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Short communication

Asymptomatic and chronic carriage of *Leptospira interrogans* serovar Pomona in California sea lions (*Zalophus californianus*)

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ABSTRACT

Since 1970, periodic outbreaks of leptospirosis, caused by pathogenic spirochetes in the genus *Leptospira*, have caused morbidity and mortality of California sea lions (*Zalophus californianus*) along the Pacific coast of North America. Yearly seasonal epizootics of varying magnitude occur between the months of July and December, with major epizootics occurring every 3–5 years. Genetic and serological data suggest that *Leptospira interrogans* serovar Pomona is the infecting serovar and is enzootic in the California sea lion population, although the mechanism of persistence is unknown. We report asymptomatic carriage of *Leptospira* in 39% (33/85) of wild, free-ranging sea lions sampled during the epizootic season, and asymptomatic seroconversion with chronic asymptomatic carriage in a rehabilitated sea lion. This is the first report of asymptomatic carriage in wild, free-ranging California sea lions and the first example of seroconversion and asymptomatic chronic carriage in a sea lion. Detection of asymptomatic chronic carriage of *Leptospira* in California sea lions, a species known to suffer significant disease and mortality from the same *Leptospira* strain, goes against widely-held notions regarding leptospirosis in accidental versus maintenance host species. Further, chronic carriage could provide a mechanism for persistent circulation of *Leptospira* in the California sea lion population, particularly if these animals shed infectious leptospires for months to years.
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1. Introduction

Leptospirosis is a disease caused by pathogenic spirochetes in the genus *Leptospira* and is common in mammals worldwide (Levett, 2001). Since 1970, periodic leptospirosis outbreaks have caused morbidity and mortality of California sea lions (*Zalophus californianus*) along the Pacific coast of North America (McIlhattan et al., 1971; Vedros et al., 1971). Cases occur year round, however most cases are observed between the months of July and December, and the magnitude of the annual outbreaks varies with major epizootics occurring every 3–5 years (Greig et al., 2005; Gulland et al., 1996). All leptospiral isolates obtained from wild California sea lions, spanning

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four decades, have been *Leptospira interrogans* serovar Pomona and genetic analysis of these isolates shows that they are almost identical and distinct from previous isolates from other host species (Zuerner and Alt, 2009). These data, combined with serological evidence showing exposure of the youngest age classes each year (Lloyd-Smith et al., 2007), suggest that serovar Pomona is enzootic in the sea lion population. Chronic, asymptomatic carriage of *Leptospira* has been proposed as a possible mechanism for the pathogen's observed long-term circulation in sea lions (Gulland et al., 1996; Lloyd-Smith et al., 2007).

Direct observation of chronic carriage is challenging due to the protected status of sea lions, which limits extended observation of infected animals in a rehabilitation setting without treatment and/or serial sampling of free-ranging sea lions, but preliminary evidence provides limited support for asymptomatic and chronic carriage. Urinary shedding of leptospires was detected by polymerase chain reaction (PCR) in 2004 in 2 sea lions in a rehabilitation facility with no clinical signs of leptospirosis (Cameron et al., 2008), providing evidence of asymptomatic carriage. Two studies report the possibility of chronic carriage after antibiotic treatment: in 1970, leptospires were detected by dark field microscopy (DFM) in the urine of a sea lion after successful leptospirosis treatment (time between admission and detection is unstated; Vedros et al., 1971); and in 1984, leptospires were detected by DFM in the urine of a recovered sea lion in a rehabilitation facility, 22 weeks after the start of successful leptospirosis treatment (Dierauf et al., 1985). Here we present case reports from 33 wild-caught sea lions that exhibit asymptomatic leptospire carriage and one stranded, rehabilitating sea lion that exhibits chronic, asymptomatic leptospire carriage. Identification of these potential and definitive chronic asymptomatic carriers provides a possible mechanism for *L. interrogans* serovar Pomona persistence in the California sea lion population and supports the hypothesis that *L. interrogans* is enzootic in this population.

2. Materials and methods

2.1. Wild-caught California sea lions

Urine and serum samples were collected from anesthetized sea lions caught on Año Nuevo Island, CA in October 2010 ($n = 34$) and September–November 2011 ($n = 51$). All wild-caught sea lion samples were collected under Marine Mammal Protection Act Permit No. 932-1905-00/MA-009526 issued by the National Marine Fisheries Service (NMFS). The wild-caught sea lion sample collection protocol was approved by the Institutional Animal Care and Use Committee of The Marine Mammal Center (Sausalito, CA; protocol # 2008-3). Blood was collected from the caudal gluteal vein, allowed to clot and centrifuged at $3000 \times g$. Serum was then separated and divided into aliquots for serum chemistry (2010 $n = 34$, 2011 $n = 51$) and serum microscopic agglutination testing (MAT; 2010 $n = 34$, 2011 $n = 51$). Serum chemistry analyses were performed within 24 h, and aliquots for serum MAT were frozen at −80 °C until analyzed. Urine was collected via sterile urinary catheterization or cystocentesis for real-time PCR for the presence of *Leptospira* spp. (2010 $n = 34$, 2011 $n = 51$) and/or culture (2010 $n = 5$, 2011 $n = 51$). Urine used for culture was immediately inoculated into media (described in Section 2.7). Urine used for real-time PCR analysis was processed as follows: if ≤4 ml urine was available, two 2 ml aliquots were stored in 2 ml cryotubes at −80 °C; if >4 ml urine was available, one uncentrifuged aliquot was stored in a 2 ml cryotube at −80 °C and the remaining urine was centrifuged at $2000 \times g$, the supernatant poured off and the pellet resuspended in 0.5–1.5 ml of the supernatant or PBS and stored in a 2 ml cryotube at −80 °C. For PCR, the resuspended pellet was prioritized over the uncentrifuged urine if both were available.

2.2. Asymptomatic stranded California sea lion

In June 2010, a yearling male sea lion was brought to the Marine Mammal Care Center Fort MacArthur (MMCC/FM, San Pedro, CA) with a healed flipper injury that precluded release back into the wild. This sea lion was housed in outdoor pens amongst other rehabilitating sea lions. None of the co-housed sea lions showed signs of leptospirosis, but during routine evaluation of the animal's health, in October 2011, an anti-*Leptospira* antibody titer was noted by serum MAT. Subsequently this sea lion's anti-*Leptospira* serum antibody titer (MAT) and urinary shedding (real-time PCR and culture) were monitored before, during and after antibiotic therapy. Antibiotic therapy (doxycycline 5 mg/kg by mouth every 12 h) commenced January 18, 2012 and was given for a total of 22 days with the exception of day 5 of treatment when a single dose of Combi-Pen-48 (Penicillin G Benzathine and Penicillin G Procaine 300,000 units/ml) was given intramuscularly. All sample collection occurred as part of the animal's routine veterinary care during stranding response activities conducted under a Stranding Agreement between NMFS and MMCC/FM under the authority of the Marine Mammal Protection Act.

2.3. Serum chemistry analyses

Serum chemistry analyses of wild-caught sea lions were performed on an ACE® Clinical Chemistry System (Alfa Wassermann, Inc., West Caldwell, NJ, USA), while those of the MMCC/FM sea lion were performed on either a VetTest® 8008 Chemistry Analyzer (IDEXX Laboratories, Inc., Westbrook, Maine, USA) or a Cobas 8000 modular analyzer (Roche Diagnostics, Indianapolis, IN, USA). In clinically healthy sea lions, blood urea nitrogen (BUN) values are 44 ± 20 mg/dl (mean ± SD) and creatinine values are 0.7 ± 0.2 mg/dl (Roletto, 1993). In sea lions with clinical signs of leptospirosis, these values are significantly elevated with BUN of 176 ± 57 mg/dl and creatinine of 7.6 ± 4.6 mg/dl in clinically confirmed cases ($n = 20$), and BUN of 182 ± 31 mg/dl and creatinine of 8.1 ± 1.5 mg/dl in suspected cases with clinical signs ($n = 357$) (Gulland et al., 1996). In this study, we classified animals as having clinical signs of leptospirosis if their BUN and creatinine values fell outside the normal range for healthy sea lions, which we defined as the mean $\pm 1.96 \times$ SD (i.e. the interval containing 95% of the

values) or 4.8–83.2 mg/dl for BUN and 0.3–1.1 mg/dl for creatinine.

2.4. Serum microscopic agglutination testing (MAT)

Microscopic agglutination testing was performed at the California Animal Health and Food Safety (CAHFS) Laboratory, Davis, CA, or at the Centers for Disease Control and Prevention (CDC), Atlanta, GA, using live cultured *L. interrogans* serovar Pomona antigen (reference strain – serovar Pomona strain Pomona). With the exception of a single serum sample from the MMCC/FM stranded sea lion for which a panel of 6 serovars was run at CAHFS, panels of 19 serovars were run at the CDC for each serum sample. For most samples tested we report results only for *L. interrogans* serovar Pomona, as historically this is almost always the strain against which there is the highest MAT titer (Lloyd-Smith et al., 2007) and it is the only serovar isolated from this species to date (Zuerner and Alt, 2009). In 3/29 instances for which MAT titers were detected and the highest titer was not against Pomona, the serovar(s) against which a higher or equivalent titer was mounted, is/are also noted. Endpoint titers were determined by starting at an initial dilution of 1:100 and using two-fold dilutions until the last well showing 50% agglutination was recorded (Faine et al., 1999). Serovars included in the 19-serovar panel were Australis, Autumnalis, Ballum, Bataviae, Bratislava, Borincana, Canicola, Celledoni, Cynopteri, Djasiman, Grippotyphosa, Icterohaemorrhagiae, Javanica, Georgia, Mankarso, Pomona, Pyrogenes, Tarassovi and Wolffi. Serovars included in the 6-serovar panel were Bratislava, Canicola, Gryppotyphosa, Hardjo, Icterohaemorrhagiae and Pomona.

2.5. Analysis of wild-caught sea lion urine using real-time PCR

Real-time PCR was performed to detect pathogenic *L. interrogans.* Extraction of genomic DNA from urine was accomplished using a QIAamp viral RNA mini kit (QIAGEN® Valencia, CA). This RNA kit is recommended for extraction of DNA from urine in order to reduce the effect of potential inhibitors present in urine. For urine samples, 1 ml of urine was concentrated by centrifugation. The resulting pellet was washed with 1 ml of PBS and then centrifuged again. Each sample was processed further according to the manufacturer's instructions and 50 μl of DNA was eluted per sample. Real-time PCR was performed in a total volume of 15 μl with forward primer (5′-GGATCCGTGTAGAAAGAATGTCGG-3′) and reverse primer (5′-GTCACCATCATCATCATCGTCC-3′) at 300 nM, probe FAM-5′-ATGCCTGACCAAATCGCCAAAGCTGCGAAA-3′ black hole quencher BHQ1 at 150 nM, 7.5 μl of TaqMan® Universal PCR master mix (Applied Biosystems®, Foster City, CA) and 1.5 μl DNA template in an Eco™ real-time PCR system (Illumina®, San Diego, CA). These probes were selected to target sequences in the gene that codes for LipL32, a major outer membrane protein that is highly conserved among pathogenic *Leptospira* spp. but absent in saprophytic *Leptospira* spp. (Haake et al., 2000). The PCR conditions were as follows: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. PCR was performed in triplicate for samples as well as for negative and positive controls. Negative controls (without DNA template) were run for all experiments to rule out any DNA contamination. Genomic DNA isolated from *L. interrogans* serovar Pomona (National Veterinary Services Laboratories reference strain Leptospira reference serovar Pomona, strain Pomona) was used as the positive control to determine the analytic sensitivity of the assay. The quantity of *L. interrogans* serovar Pomona genomic DNA was estimated by measuring absorbance of DNA using the NanoDrop 1000 Spectrophotometer (Thermo Scientific®, Wilmington, DE). 5.07 fg DNA, equivalent to one genome copies assumed a genome size of 4.627 Mb (Xue et al., 2009). Limit of detection was three genome copies per reaction.

2.6. Analysis of rehabilitated sea lion urine using real-time PCR

Urine samples from the rehabilitated sea lion were sent to the Colorado State University College of Veterinary Medicine and Biomedical Sciences Veterinary Diagnostic Laboratories for real-time PCR to detect pathogenic *Leptospira* DNA by using probes that target sequences in the LipL32 gene. Real-time PCR was performed using the PrimerDesign™ genesig Kit for Leptospirosis Genomes (PrimerDesign Ltd., Southampton, UK) according to the manufacturer's instructions.

2.7. Culture and isolation of Leptospira

Urine was added to EMJH medium (Ellinghausen and McCullough, 1965; Johnson and Harris, 1967) at a 1:10 dilution and 100–500 μl of this dilute urine were inoculated into either T80/40/LH, a modified semi-solid EMJH medium (Zuerner, 2006) supplemented with 0.4% heat inactivated rabbit sera (NADC) or EMJH supplemented with 5-fluorouracil (CDC). These cultures were held in a 30 °C incubator at TMMC for 1–14 days prior to overnight shipment to the National Animal Disease Center (NADC) or the CDC for isolation. Samples from MMCC/FM were held at room temperature for 2–7 days prior to overnight shipment to the CDC. Once at CDC or NADC, cultures were incubated at 29 °C for up to 6 months and received periodic examination using DFM before being considered negative for *Leptospira*.

2.8. Pulsed-field gel electrophoresis (PFGE) of isolates

Pulsed-field gel electrophoresis was performed as previously described to identify *Leptospira* isolates to the serovar level (Galloway and Levett, 2008, 2010). Two of the 7 isolates grew well enough to yield sufficient DNA for PFGE analysis.

3. Results

3.1. Urinary shedding in asymptomatic, wild-caught free-ranging California sea lions

Urinary shedding of leptospires was detected in 33/85 (32/85 by real-time PCR and 7/56 by culture) wild-caught,

Table 1
Serum MAT endpoint titers against *Leptospira interrogans* serovar Pomona, BUN, serum creatinine, and *Leptospira* detection assays for 33 free-ranging California sea lions found to be shedding *Leptospira* spp. via culture or PCR. The normal ranges in healthy sea lions are 4.8–83.2 mg/dl for BUN and 0.3–1.1 mg/dl for creatinine.

ID	Date	Serum			Urine
		MAT	BUN	Creatinine	Assay
WCSL10-66	6-Oct-10	1:3200	24	0.5	PCR and culture[a]
WCSL10-72	11-Oct-10	1:51,200	37	0.4	PCR[c]
WCSL10-73	11-Oct-10	1:3200	29	0.6	PCR[c]
WCSL10-79	11-Oct-10	1:51,200	19	0.7	PCR[c]
WCSL 209-11	19-Sep-11	1:25,600	38	0.6	PCR and culture[a]
WCSL 215-11	19-Sep-11	1:6400[d]	27	0.7	PCR and culture
WCSL 5651-11	3-Oct-11	1:6400	28	0.8	PCR and culture
WCSL 5652-11	3-Oct-11	1:102,400	43	0.7	PCR and culture
WCSL 5660-11	3-Oct-11	<1:100	24	0.8	PCR
WCSL 6264-11	17-Oct-11	1:400[e]	30	0.4	PCR
WCSL 6265-11	17-Oct-11	<1:100	52	0.4	PCR
WCSL 6267-11	17-Oct-11	1:102,400	54	0.4	PCR
WCSL 6268-11	17-Oct-11	1:102,400	60	0.8	PCR
WCSL 6269-11	21-Oct-11	1:25,600	33	0.8	Culture[b]
WCSL 6271-11	21-Oct-11	1:51,200	62	0.5	PCR
WCSL 6274-11	21-Oct-11	1:12,800	36	0.6	PCR
WCSL 6432-11	31-Oct-11	<1:100	21	0.5	PCR
WCSL 6433-11	31-Oct-11	<1:100	19	0.5	PCR
WCSL 6434-11	31-Oct-11	1:6400	23	0.7	PCR and culture
WCSL 6436-11	31-Oct-11	1:400	36	0.6	PCR
WCSL 6437-11	31-Oct-11	<1:100	19	0.6	PCR
WCSL 6438-11	31-Oct-11	1:51,200	37	0.8	PCR
WCSL 6439-11	31-Oct-11	1:1600	21	0.9	PCR
WCSL 6441-11	31-Oct-11	1:204,800	38	0.9	PCR
WCSL 6442-11	31-Oct-11	<1:100	22	0.7	PCR
WCSL 6443-11	31-Oct-11	1:204,800	79	1.1	PCR
WCSL 6431-11	4-Nov-11	1:102,400	32	1	PCR
WCSL 6445-11	4-Nov-11	1:3200	31	0.7	PCR
WCSL 6446-11	4-Nov-11	1:204,800	50	0.5	PCR
WCSL 6447-11	4-Nov-11	1:51,200	68	0.9	PCR
WCSL 6450-11	4-Nov-11	1:800[f]	34	0.7	PCR
WCSL 6452-11	4-Nov-11	1:51,200	23	0.6	PCR
WCSL 6453-11	4-Nov-11	1:25,600	53	0.6	PCR

[a] Isolates were classified to the serogroup Pomona by PFGE.
[b] Urine from this animal was PCR negative for *Leptospira* DNA.
[c] Culture was not performed on these urine samples.
[d] Highest MAT titer is 1:12,800 against Autumnalis; MAT titer of 1:6400 against Bratislava.
[e] Highest MAT titer is 1:3200 against Djasiman, Icterohaemorrhagiae and Mankarso; MAT titer of 1:1600 against Bratislava, Autumnalis and Grippotyphosa; 1:800 against Canicola and Cynopteri; and 1:400 against Australis.
[f] Highest MAT titer is 1:3200 against Autumnalis; MAT titer of 1:800 against Bratislava, Cynopteri and Djasiman.

free-ranging sea lions (Table 1). All sea lions testing positive were asymptomatic for leptospirosis as determined by serum chemistry, had no abnormalities noted during capture or on physical exam during sample collection, and 27 of the 33 leptospiruric sea lions had detectable serum anti-*Leptospira* antibodies by MAT (Table 1). Three of these leptospiruric MAT positive sea lions had positive MAT titers that were highest against *Leptospira* serovars other than Pomona (Table 1). Two of the 48 non-leptospiruric sea lions had *L. interrogans* serovar Pomona MAT titers (1:100 and 1:6400). One sea lion was culture positive but PCR negative. The two isolates for which PFGE was performed, sampled from sea lions captured in October 2010 and September 2011, were identified as *L. interrogans* serovar Pomona.

3.2. Seroconversion and urinary shedding without symptoms in a captive California sea lion

Seroconversion was detected in a sea lion admitted to MMCC/FM with a healed flipper injury that precluded release back into the wild (Table 2). The animal was admitted in June 2010 and had no detectable anti-leptospiral antibodies 22 days later in July 2010, but had seroconverted by October 2011; this animal was assumed to have been exposed to an infected animal at MMCC/FM. The veterinarian and the animal care staff monitored the animal daily and never noted any abnormalities in behavior suggestive of leptospirosis (typical clinical signs of leptospirosis in sea lions include unusual behaviors such as polydipsia, reluctance to use hind flippers and adoption of a hunched position while holding the hind flippers over the abdomen (Gulland et al., 1996)); no such signs were observed in any of the possible source animals either. Serum chemistry results from when anti-*Leptospira* antibodies were first detected, as well as from the following two blood draws, showed BUN values (23–36 mg/dl) well within the normal range (4.8–83.2 mg/dl) and serum creatinine values at the high end of or just above (1.0–1.2 mg/dl) the normal range (0.3–1.1 mg/dl), but still much lower than creatinine levels observed in cases with clinical signs of leptospirosis. BUN and serum creatinine results

Table 2
Serum MAT endpoint titers against *Leptospira interrogans* serovar Pomona, BUN, serum creatinine, and urine real-time PCR and culture results for a California sea lion that seroconverted during rehabilitation for a flipper injury.

Weeks (days) at MMCC/FM	Serum			Urine	
	MAT	BUN	Creatinine	PCR	Culture
3 (22)	<1:100	32	0.3	NA	NA
69 (486)	1:12,800[a]	36	1	NA	NA
73 (514)	1:3200	23	1.2	NA	NA
78 (547)	1:800	25	1.2	Positive	Negative
81 (568)	1:800	30	0.9	Negative	Negative
83 (581)[b]	1:800	26	0.9	Negative	Negative
85 (596)[b]	1:800	27	1	Negative	Negative

[a] MAT result from CAHFS where only a 6 serovar panel was run. All other MAT analyses performed at both the CDC and CAHFS. In all cases CAHFS and CDC anti-Pomona antibody titers were equal.
[b] Antibiotic treatment began on day 577 and continued to day 599.

from all subsequent blood draws were within the normal range. Urinary shedding was detected via real-time PCR of urine collected 61 days after the first detection of antibodies (hence exposure must have occurred ≥61 days prior to detection of shedding), but not in urine collected subsequently, including two samples collected after antibiotic treatment had commenced (Table 2).

4. Discussion

We have assessed asymptomatic and chronic carriage of *L. interrogans* serovar Pomona in sea lions, to determine whether individual sea lions exhibit traits classically associated with *Leptospira* maintenance hosts (i.e. exhibiting few or no clinical signs while shedding infectious leptospires in their urine for months to years), and to investigate our hypothesis that chronic carriers enable persistent circulation of the pathogen in the sea lion population. Using real-time PCR and culture of urine, we definitively identified asymptomatic carriage in 33 wild, free-ranging sea lions. Six of these leptospiruric wild, free-ranging sea lions had no detectable antibodies suggesting that these animals were sampled during the early stages of infection prior to mounting an immune response, that these animals were chronically infected and their antibody titers had declined below the detectable limit, or that no detectable immune response had been or would be mounted. The latter scenario could be due to an immunocompromised state, or it could mirror the low response seen for classical *Leptospira* serovar/maintenance host infections (Ellis et al., 1981; Thiermann, 1983; Vinetz et al., 1996). Three wild, free-ranging sea lions had MAT titers that were highest against serovars other than Pomona. Heterologous reactions are common in MAT analyses (Turner, 1968) and are thought to be due to cross-reactive antibodies; it is noteworthy that the serovars associated with highest titers in these three animals appeared frequently as cross-reactions in the 26 sea lions with highest titer against Pomona. Therefore, given the fact that all past isolates as well as those reported in this study were serovar Pomona, the infecting serovar in these three cases was most likely Pomona. However, as no isolates were obtained from these animals we cannot rule out the possibility of infection with a non-Pomona serovar. One wild, free-ranging sea lion had a serum MAT of 1:25,600 against serovar Pomona and was positive for leptospire shedding by culture, but not by PCR. Low urine leptospire concentration can lead to negative urine PCR results in an actively shedding animal, especially if the urine sample volume is small, as was the case for this animal (<0.5 ml urine). Uneven distribution of leptospires, coupled with the low sample volume and/or PCR inhibitors that may be present in urine (Rojas et al., 2010; Stoddard et al., 2009; Tulsiani et al., 2011), may have led to a sample that included detectable infectious leptospires in the culture medium but not in the sample set aside for PCR. In addition, storage at −80 °C for extended periods can reduce PCR amplification potential for urine samples (Cameron et al., 2008), and this sample was stored for almost 8 months. Two wild, free-ranging sea lions had positive serum MAT titers but were not found to be shedding. These animals may have recovered from infection and cleared the leptospires from their kidneys; or leptospiruria may not have been detected due to intermittency in shedding or imperfect assays.

Using real-time PCR of urine we evaluated longitudinal measurements from a sea lion in rehabilitation and demonstrated that chronic shedding of leptospiral DNA for at least 9 weeks post-infection is possible. This MMCC/FM animal never exhibited overt clinical signs (despite being under observation during all stages of infection, including initial exposure and seroconversion), and had not been treated with antibiotics prior to detection of shedding. Hence this case history represents a possible course of infection for untreated sea lions, suggesting that some of the asymptomatic carriers we identified in the wild may have been, or may have become, chronic asymptomatic carriers.

Chronic carriage in maintenance hosts is typically believed to persist for months to years, depending on the lifespan of the host species in question. The maximum duration of carriage observed in this study was 9 weeks, but several factors make our observed duration a minimum bound and the true duration of carriage could be much longer. Observation of chronic carriage was both left- and right-censored for the MMCC/FM rehabilitated animal. Substantial underestimation of shedding duration may have arisen because the time of initial exposure was unknown (i.e. observations were left-censored), and may have occurred any time during the 66 weeks since the last negative MAT result. The first measured titer of 1:12,800 is far below peak values (≥1:204,800) commonly observed in

sea lions, which given the estimated titer half-life of 3 weeks could indicate exposure several months prior to detection (Lloyd-Smith et al., 2007), though we emphasize that peak titers and decay rates in asymptomatic and chronic infections are unknown. In addition, further underestimation may have occurred as antibiotic treatment was initiated prior to the final two urine samples, and may have reduced shedding prematurely (i.e. caused right-censoring of the shedding duration). Intermittent shedding and imperfect assays, combined with limited opportunities for sampling, may also have contributed to underestimation of shedding duration. PCR and culture assays for detection are not 100% sensitive, especially when urine leptospire concentration is low and because urine may contain PCR inhibitors (Rojas et al., 2010; Stoddard et al., 2009; Tulsiani et al., 2011). On the contrary, it is possible that PCR-based detection could lead to overestimation of shedding duration, because PCR could detect non-viable leptospires that could be shed for a brief period following resolution of *Leptospira* infection and kidney colonization; therefore, confirmation of chronic carriage by culture remains an important priority.

5. Conclusion

Here we report, for the first time, asymptomatic shedding of leptospires in free-ranging wild California sea lions and asymptomatic seroconversion and chronic asymptomatic shedding in a rehabilitated sea lion. This study adds to the evidence that sea lions infected with *L. interrogans* serovar Pomona do not conform to the dichotomous view of maintenance versus accidental hosts for pathogenic *Leptospira* spp., reinforcing doubts raised elsewhere about this paradigm (Ko et al., 2009). We have identified asymptomatic carriage in numerous animals, and chronic asymptomatic carriage for at least 9 weeks in 1 animal, providing a stark contrast to the severe and lethal infections commonly observed in this system. Our findings also provide information on the course of infection in naturally infected individuals, including leptospiral carriage and shedding, measures of renal function, and antibody titer decline. Identification of chronic carrier animals provides a potential mechanism for persistent circulation of *L. interrogans* serovar Pomona in the sea lion population, but confirming their epidemiological role will require further research to characterize the prevalence of chronic carriage (particularly in the period between seasonal outbreaks), as well as the duration, intensity and intermittency of urinary shedding by chronic carriers.

Conflict of interest statement

All authors declare that there are no financial or other relationships that might lead to a conflict of interest.

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