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Colony-level assessment of *Brucella* and *Leptospira* in the Guadalupe fur seal, Isla Guadalupe, Mexico

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ABSTRACT: The relatively small population size and restricted distribution of the Guadalupe fur seal *Arctocephalus townsendi* could make it highly vulnerable to infectious diseases. We performed a colony-level assessment in this species of the prevalence and presence of *Brucella* spp. and *Leptospira* spp., pathogenic bacteria that have been reported in several pinniped species worldwide. Forty-six serum samples were collected in 2014 from pups at Isla Guadalupe, the only place where the species effectively reproduces. Samples were tested for *Brucella* using 3 consecutive serological tests, and for *Leptospira* using the microscopic agglutination test. For each bacterium, a Bayesian approach was used to estimate prevalence to exposure, and an epidemiological model was used to test the null hypothesis that the bacterium was present in the colony. No serum sample tested positive for *Brucella*, and the statistical analyses concluded that the colony was bacterium-free with a 96.3% confidence level. However, a *Brucella* surveillance program would be highly recommendable. Twelve samples were positive (titers 1:50) to 1 or more serovars of *Leptospira*. The prevalence was calculated at 27.1% (95% credible interval: 15.6–40.3%), and the posterior analyses indicated that the colony was not *Leptospira*-free with a 100% confidence level. Serovars Icterohaemorrhagiae, Canicola, and Bratislava were detected, but only further research can unveil whether they affect the fur seal population.

KEY WORDS: *Arctocephalus townsendi* · Infectious diseases · Epidemiology · Introduced species · Isla Guadalupe · Serology · Bayesian approach

INTRODUCTION

The Guadalupe fur seal *Arctocephalus townsendi* was historically distributed in the eastern Pacific, from Islas Revillagigedo, Mexico, to Monterey Bay, California, USA (Townsend 1924, Repenning et al. 1971). After being hunted in the 19th century, its population declined drastically, and the species was confined to Isla Guadalupe, Baja California, Mexico (Hubbs 1956) (Fig. 1), until 1997, when a colony of 300 individuals was discovered at Islas San Benito, Baja California (Maravilla-Chávez & Lowry 1999). In 2010, the Isla Guadalupe colony was estimated at about 17 000 individuals, and the Islas San Benito colony at 2500 (García-Capitanachi 2011). However, the reproductive output of the Islas San Benito colony is negligible (<20 pups yr^{-1}), and its increment is the result of immigration from Isla Guadalupe (Aurioles-Gamboa et al. 2010). Given their relatively small population size and restricted distribution, Guadalupe fur seals are highly vulnerable to potential threats, such as infectious diseases (Aurioles-Gamboa 2015).

Two zoonotic bacterial genera known to infect pinnipeds are *Brucella* and *Leptospira* (Waltzek et al. 2012). *Brucella* was reported for the first time in marine mammals in 1994 (Ross et al. 1994), and since then it has been isolated from a wide range of pinniped

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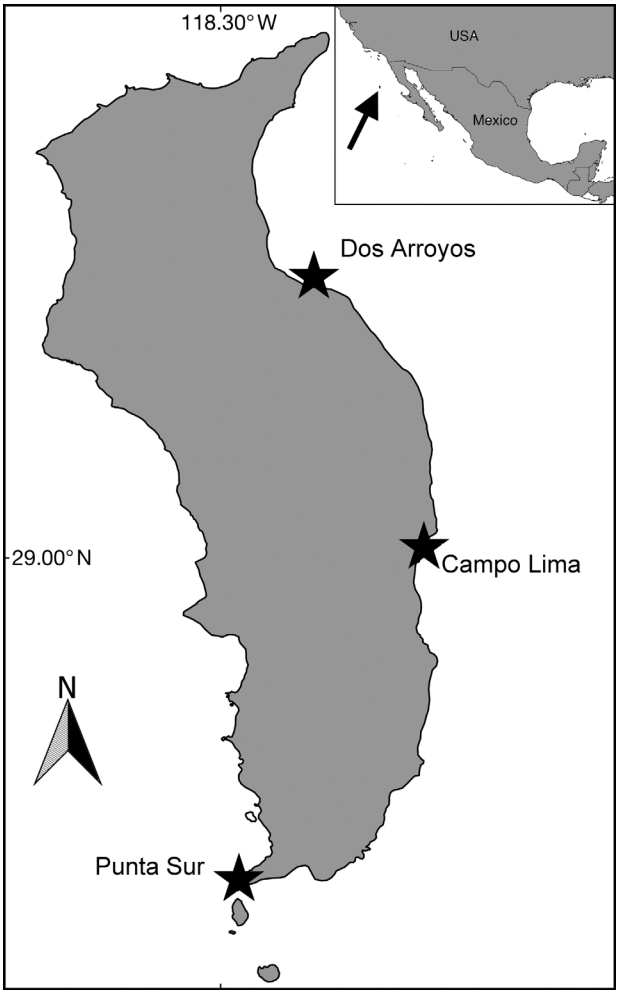

Fig. 1. Sampling sites of Guadalupe fur seal *Arctocephalus townsendi* pups in August 2014 at Isla Guadalupe, Mexico

species around the world (Nymo et al. 2011). Strains of *Brucella* are divided according to their lipopolysaccharide (LPS) phenotype as smooth (SLPS) or rough (RLPS), and because the bacterium is adapted to specific hosts, its strains have been classified as species based on their preferred host (Corbel & Brinley-Morgan 1984). Smooth strains include *B. melitensis*, *B. abortus*, *B. suis*, *B. neotomae*, *B. microti* (Corbel & Brinley-Morgan 1984, Scholz et al. 2008), and the 2 marine mammal strains, *B. pinnipedialis* and *B. cetis* (Foster et al. 2007). Rough strains are *B. canis* and *B. ovis* (Moreno et al. 1984). The virulence of the strains is associated with their LPS phenotype (Rittig et al. 2003), and most of the SLPS *Brucella* are capable of infecting different species (Nielsen et al. 2005a).

Only SLPS have been detected in pinnipeds, e.g. from a placenta of California sea lion *Zalophus californianus* (Goldstein et al. 2009) and in serological surveys of several species such as the Pacific harbor seal *Phoca vitulina richardii* (Nielsen et al. 2001, Lambourn et al. 2013, Greig et al. 2014). The pathology associated with *Brucella* infection could affect the reproductive potential in pinnipeds (Zarnke et al. 2006, Goldstein et al. 2009), as it does in terrestrial mammals. The modes of transmission of *Brucella* in pinnipeds are not clearly established yet. It has been suggested that, like in other mammals, it could be transmitted vertically (Zarnke et al. 2006), while horizontal transmission could occur by contact with infected aborted fetuses or placental tissues (Nymo et al. 2011). Lungworms have been proposed as vectors, and involve pinnipeds and coprophagic fishes (Howard et al. 1983, Garner et al. 1997).

Most mammals can become infected with *Leptospira*, and this genus contains hundreds of pathogenic serovars that are associated with 1 or more maintenance hosts, which serve as long-term reservoirs (Bolin 2003). The best known mammalian hosts are rodents, insectivores, dogs, pigs, and cattle, but serovars have the capability to adapt to new hosts (Hartskeerl et al. 2011). Several pathogenic *Leptospira* serovars can infect pinnipeds, and their presence varies geographically. Serovars detected in pinnipeds of the northeastern Pacific include Pomona, which has been reported commonly from otariids (Smith et al. 1977, Gulland et al. 1996, Colagross-Schouten et al. 2002, Zuerner et al. 2009, Prager et al. 2013), while serovars Bratislava, Canicola, Hardjo, Icterohaemorragiae, and Grippotyphosa have been detected in phocids (Stamper et al. 1998, Colegrove et al. 2005, Delaney et al. 2014, Greig et al. 2014).

Leptospira infection causes acute renal failure (Gulland et al. 1996, Delaney et al. 2014), and cyclic epizootic events in California sea lions cause high mortality (Colagross-Schouten et al. 2002). The route of *Leptospira* transmission in pinnipeds is unknown. In most mammals, vertical transmission could occur (Ellis et al. 1985). Horizontal transmission involves the oral ingestion of contaminated urine or water (Leighton & Kuiken 2001, Greig et al. 2005), and transmission from unrelated adults to pups could occur in the California sea lions during the breeding season (Avalos-Téllez et al. 2016).

Guadalupe fur seals are gregarious and spend much time at rocky breeding and hauling-out sites year round (Gallo-Reynoso 1994), which would allow for extended opportunities for pathogen transmission. The aim of this study was to evaluate the status of the fur seal colony of Isla Guadalupe regarding the prevalence and presence of *Brucella* spp. and *Lep-*

tospira spp. We assessed the status of the colony rather than the status of each individual within the colony. For each bacterium, we estimated prevalence to exposure based on serum antibodies from free-ranging pups, and tested the null hypothesis that the bacterium was present in the colony.

MATERIALS AND METHODS

Sampling and serology

Fieldwork was carried out on Isla Guadalupe, 260 km west of Baja California (Fig. 1), in August 2014, at the end of the Guadalupe fur seal breeding season. Live pups (1 to 2 mo old, n = 46) in apparently good physical condition were captured opportunistically and restrained manually at 3 sites on the island (Fig. 1): 14 at Punta Sur (28° 52′ N, 118° 17′ W), 17 at Campo Lima (29° 0′ N, 118° 13′ W), and 15 at Dos Arroyos (29° 6′ N, 118° 16′ W). Blood samples of 3 to 5 ml were collected following standard venipuncture techniques, and serum was stored at −20°C until lab analyses.

No serological test is 100% accurate for the detection of *Brucella*, and it is recommended that sera be tested through several procedures (Nielsen & Yu 2010). We therefore used the Rose Bengal test (RBT), the rivanol precipitation test (RIV), and the fluorescent polarization assay (FPA) to detect SLPS. Both the RBT and the RIV are agglutination tests, and because common epitopes are present in all SLPS, antibodies of these strains can be detected serologically using *B. abortus* antigen (Nielsen et al. 2001); on the other hand, the FPA is a robust primary binding assay capable of detecting antibodies in several host species (Nielsen et al. 2005a), including pinnipeds (Nielsen et al. 2005b, Lynch et al. 2011). These tests were performed following standard protocols (Nielsen 2002, OIE 2015) at the Instituto de Investigaciones en Ciencias Veterinarias (Universidad Autónoma de Baja California, Mexicali, BC). Samples were considered suspicious if the RBT detected antibodies against *B. abortus*, but they were considered positive only if they were also positive by the RIV and FPA.

The microscopic agglutination test (MAT) was performed following the standard protocol (OIE 2015) to test for antibodies against 9 pathogenic serovars of *Leptospira*: Bratislava, Canicola, Hardjo, Icterohaemorrhagiae, Pomona, Wolfii, Grippotyphosa, and Tarassovi. These tests were performed at the Centro Nacional de Investigación Disciplinaria en Microbiología Animal (Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias, Mexico City). The MAT has been validated for the California sea lion, and titers ≥1:100 are considered positive for exposure (Colagross-Schouten et al. 2002). Nevertheless, to determine the cut-off point used in our study, we followed the recommendations of the World Organisation for Animal Health (OIE), which establishes that given the high specificity of MAT, titers <1:100 can be taken as evidence of previous exposure (OIE 2015). Dilutions started at 1:50, and the endpoint was determined when 50% agglutination was recorded. Thus, in our study, titers as low as 1:50 were considered positive for exposure.

Data analysis

Prevalence (π) is defined as the frequency of a population attribute, such as exposure, infection, or disease (Greiner & Gardner 2000). We used Bayesian inference for binomial proportions to estimate the prevalence to exposure of both bacteria. The method is based on Bolstad (2007) and was computed in the R platform (R Development Team).

The conditional distribution of y, the total number of positive reactors (i.e. exposed animals), in n samples given the prevalence π is $binomial(n, \pi)$. Bayes' theorem postulates that the posterior distribution of π, $g(\pi|y)$, is proportional to the prior distribution, $g(\pi)$, times likelihood, $f(y|\pi)$. If prior information in the form of beta distribution with parameters (a, b) is assumed, then the posterior is beta with parameters $a' = a + y$ and $b' = b + n - y$, and the posterior mean equals

$$\pi = \frac{a'}{a' + b'} \quad (1)$$

with posterior standard deviation

$$s = \sqrt{\frac{a'b'}{(a' + b')^2(a' + b' + 1)}} \quad (2)$$

Because we had no prior knowledge about π, we used the uniform prior distribution $beta(1,1)$. Since $(\pi|y)$ is approximately $normal(\pi,s)$, the $(1 - \alpha) \times 100\%$ credible region for π is approximately $\pi \pm z_{\alpha/2}s$, where for a 95% credible interval (95% CrI), $z_{0.025} = 1.96$.

For each bacterium, to test the null hypothesis that it was present in the colony, the minimum sample size and the exact probability of detecting positive reactors, $P(T^+ = y)$, were calculated applying the epidemiological model described by Cameron & Baldock (1998) and Cameron (1999), and using the free available utility FreeCalc (Sergeant 2016). The calcu-

lation is based on the assumption that if the pathogen is present in the population, it will be present at given prevalence π; then H_0: $P(T^+ = y) \geq \pi$.

The minimum sample size was calculated by iterations on the basis of the cutpoint (see below) considering the population size, and the diagnosis test sensitivity (Se) and specificity (Sp), with $\alpha = 0.05$. Because we used a serial strategy for the detection of *Brucella*, conditional dependence for the RBT/FPA combination was assessed using the method described by Gardner et al. (2000) (see Appendix). This combination was chosen because the RBT is considered a suitable test for screening individual animals (Gall & Nielsen 2004, Nielsen et al. 2005a), while the FPA is a rugged test proposed as an alternative for otariids (Lynch et al. 2011). The expected Se after serial testing equals 89.6%, and expected Sp is 99.8% (see Appendix, Table A1). These values were used in the epidemiological model. For the detection of *Leptospira* we used the MAT, which is considered the 'gold standard' for the laboratory diagnosis both for human and animals, and the Se and Sp values used were 90% and 99%, respectively (Hartskeerl et al. 2011).

The cutpoint is the maximum number of positive reactors that can be observed with $1 - \beta$ probability if the population is pathogen free, where β is the probability of type II error. The minimum sample size is the value at which the number of reactors at the cutpoint with a probability of $1 - \beta$ from the distribution with zero prevalence is equivalent to the number of reactors occurring with probability α at the left tail of the distribution with prevalence π.

The probability of detecting y positive reactors, $P(T^+ = y)$, when testing n animals from an infinite population with prevalence π, is given by the binomial distribution as

$$P(T^+ = y) = \binom{n}{y}[\pi\mathrm{Se} + (1-\pi)(1-\mathrm{Sp})]^y[\pi(1-\mathrm{Se}) + (1-\pi)\mathrm{Sp}]^{n-y} \quad (3)$$

where $\pi\mathrm{Se} + (1 - \pi)(1 - \mathrm{Sp})$ is the probability of getting a positive result when testing a single animal, and $\pi(1 - \mathrm{Se}) + (1 - \pi)\mathrm{Sp}$ is the probability of getting a negative result.

RESULTS

The sample size in our study was sufficient for the calculation of prevalence of both bacteria because it represents >90% of the calculated equivalent sample size (i.e. the amount of information about the parameter from the prior equivalent to the amount from a random sample of that size) (Table 1). Five samples were detected as positive for *Brucella* by the RBT, but positive reactors were not confirmed by either the RIV or the FPA, and the prevalence was calculated at 2.1% (95%CrI: 0.1–7.5%; Table 1, Fig. 2A).

Twelve positive reactors were found for *Leptospira* spp.: 4 at Punta Sur (35.7%, 95%CrI: 13.9–61.4%), 3 at Campo Lima (28.6%, 95%CrI: 9.1–53.8%), and 5 at Dos Arroyos (42.9%, 95%CrI: 19.2–68.4%). The overall prevalence was calculated at 27.1% (95%CrI: 15.6–40.3%; Table 1, Fig. 2B). No titers >1:50 were recorded. Three serovars were detected (Table 2): Icterohaemorrhagiae, Canicola, and Bratislava. One sample that tested positive for Icterohaemorrhagiae was also positive for the Palo Alto strain of the same serovar. Three samples had reactions against 2 serovars: 2 were reactive to Bratislava and Canicola, and 1 to Canicola and Icterohaemorrhagiae.

The sample size used to test the null hypothesis that the colony is non-*Brucella* free was too small in relation to the calculated minimum sample size

Table 1. Prevalence of *Brucella* and *Leptospira* based on serum samples of free-ranging Guadalupe fur seal *Arctocephalus townsendi* pups from Isla Guadalupe, Mexico, in August 2014

	Brucella	*Leptospira*
Sample size (n)	46	46
Positive reactors (y)	0	12
Posterior	*beta*(13, 47)	*beta*(13, 35)
Equivalent sample size	49	49
Prevalence (π)	0.021	0.271
Standard deviation (s)	0.020	0.063
95% credible interval	0.001–0.075	0.156–0.403

Table 2. Microscopic slide agglutination test results (negative: titers < 1:50; positive: titers ≥ 1:50) for *Leptospira* serovars of serum samples of 46 free-ranging Guadalupe fur seal *Arctocephalus townsendi* pups from Isla Guadalupe, Mexico, in August 2014. CrI: credible interval

Serovar	Neg.	Pos.	Frequency (95%CrI)
Bratislava	44	2	16.7 (3.8–36.4)
Canicola	39	7	44.4 (22.9–67.1)
Hardjo	46	0	
Icterohaemorrhagiae	39	7[a]	44.4 (22.9–67.1)
Pomona	46	0	
Pyrogenes	46	0	
Wolfii	46	0	
Grippotyphosa	46	0	
Tarassovi	46	0	
Sum	398	16	

[a]Including 1 sample that also tested positive for the Palo Alto strain

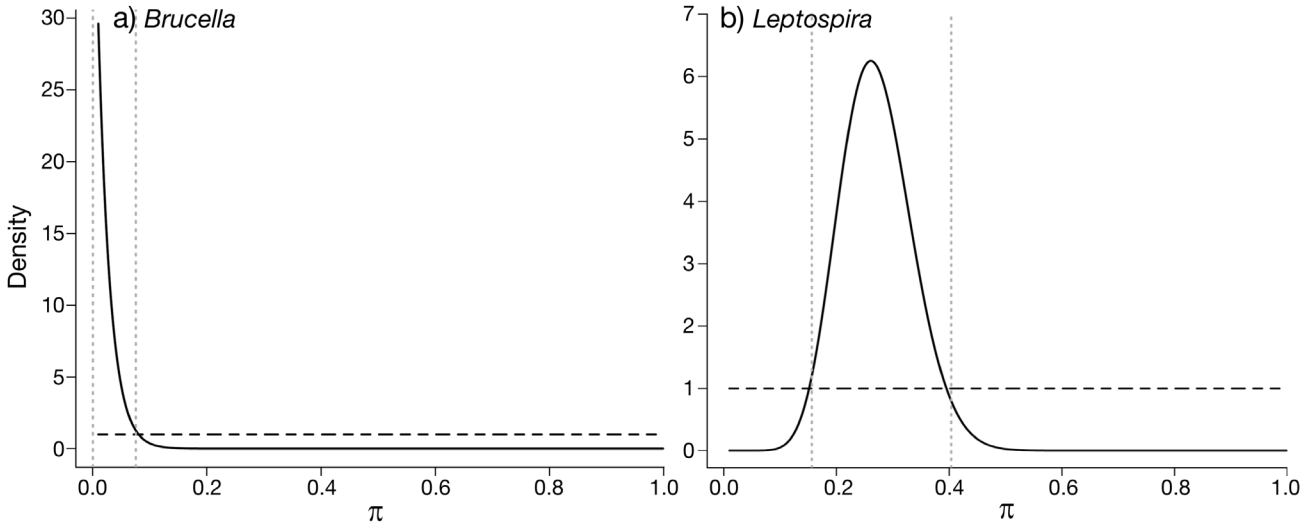

Fig. 2. Prior (dashed line) and posterior (solid line) distributions of the prevalence (π) of (a) *Brucella* and (b) *Leptospira* based on sera from 46 free-ranging Guadalupe fur seal *Arctocephalus townsendi* pups. Vertical, dotted lines are the limits of the credible intervals

(Table 3), and under this condition the test was unable to distinguish between an infected and uninfected population, with a negligible prevalence of 2.1%. However, if the upper limit of the 95% credible interval is used, i.e. assuming a prevalence of 7.5% (see Table 1), both the cutpoint and the minimum sample size are reduced, and the probability of observing 0 positive reactors in a sample of 46 individuals equals 0.037 (Table 3). These results are adequate to reject the null hypothesis and conclude that the colony is *Brucella*-free, with 96.3% confidence level.

In the case of *Leptospira*, the minimum sample size estimated was lower than our real sample size, and the probability of observing 12 or fewer positive reactors in a sample of 46 individuals was higher than the prevalence (Table 3). These results are not adequate to reject the null hypothesis at the observed prevalence of 27.1%, and it is possible to conclude that the bacterium is present in the colony with a 100% confidence level.

Table 3. Probability of observing positive reactors, $P(T^+ = y)$, to *Brucella* and *Leptospira* in the Guadalupe fur seal *Arctocephalus townsendi* colony of Isla Guadalupe in August 2014

	Brucella	*Leptospira*
Test sensitivity (%)	89.6	90
Test specificity (%)	99.8	99
Observed positive reactors (y)	0	12
Prevalence (%)	2.1–7.5[a]	27.1
Cutpoint	2–1[a]	1
Minimum sample size	301–67[a]	17
$P(T^+ = y)$	0–0.037[a]	0.635

[a]95% credible interval

DISCUSSION

The gregarious behavior on land of Guadalupe fur seals could represent an opportunity for the horizontal transmission of *Brucella* spp. and *Leptospira* spp.; furthermore, it is likely that maternal transfer occurs congenitally or during lactation. Thus, serum antibodies of pups may reflect the maternal antibodies or could be the result of exposure to infected fomites and/or individuals (conspecific or not) in their terrestrial habitat. In this study, we assessed the prevalence of both bacteria through the analysis of serum samples of pups. Freedom from each bacterium at the colony level was tested using an epidemiological model, which provides probability statements about its presence. Our results indicate that the fur seal colony of Isla Guadalupe is *Brucella*-free, but *Leptospira* is present.

Our analyses suggested that the colony of Isla Guadalupe is free of smooth strains of *Brucella*. Although the serological tests applied have not been validated for pinnipeds, detection of seropositive individuals through conventional tests has been confirmed (Hernández-Mora et al. 2013), especially through the FPA (Nielsen et al. 2005b, Lynch et al. 2011). Estimated prevalence was very low, and previ-

ous studies with otariids (e.g. Burek et al. 2005, Mackereth et al. 2005, Tryland et al. 2012), have provided results similar to ours, suggesting that otariids may have low susceptibility to *Brucella* spp. infection (Jensen et al. 2013).

In some pinniped species, no differences in *Brucella* antibody prevalence have been detected among sex and age classes (e.g. Nielsen et al. 2001); Australian fur seals *Arctocephalus pusillus doriferus* show an age-related progressive increase (Lynch et al. 2011). We only tested sera from pups, and it is likely that the lack of antibodies could be related to their age. Relative immunocompetence has been observed in harbor seal pups (Ross et al. 1994), and some studies with pinnipeds (e.g. Lynch et al. 2011, Nymo et al. 2013) suggest that antibody conversion could occur after weaning. Therefore, testing sera from animals of other sex and age classes, especially adult females, is highly recommended.

Serological evidence of *Leptospira* spp. based on the MAT has been reported in several pinnipeds. The calculated prevalence in our study is moderate (27.1%, 95%CrI: 15.3–40.3%); nevertheless, even though pups are a good indicator of bacterium presence, it is feasible that the prevalence might be higher if samples from animals in other sex and age classes were analyzed. Our results show that the fur seal colony of Isla Guadalupe should not be considered bacterium-free. However, in the absence of clinical reports (there is no disease surveillance program), it cannot be concluded whether the bacterium is actually causing health problems in the population.

Serovars detected in the fur seals were Icterohaemorrhagiae, Canicola, and Bratislava. Isla Guadalupe is also inhabited by California sea lions and northern elephant seals *Mirounga angustirostris*. Surprisingly, serovar Pomona was not detected, even though it has been reported commonly in sea lions along the California coast since the 1970s (McIlhattan et al. 1971, Gerber et al. 1993, Gulland et al. 1996, Colagross-Schouten et al. 2002, Lloyd-Smith et al. 2007), and, along with Wolfii, is the most frequent serovar in sea lion pups from islands off the west coast of Baja California (Avalos-Téllez et al. 2016). Absence of serovars Pomona and Wolfii is probably due to the segregation of terrestrial habitat between fur seals and sea lions (García-Aguilar et al. 2013), which, in addition to reducing interference and exploitation competition, may act as a non-physical barrier for disease transmission.

Our results from fur seals partially coincide with serovars found in live-stranded elephant seals along the coast of California. As intraspecific transmission between otariids (sea lion) and phocids (elephant seal) is possible (Delaney et al. 2014), it is likely that the transmission between fur seals and elephant seals occurs. On the other hand, observed exposure to *Leptospira* in fur seals could also be due to the introduction of exotic mammals to Isla Guadalupe. Serovar Icterohaemorrhagiae is associated with rodents (Matthias & Levett 2002), especially house mice *Mus musculus* (Collares-Pereira et al. 2000), while Canicola is the commonest serovar in dogs *Canis familiaris* (Faine 1994). Serovar Bratislava is associated mainly with rats (*Rattus* spp.), but has also been reported as common in dogs (Scanziani et al. 2002). House mice and dogs were introduced to Isla Guadalupe in the 19th and 20th centuries (Morán 1996). House mice remain on the island, whereas dogs were eradicated in 2007 (Aguirre-Muñoz et al. 2013).

The sources of exposure of Guadalupe fur seals to *Leptospira* cannot be determined from the information at hand. Because all elephant seal samples tested have been taken from stranded animals, the geographic area of origin (i.e. the provenance colony) is unknown; hence, it is not possible to determine whether the fur seals of Isla Guadalupe have been in contact with infected elephant seals. On the other hand, neither the dogs, before they were eradicated from the island, nor the mice, in the past or currently, have ever been tested; thus, the presence of the bacterium (and its serovars) in these populations is also unknown. Finally, pinnipeds can act as maintenance hosts, like the California sea lion for serovar Pomona (Zuerner et al. 2009, Prager et al. 2013), and the fur seal could also be a maintenance host. Nevertheless, to discern between maintenance and accidental host requires data that are nonexistent for the Guadalupe fur seal.

The data obtained in this first assessment indicate that the fur seal colony on Isla Guadalupe is free of *Brucella*, but a surveillance program is recommended to further our knowledge on the subject. We demonstrated that the colony has been exposed to *Leptospira*; additional work would clarify the sources of the bacterium and its impacts over the Guadalupe fur seal population.

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Appendix

Analysis of conditional dependence of the Rose Bengal tests/fluorescent polarization assay (RBT/FPA) serial combination for the diagnosis of *Brucella*

Conditional dependence of the RBT/FPA combination was assessed based on the method of Gardner et al. (2000). Sensitivity (Se) and specificity (Sp) values of the RBT and FPA were taken from Gall & Nielsen (2004) and Nielsen et al. (2005a). The conditional covariance for sensitivity (γ_{Se}) was calculated as $\gamma_{Se} = p - Se_1Se_2$, where p is the observed proportion of animals that are positive in both tests, and Se_1 and Se_2 are the individual sensitivities of the tests used. The γ_{Se} was estimated with its corresponding 95% confidence interval (CI). Conditional dependence was considered significant if 0 is excluded from the γ_{Se} 95%CI, and the degree of dependence (ψ) was calculated as a proportion of the γ_{Se} obtained with respect to the maximal γ_{Se} value possible. If $\psi = 1$, then a complete dependence exists between tests. If tests are conditionally dependent, the expected sensitivity is $Se_{exp} = 1 - (1 - Se_1)(1 - Se_2) - \gamma_{Se}$; if test sensitivities are conditionally independent, the expected sensitivity is $Se_{exp} = 1 - (1 - Se_1)(1 - Se_2)$. Similarly expressions were applied to assess dependence in test specificities.

Table A1. Expected sensitivity (Se_{exp}) and specificity (Sp_{exp}) for the serial combination RBT/FPA. RBT: Rose Bengal tests; FPA: fluorescent polarization assay; CI: confidence interval; Se_{exp}: expected sensitivity

Test	Sensitivity	Specificity
RBT (%)	81.2	86.3
FPA (%)	97.5	98.9
RBT/FPA serial combination covariance (95%CI)	γ_{Se} = 0.10 (0.02 to 0.18)	γ_{Sp} = 0.04 (0.06 to −0.02)
Degree of dependence (ψ, %)	54	27
Expected value (%)	Se_{exp} = 89.6	Sp_{exp} = 99.8

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