1–2 yr (Glynn and Lynn, 2008). Longitudinal data were only available from one individual in our study, and this adult female changed from antibody-positive to antibody-negative over 6 yr. This suggests that antibodies elicited by *Brucella* exposure in Australian fur seals are not longer lasting than those in terrestrial animals and that this animal had not repeatedly been exposed to infection. However, the finding that older adult females were not more likely to be antibody-negative suggests that exposure to *Brucella* could occur at any life stage postweaning, by maintenance of infection within the population (enzootic disease) or by repeated exposure to an infection source.

If antibody prevalence in the seal population is maintained by repeated exposure to *Brucella* in some prey species, then environmental factors affecting prey densities could influence frequency of exposure to seals. In northern Bass Strait, interannual fluctuations in sea-surface temperature have been correlated with broad fluctuations in the prey composition of Australian fur seals (Kirkwood et al., 2008). The increase in *Brucella* prevalence between 2003 and 2005 corresponded with a protracted period of cooler-than-average sea surface temperatures and higher-than-average proportions of the fish redbait (*Emmelichthys nitidus*) in the diet of Australian fur seals in northern Bass Strait. Investigators conducting future studies to isolate the source of *Brucella* infection for Australian fur seals should consider investigating infection in selected prey species.

The finding of a relationship between pregnancy and *Brucella* antibody in Australian fur seals is not consistent with findings in many terrestrial species in which antibody-positive individuals have

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a higher rate of infertility (Glynn and Lynn, 2008; Rhyan et al., 2009; Neta et al. 2010). Infertility in terrestrial species is due to chronic infection with *Brucella,* and presence of antibody is positively correlated with the likelihood of isolating the pathogen (Roffe et al., 1999). The infection status for Australian fur seal females sampled in our study is unknown; therefore, interpretation of the relationship between pregnancy and antibody status requires further investigations.

Gestational failure in fur seals is believed to be a physiologic outcome related to interannual variation in food availability (Guinet et al., 1998; Gibbens and Arnould, 2009). Our finding of inflammatory lesions in fetal tissues suggests that infectious agents are likely involved in some instances of gestational failure. Suppurative lesions consistent with *Brucella* or other bacterial infection (Anderson et al., 1986; Neta et al., 2010) were seen in four individuals: one fetus had placentitis, two had pericarditis, and the fourth had lymphadenitis. However, the diffuse, interstitial pneumonia with a mononuclear infiltrate seen in six individuals is not typical for *Brucella,* for which suppurative pleuritis and bronchointerstitial pneumonia are expected to be the predominant lesions (Neta et al., 2010). In addition, the failure to isolate or detect *Brucella* spp. by molecular means from fetal tissues does not support the involvement of this pathogen in the aborted fetuses examined.

Failure to isolate *Brucella* or detect infection with PCR from tissues, milk, swabs of the gastrointestinal system, or lungworms suggests that the proportion of infected Australian fur seals is low, despite the high prevalence of antibody. A limitation to this aspect of the study was the predominant use of frozen tissues for culture, which would be expected to reduce the number of viable organisms in samples. In addition, fresh tissues were cultured only on Farrell's medium for *Brucella* investigation, and some marine mammal isolates grow poorly and slowly or not at all on this substrate (Foster et al., 2002). However, the factors that limited culture success would not affect the ability of PCR to detect organisms if present in the tissues examined. Tissues chosen for *Brucella* investigation were based on those found most commonly to produce isolates in phocid seals from the Northern Hemisphere (Garner et al., 1997; Foster et al., 2002; Prenger-Berninghoff et al., 2008), and it is possible that sites of infection are different for otariid seals. Furthermore, if the proportion of animals harboring infection is low compared with those with antibodies, then the numbers of animals, tissues, swabs, and lungworms examined might have been inadequate to detect a single infected animal. The prevalence of infected animals in other pinniped species is mostly unknown, although in one study, isolates were made from 3% (1 of 34) of the grey seals (*Halichoerus grypus*) and 11% (47 of 426) of the harbor seals (Prenger-Berninghoff et al., 2008).

Although further work would be needed to validate serologic assays for *Brucella* in Australian fur seals, we believe that the FPA test is probably the most suitable screening test but that cELISAs are adequate for this purpose. Prevalence of *Brucella* antibodies varied between age classes in Australian fur seals, and individuals are probably not exposed to infection until postweaning. Although many juveniles and adult Australian fur seals carry antibodies to a *Brucella* sp., it is likely that the prevalence of active infection is low. We found no conclusive evidence that *Brucella* causes gestational failure in Australian fur seals, but it is likely that infectious agents are involved in some cases of abortion.

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LITERATURE CITED

ABALOS, P., P. RETAMAL, O. BLANK, D. TORRES, AND V. VALDENEGRO. 2009. Brucella infection in marine mammals in Antarctica. Veterinary Record 164: 250–250.

ABRAMSON, J. H. 2004. WINPEPI (PEPI-for-Windows): Computer programs for epidemiologists. Epidemiological Perspectives and Innovations 1: 6.

AGUIRRE, A. A., T. J. KEEFE, J. S. REIF, L. KASHINSKY, P. K. YOCHEM, J. T. SALIKI, J. L. STOTT, T. GOLDSTEIN, J. P. DUBEY, R. BRAUN, AND G. ANTONELIS. 2007. Infectious disease monitoring of the endangered Hawaiian monk seal. Journal of Wildlife Diseases 43: 229–241.

ANDERSON, T. D., V. P. MEADOR, AND N. F. CHEVILLE. 1986. Pathogenesis of placentitis in the goat inoculated with *Brucella abortus*. 1. Gross and histologic lesions. Veterinary Pathology 23: 219–226.

BLANK, O., P. RETAMAL, P. ABALOS, AND D. TORRES. 2001. Additional data on anti-*Brucella* antibodies in *Arctocephalus gazella* from Cape Shirreff, Livingston Island, Antarctica. CCAMLR Science 8: 147–154.

———, ———, ———, AND ———. 2002. Detection of anti-brucella antibodies in Weddell seals (*Leptonychotes weddellii*) from Cape Shirref, Antarctica. Archivos De Medicina Veterinaria 34: 117–122.

CLOECKAERT, X., M. GRAYON, AND O. GREPINET. 2000. An IS711 element downstream of the *bp26* gene is a specific marker of *Brucella* spp. isolated from marine mammals. Clinical and Diagnostic Laboratory Immunology 7: 835–839.

CORNER, L. A., AND G. G. ALTON. 1987. Bovine brucellosis—Standard bacteriological techniques. Australian Diagnostic Techniques for Animal Diseases Series, No. 4, L. A. Corner and T. J. Bagust (eds.). CSIRO Publishing, East Melbourne, Australia, 14 pp.

DAGLEISH, M. P., J. BARLEY, J. FINLAYSON, R. J. REID, AND G. FOSTER. 2008. *Brucella ceti* associated pathology in the testicle of a harbour porpoise (*Phocoena phocoena*). Journal of Comparative Pathology 139: 54–59.

DAILEY, M. D. 1970. Transmission of *Parafilaroides decorus* (Nematoda-Metastrongyloidea) in California sea lion (*Zalophus californianus*). Proceedings of the Helminthological Society of Washington 37: 215–222.

DAVISON, N. J., M. P. CRANWELL, L. L. PERRETT, C. E. DAWSON, R. DEAVILLE, E. J. STUBBERFIELD, D. S. JARVIS, AND P. D. JEPSON. 2009. Meningoencephalitis associated with *Brucella* species in a live-stranded striped dolphin (*Stenella coeruleoalba*) in south-west England. Veterinary Record 165: 86–89.

DAWSON, C. E. 2005. Anti-*Brucella* antibodies in pinnipeds of Australia. Microbiology Australia 26: 87–89.

———, L. L. PERRETT, E. J. STUBBERFIELD, J. A. STACK, S. S. J. FARRELLY, W. A. COOLEY, N. J. DAVISON, AND S. QUINNEY. 2008a. Isolation and characterization of Brucella from the lungworms of a harbor porpoise (*Phocoena phocoena*). Journal of Wildlife Diseases 44: 237–246.

———, E. J. STUBBERFIELD, L. L. PERRETT, A. C. KING, A. M. WHATMORE, J. B. BASHIRUDDIN, J. A. STACK, AND A. P. MACMILLAN. 2008b. Phenotypic and molecular characterisation of *Brucella* isolates from marine mammals. BMC Microbiology 8: 224–231.

EL-TRAS, W. F., A. A. TAYEL, M. M. ELTHOLTH, AND J. GUITIAN. 2010. *Brucella* infection in fresh water fish: Evidence for natural infection of Nile catfish, *Clarias gariepinus*, with *Brucella melitensis*. Veterinary Microbiology 141: 321–325.

FARRELL, I. D., AND L. ROBERTSON. 1972. Comparison of various selective media, including a new selective medium for isolation of brucellae from milk. Journal of Applied Bacteriology 35: 625–630.

FOSTER, G., A. P. MACMILLAN, J. GODFROID, F. HOWIE, H. M. ROSS, A. CLOECKAERT, R. J. REID, S. BREW, AND I. A. P. PATTERSON. 2002. A review of *Brucella* sp. infection of sea mammals with particular emphasis on isolates from Scotland. Veterinary Microbiology 90: 563–580.

———, B. S. OSTERMAN, J. GODFROID, I. JACQUES, AND A. CLOECKAERT. 2007. *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. International Journal of Systematic and Evolutionary Microbiology 57: 2688–2693.

GARNER, M. M., D. M. LAMBOURN, S. J. JEFFRIES, P. B. HALL, J. C. RHYAN, D. R. EWALT, L. M. POLZIN, AND N. F. CHEVILLE. 1997. Evidence of *Brucella* infection in Parafilaroides lungworms in a Pacific harbor seal (*Phoca vitulina richardsi*). Journal of Veterinary Diagnostic Investigation 9: 298–303.

GIBBENS, J., AND J. P. Y. ARNOULD. 2009. Interannual variation in pup production and the timing of

Downloaded from http://meridian.allenpress.com/jwd/article-pdf/47/2/352/2238476/0090-3558-47_2_352.pdf by guest on 14 September 2024

breeding in benthic foraging Australian fur seals. Marine Mammal Science 25: 573–587.

———, L. J. Parry, and J. P. Y. Arnould. 2010. Influences on fecundity in Australian fur seals (*Arctocephalus pusillus doriferus*). Journal of Mammalogy 91: 510–518.

Glynn, M. K., and T. V. Lynn. 2008. Brucellosis. Journal of the American Veterinary Medical Association 233: 900–908.

Goldstein, T., T. S. Zabka, R. L. Delong, E. A. Wheeler, G. Ylitalo, S. Bargu, M. Silver, T. Leighfield, F. Van Dolah, G. Langlois, I. Sidor, J. L. Dunn, and F. M. D. Gulland. 2009. The role of domoic acid in abortion and premature parturition of California sea lions (*Zalophus californianus*) on San Miguel Island, California. Journal of Wildlife Diseases 45: 91–108.

Gonzalez-Barrientos, R., J. A. Morales, G. Hernandez-Mora, E. Barquero-Calvo, C. Guzman-Verri, E. Chaves-Olarte, and E. Moreno. 2010. Pathology of striped dolphins (*Stenella coeruleoalba*) infected with *Brucella ceti*. Journal of Comparative Pathology 142: 347–352.

Guinet, C., J. P. Roux, M. Bonnet, and V. Mison. 1998. Effect of body size, body mass, and body condition on reproduction of female South African fur seals (*Arctocephalus pusillus*) in Namibia. Canadian Journal of Zoology-Revue Canadienne De Zoologie 76: 1418–1424.

Hernandez-Mora, G., R. Gonzalez-Barrientos, J. A. Morales, E. Chaves-Olarte, C. Guzman-Verri, E. Barquero-Calvo, M. J. De-Miguel, C. M. Marin, J. M. Blasco, and E. Moreno. 2008. Neurobrucellosis in stranded dolphins, Costa Rica. Emerging Infectious Diseases 14: 1430–1433.

Hume, F., J. P. Y. Arnould, R. Kirkwood, and P. Davis. 2001. Extended maternal dependence by juvenile Australian fur seals (*Arctocephalus pusillus doriferus*). Australian Mammalogy 23: 67–70.

Kirkwood, R., F. Hume, and M. Hindell. 2008. Sea temperature variations mediate annual changes in the diet of Australian fur seals in Bass Strait. Marine Ecology–Progress Series 369: 297–309.

———, D. Pemberton, R. Gales, A. Hoskins, A. Mitchell, P. D. Shaughnessy, and J. P. Y. Arnould. 2010. Continued population recovery by Australian fur seals. Marine and Freshwater Research 61: 1–7.

Kittelberger, R., F. Hilbink, M. F. Hansen, G. P. Ross, M. A. Joyce, S. Fenwick, J. Heesemann, H. Wolfwatz, and K. Nielsen. 1995. Serological crossreactivity between *Brucella abortus* and *Yersinia enterocolitica* 0:9. 2. The use of *Yersinia* outer proteins for the specific detection of *Yersinia enterocolitica* infections in ruminants. Veterinary Microbiology 47: 271–280.

McDonald, W. L., R. Jamaludin, G. Mackereth, M. Hansen, S. Humphrey, P. Short, T. Taylor, J. Swingler, C. E. Dawson, A. M. Whatmore, E. Stubberfield, L. L. Perrett, and G. Simmons. 2006. Characterization of a *Brucella* sp. strain as a marine-mammal type despite isolation from a patient with spinal osteomyelitis in New Zealand. Journal of Clinical Microbiology 44: 4363–4370.

Miller, W. G., L. G. Adams, T. A. Ficht, N. F. Cheville, J. P. Payeur, D. R. Harley, C. House, and S. H. Ridgway. 1999. *Brucella*-induced abortions and infection in bottlenose dolphins (*Tursiops truncatus*). Journal of Zoo and Wildlife Medicine 30: 100–110.

Neta, A. V. C., J. P. S. Mol, M. N. Xavier, T. A. Paixao, A. P. Lage, and R. L. Santos. 2010. Pathogenesis of bovine brucellosis. Veterinary Journal 184: 146–155.

Nicoletti, P. 1980. The epidemiology of bovine brucellosis. Advances in Veterinary Science and Comparative Medicine 24: 69–98.

Nielsen, K. 1990. The serological response of cattle immunized with *Yersinia enterocolitica* O:9 or O:16 to *Yersinia* and *Brucella abortus* antigens in enzyme immunoassays. Veterinary Immunology and Immunopathology 24: 373–382.

Nielsen, O., R. E. A. Stewart, K. Nielsen, L. Measures, and P. Duignan. 2001. Serologic survey of *Brucella* spp. antibodies in some marine mammals of North America. Journal of Wildlife Diseases 37: 89–100.

———, K. Nielsen, R. Braun, and L. Kelly. 2005. A comparison of four serologic assays in screening for *Brucella* exposure in Hawaiian monk seals. Journal of Wildlife Diseases 41: 126–133.

Ohishi, K., R. Zenitani, T. Bando, Y. Goto, K. Uchida, T. Maruyama, S. Yamamoto, N. Miyazaki, and Y. Fujise. 2003. Pathological and serological evidence of *Brucella*-infection in baleen whales (Mysticeti) in the western North Pacific. Comparative Immunology Microbiology and Infectious Diseases 26: 125–136.

Prenger-Berninghoff, E., U. Siebert, M. Stede, A. Koenig, R. Weiss, and G. Baljer. 2008. Incidence of *Brucella* species in marine mammals of the German North Sea. Diseases of Aquatic Organisms 81: 65–71.

Ray, W. C., R. R. Brown, D. A. Stringfellow, P. R. Schnurrenberger, C. M. Scanlan, and A. I. Swann. 1988. Bovine brucellosis: An investigation of latency in progeny of culture-positive cows. Journal of the American Veterinary Medical Association 192: 182–186.

Retamal, P., O. Blank, P. Abalos, and D. Torres. 2000. Detection of anti-*Brucella* antibodies in pinnipeds from the Antarctic territory. Veterinary Record 146: 166–167.

Rhyan, J. C., K. Aune, T. Roffe, D. Ewalt, S. Hennager, T. Gidlewski, S. Olsen, and R. Clarke. 2009. Pathogenesis and epidemiology of

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brucellosis in Yellowstone bison: Serologic and culture results from adult females and their progeny. Journal of Wildlife Diseases 45: 729–739.

ROFFE, T. J., J. C. RHYAN, K. AUNE, L. M. PHILO, D. R. EALT, T. GIDLEWSKI, AND S. G. HENNAGER. 1999. Brucellosis in Yellowstone National Park bison: Quantitative serology and infection. Journal of Wildlife Management 63: 1132–1137.

ROSS, H. M., G. FOSTER, R. J. REID, K. L. JAHANS, AND A. P. MACMILLAN. 1994. *Brucella* species infection in sea-mammals. Veterinary Record 134: 359–359.

SAMARTINO, L., D. GALL, R. GREGORET, AND K. NIELSEN. 1999. Validation of enzyme-linked immunosorbent assays for the diagnosis of bovine brucellosis. Veterinary Microbiology 70: 193–200.

SOHN, A. H., W. S. PROBERT, C. A. GLASER, N. GUPTA, A. W. BOLLEN, J. D. WONG, E. M. GRACE, AND W. C. MCDONALD. 2003. Human neurobrucellosis with intracerebral granuloma caused by a marine mammal *Brucella* spp. Emerging Infectious Diseases 9: 485–488.

TRYLAND, M., K. K. SORENSEN, AND J. GODFROID. 2005. Prevalence of *Brucella pinnipediae* in healthy hooded seals (*Cystophora cristata*) from the North Atlantic Ocean and ringed seals (*Phoca hispida*) from Svalbard. Veterinary Microbiology 105: 103–111.

VAN CAMPEN, H., AND J. RHYAN. 2010. The role of wildlife in diseases of cattle. Veterinary Clinics of North America Food Animal Practice 26: 147–161.

WARNEKE, R. M., AND P. D. SHAUGHNESSY. 1985. *Arctocephalus pusillus*, the South African and Australian fur seal: Taxonomy, evolution, biogeography, and life history. *In* Studies of sea mammals in south latitudes, J. K. Ling, and M. M. Bryden (eds.). South Australian Museum, Adelaide, Australia. pp. 53–77.

ZARNKE, R. L., J. T. SALIKI, A. P. MACMILLAN, S. D. BREW, C. E. DAWSON, J. M. V. HOEF, K. J. FROST, AND R. J. SMALL. 2006. Serologic survey for *Brucella* spp., phocid herpesvirus-1, phocid herpesvirus-2, and phocine distemper virus in harbor seals from Alaska, 1976–1999. Journal of Wildlife Diseases 42: 290–300.

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