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Seroprevalence of *Brucella* antibodies in harbor seals in Alaska, USA, with age, regional, and reproductive comparisons

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ABSTRACT: Populations of harbor seal *Phoca vitulina* in the Gulf of Alaska have dramatically declined during the past 4 decades. Numbers of seals in Glacier Bay, in southeast Alaska, USA, have also declined despite extensive protection. Causes of the declines and slow recovery are poorly understood. Brucellosis is a zoonotic disease that adversely affects reproduction in many domestic species. We measured the seroprevalence of *Brucella* antibodies in 554 harbor seals in 3 Alaska locations: Prince William Sound (PWS), Glacier Bay (GB), and Tracy Arm Fords Terror (TAFT) Wilderness Area. Objectives included testing for regional, sex, age, and female reproductive state differences in *Brucella* antibody seroprevalence, persistence in titers in recaptured seals, and differences in titers between mother seals and their pups. Overall, 52% of adults (AD), 53% of subadults (SA), 77% of yearlings (YRL), and 26% of <5 mo old pups were seropositive. Matched mother–pup samples were consistent with dependent pups acquiring maternal passive immunity to *Brucella*. Results show higher seroprevalence (64%) for AD and SA seals in the depressed and declining populations in PWS and GB than in TAFT (29%). Lactating females were less likely to be seropositive than other AD females, including pregnant females. Further research is needed to seek evidence of *Brucella* infection in Alaskan harbor seals, identify effects on neonatal viability, and assess zoonotic implications for Alaska Natives who rely on harbor seals for food.

KEY WORDS: *Phoca vitulina* · *Brucella* · Harbor seal · Seroprevalence · Reproduction · Alaska

INTRODUCTION

Populations of harbor seal *Phoca vitulina* in the Gulf of Alaska, Aleutian Islands, and Glacier Bay, USA, have experienced dramatic population declines during the past 4 decades (Pitcher 1990, Frost et al. 1999, Small et al. 2003, 2008, Mathews & Pendleton 2006, Womble et al. 2010). Monitored infectious diseases, including brucellosis, have not been considered a significant mortality factor (Zarnke et al. 2006), but high seroprevalence of *Brucella* antibodies in Alaskan harbor seals detected by Zarnke et al. (2006) in conjunction with widespread population declines, and effects of brucellosis on health and reproduction in other species (Olsen & Palmer 2014) invite further consideration.

Brucellosis, a disease caused by infection with a *Brucella* species bacteria, affects wild and domestic mammals and humans. *Brucella* species are gram-negative coccobacilli that preferentially target the reticuloendothelial and reproductive systems, resulting in reduced reproductive success in numerous species (Corbel 1997). At least 9 terrestrial species of *Brucella* have been recognized and are associated

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with a host preference and zoonotic potential (Olsen & Palmer 2014, Whatmore et al. 2014). Consequences of *Brucella* infections vary greatly by species of *Brucella* and host. Among terrestrial species and hosts, brucellosis can result in diminished reproduction in females by causing infertility, spontaneous abortion, and weak or moribund offspring, while males may have inferior semen quality (Olsen & Palmer 2014). Effects may be chronic, lasting months or years (Olsen & Palmer 2014). *Brucella* can also infect other systems such as the nervous and musculoskeletal systems and has complex zoonotic potential (Nymo et al. 2011).

In 1994, *Brucella* was cultured from marine mammals (Ewalt et al. 1994, H. M. Ross et al. 1994). Since then, *B. ceti* (primarily found in porpoises and dolphins) and *B. pinnipedialis* (found in pinnipeds) have been recognized as species, each having several molecular subgroups (Maquart et al. 2009a, Hernández-Mora et al. 2013, Duncan et al. 2014, Olsen & Palmer 2014).

Phenotypic characterization and pathology associated with marine *Brucella* species, including strains of *B. ceti* and *B. pinnipedialis*, are complex and are still being described (Maquart et al. 2009a). Pinniped and cetacean species affected by *Brucella* are widely distributed in the Atlantic and Pacific Oceans in the northern and southern hemispheres (Lynch et al. 2011, Olsen & Palmer 2014). Recently, in California, a *Brucella* variant, with biochemical characteristics of both *B. ceti* and *B. pinnipedialis*, was found to cause osteolytic lesions in a sea otter *Enhydra lutris* (Miller et al. 2017). Marine *Brucella* have been isolated from all major body tissues of marine mammals, including male and female reproductive organs, mammary glands, lungs, spleen, kidneys, liver, lymph nodes, central nervous system, blood, and feces (Maratea et al. 2003, Sidor et al. 2013, Olsen & Palmer 2014). Consequences of brucellosis are most commonly observed in cetaceans, with differing pathology expressed among dolphins, porpoises, and whales (Guzmán-Verri et al. 2012, Meegan et al. 2012).

Among pinnipeds, pathology associated with *Brucella* infections differs between otariids and phocid seals, with stronger responses observed in otariids (Guzmán-Verri et al. 2012, Lambourn et al. 2013, Duncan et al. 2014). Most research has been conducted on *Brucella* species and phocid seals in the North Atlantic. *Brucella* strains isolated from hooded seals *Cystophora cristata* produced the least virulent infection response among 3 identified *B. pinnipedia* IS*711* gene insertion mutation strains (Maquart et al. 2009a,b, Larsen et al. 2013, 2016, Nymo et al. 2016). No intracellular multiplication of *Brucella* isolated from hooded seals and common seals *P. vitulina* has been documented in hooded seal epithelial cells or alveolar macrophages, human or murine macrophages, or in standardized pathogenicity experiments with *B. pinnipedialis* in established BALB/c mouse models. These findings indicate exposure responses are consistent with a mild, acute, and transient infection in these cell types (Maquart et al. 2009b, Larsen et al. 2013, 2016, Nymo et al. 2016).

Less is known about *B. pinnipedialis* virulence in the North Pacific. In Washington state, inoculation of pregnant cattle with *Brucella* isolated from a local harbor seal appeared less pathogenic than inoculation with *B. abortus*, but resulted in seroconversion in all cattle and abortion in some (Rhyan et al. 2001). In Alaska, evidence of *Brucella* infection has been found in the declining eastern Pacific stock of northern fur seals *Callorhinus ursinus*, of which 5% of placentae tested were positive for *Brucella* by polymerase chain reaction (PCR) and 1 fur seal exhibited severe placentitis (Duncan et al. 2014). However, no virulence assessments of *Brucella* strains have been published for harbor seals in Alaska. Serology assessments, as presented in this paper, only indicate past exposure to *Brucella* sufficient to generate antibodies; seropositvity does not confirm ongoing exposure or infection. Nonetheless, differences in titers among age and sex groups over time and across regions help identify particular phases in the life history of seals where exposure may have consequences.

This study investigated the seroprevalence of *Brucella* antibodies in harbor seals of different age, sex, and reproductive categories captured in 3 geographic regions in southcentral and southeast Alaska. Study areas included (1) Prince William Sound (PWS), (2) Glacier Bay (GB), and (3) Tracy Arm Fords Terror (TAFT) Wilderness Area (Fig. 1). Population trends among those regions have not been equivalent over time. The numbers of harbor seals in PWS experienced a 63% decline between 1984 and 1997 (Frost et al. 1999) that continued through at least 1999 (Ver Hoef & Frost 2003). Unpublished surveys indicate the population stabilized around 2002 and has likely increased since then (Allen & Angliss 2015). In GB, numbers of seals were thought to be increasing until 1992 (Mathews & Pendleton 2006). From 1992 to 2008, numbers of seals counted during the molting period in GB declined by 8.2% yr^{-1} at glacial sites and 12.4% yr^{-1} at terrestrial haulouts (Womble et al. 2010). Population trends of harbor seals in TAFT have not

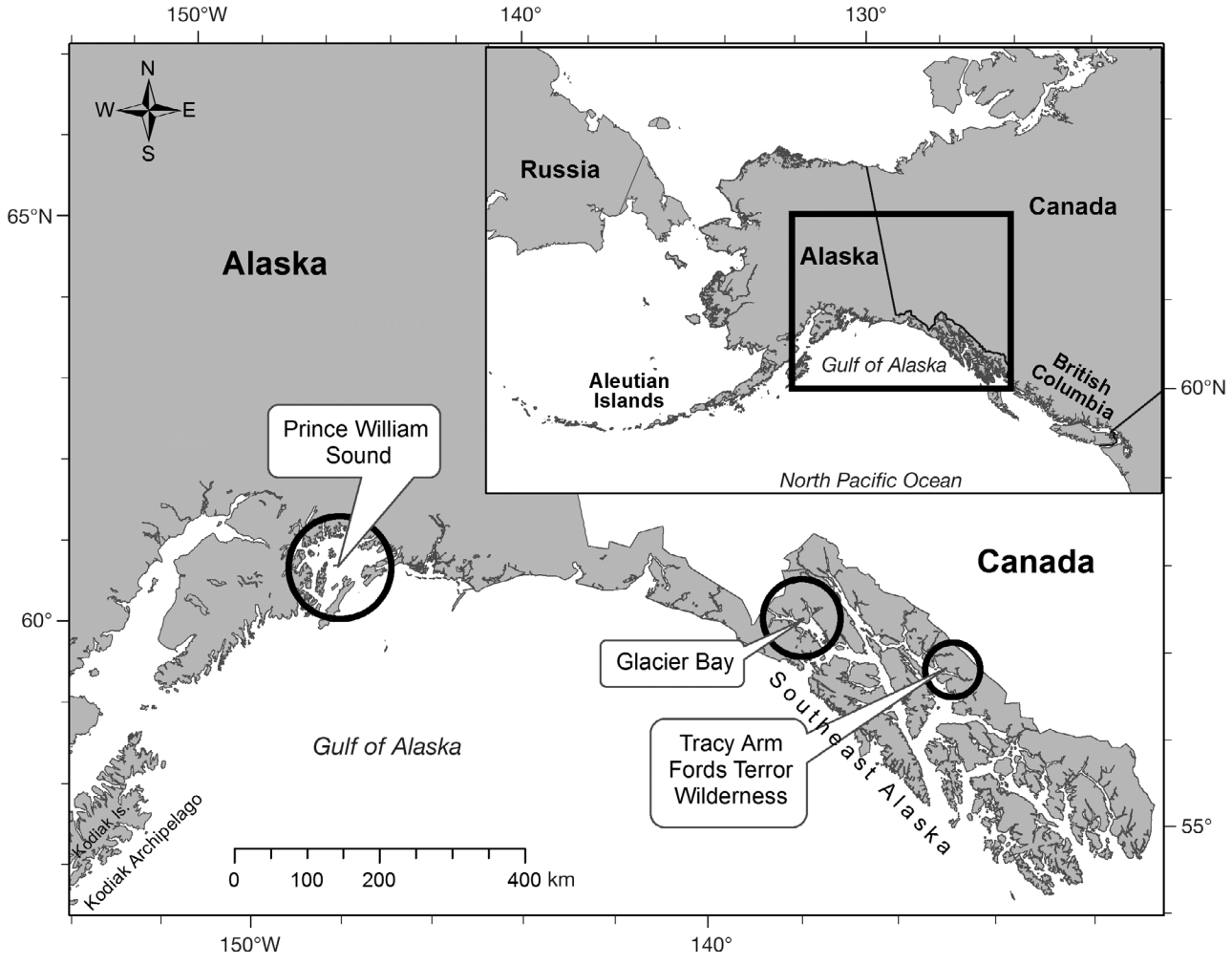

Fig. 1. Locations of the 3 harbor seal *Phoca vitulina* sampling areas compared in this study

been quantitatively determined; however, with the exception of GB, population trends of seals at most monitored locations in southeast Alaska have been stable or increasing (Small et al. 2003, Allen & Angliss 2015).

The overall goals of this study were to assess whether exposure to marine *Brucella*, as indicated by seropositive harbor seals, has been equivalent across regions and to identify characteristics of seropositive seals in relation to age, sex, region, and reproductive status. Specific objectives included (1) testing for regional, sex, and age specific differences in seroprevalence rates of harbor seals in southcentral and southeast Alaska; (2) testing for differences in *Brucella* seroprevalence in pregnant and lactating female seals relative to regional prevalence; (3) assessing temporal changes in *Brucella* seroprevalence of pups during their first year of life; and (4) measuring changes in seroprevalence over time in recaptured seals.

MATERIALS AND METHODS

Seal capture and handling

In conjunction with multiple research projects conducted between 2003 and 2010, harbor seals were captured in PWS in southcentral Alaska, GB in southeast Alaska, and TAFT southeast Alaska (Fig. 1). Seals in PWS were captured in 2003 to 2005 at terrestrial sites that were predominantly rocky reefs; in GB, seals were captured in 2004 to 2006 at both terrestrial sites and within a glacial ice habitat in Johns Hopkins Inlet; seals in TAFT were captured from 2008 to 2010 in glacial ice habitats. A multifilament seine net was used for terrestrial captures (Small et al. 2005), and monofilament gill nets were used for capturing seals at the glacial ice sites (Blundell et al. 2011). Following capture, seals were transported to a research vessel where sex, standard length (to the nearest cm), and body mass (to the nearest 0.1 kg) were

determined, blood was collected, and passive integrated transponder (PIT) and rear flipper tags were applied prior to release. Methods of capture and sample collection were reviewed and approved through multiple institutional animal care and use committees.

Sample collection

Harbor seal blood was collected from the extradural vein into serum separator Vacutainer® tubes, allowed to clot for at least 30 min, then centrifuged at 1500 × *g* for 10 min. Serum was removed and frozen at approximately −10ºC in the field (≤14 d) and subsequently transferred to a −80ºC freezer for storage.

Progesterone assays

In conjunction with other research, serum progesterone concentrations (P4) were measured in 53 adult (AD) and subadult (SA) female seals sampled from mid-April to mid-July in GB and PWS (not in TAFT). Progesterone measurements were used to identify pregnant seals and assist with identifying reproductively mature seals (evidenced by pregnancy), which morphometrics classified as subadult.

Progesterone assays used a previously validated radioimmunoassay (RIA) protocol (Greig et al. 2007, Villegas Amtmann & Costa 2010). Briefly, serial dilutions of pooled samples were run in the assay to determine pool displacement relative to the standard curve, and assay accuracy was determined by combining 50% of the total assay volume of a known mass of hormone with 50% of a pooled sample and run in each assay. Recovery (%) was determined for each assay and was calculated from recovered amounts of known hormone as demonstrated by the accuracy curve. The lower limit of sensitivity for P4 was 0.1 ng ml^{-1}. Samples were assayed in 2 assays and intra-assay coefficients of variation were <5%. Inter-assay coefficients of variation were 1.69 and 3.52% for the P4 high and low internal controls, respectively. All RIAs were conducted at the University of Alaska Endocrine Laboratory. Among samples obtained from April to June, P4 values >26 nmol l^{-1} (7 ng ml^{-1}) were interpreted as indicating a 95% probability of being pregnant (Greig 2002). Pregnancy detection analysis (positive or negative) was conducted for AD females sampled during the last 3 mo of pregnancy (April to June), based on that threshold.

Age and sex classifications

Age categories were determined from observations in the field, which visually distinguished pups from yearlings (YRL), and from a morphometric-based model derived by Blundell & Pendleton (2008) that distinguished SA, which included 2 to 5 yr old seals, from older AD seals.

Pups sampled from June to November were grouped into 2 categories: near-weaning and post-weaning. Near-weaning pups were captured in June and July and represent pups that were still nursing or had been recently weaned and were relying on milk or their blubber for sustenance. Near-weaning pups had not transitioned to effective foraging, although they may have caught and ingested fish or invertebrates while exploring. Post-weaning pups, captured from September to October of the pup's first year, were independent of their mother and foraged for food. Pups sampled from February to May were classified as spring pups and represented pups that sustain themselves completely through foraging for a prolonged period of time.

Reproductive status

Female harbor seals classified as AD or that were morphometrically classified as SA but showed evidence of reproduction were considered reproductively mature and expected to reproduce on an annual basis (Bigg 1969). Mature female harbor seals captured from April to July, when reproductive status could be most accurately assessed, were classified by reproductive status. Females known to be pregnant or lactating were classified as reproductive females, females lacking evidence of reproduction were classified as not reproductive.

The reproductive status of female seals captured from April to July was determined based on visual observations taken in the field and, in April to June, serum progesterone concentrations. Visual determination of pregnancy in the field from April to June was based on rotund appearance, fetal movements, or presence of a vaginal mucus plug (a vaginal barrier sustained during pregnancy). In addition, seals that were not classified as pregnant in the field but exhibited serum progesterone concentrations exceeding 26 nmol l^{-1} (7 ng ml^{-1}) were categorized as pregnant (Greig 2002). In the absence of progesterone assays, seals (n = 4) that were of adult length (>142 cm; Blundell & Pendleton 2008) and exhibited mass/standard length ratios less than 0.5 kg cm^{-1}

were classified as not pregnant (in contrast to known pregnant seals, all of which exceeded 0.67 kg cm^{-1}). In late June and July, seals were categorized as reproductive if they were observed with a pup or if evidence of lactation or recent weaning of young was recorded. In this paper, females showing evidence of caring for a pup are labeled as 'lactating' seals.

Seals that were identified as not reproductive may have included seals that did not breed, had failed pregnancies, or had successful pregnancies that we did not detect. As noted above, seals captured from September to March were excluded from reproductive analysis due to ambiguities in physical appearance and progesterone concentrations (Greig 2002).

Brucella assay

Harbor seal serum samples were analyzed for *Brucella* antibodies at Mystic Aquarium (Mystic, Connecticut) using a competitive enzyme-linked immunosorbent assay (cELISA) based on a whole cell antigen from a *Brucella*-infected harbor seal (Meegan et al. 2010). This assay was blindly and successfully compared with previous results achieved using multiple Hawaiian monk seal samples (Nielsen et al. 2005). Based on a consensus method involving multiple assay analysis of samples, Meegan et al. (2010) classified ≥30% inhibition as positive, which reflected samples that tested positive and showed agreement among all assays; samples with 25 to 29.99% inhibition were classified as suspect and had less than 100% agreement among assays; samples with <25% inhibition were classified as negative and were negative among all assays. Antibody responses of samples in our study were compared among age and sex categories and location of capture.

Statistical analysis

Seroprevalence. Seroprevalence based on cELISA assays was initially classified as positive (≥30% inhibition), suspect (25 to 29.99% inhibition), and negative (<25% inhibition). Bivariate models used for statistical analysis classified *Brucella* titers binomially as positive (% inhibition ≥ 30) and negative (% inhibition < 30).

Seroprevalence among regions, ages, and sex categories. Identification of covariates associated with the prevalence of *Brucella* seropositive seals among region, age, and sex categories were accomplished using generalized linear models (GLM) with a binomial distribution and logit link. Models included the binomial designation for *Brucella* titers. Covariates of *Brucella* seroprevalence were also assessed for all samples. Initial covariates included region (PWS, GB, TAFT), age (AD, SA, YRL, pup), and sex (male, female). Covariates that did not significantly contribute to the model were sequentially removed, in order of least significant contribution, and the model was rerun. Once all variables significantly contributed to the model, least-squares means contrasts were used to compare differences among variables within covariate categories. In this analysis, contrast coefficients were normalized to make their sum zero and their absolute sum equal to 2. The overall test was a joint *F*-test (SAS Institute 2014).

Temporal changes in seroprevalence among pups and yearlings were assessed using nominal logistic fits of *Brucella* titer status (positive, negative) for each age class (pre-weaning pup, post-weaning pup, spring pup, YRL). Region (PWS, GB, TAFT) and sex (male, female) were also initially included as potential covariates affecting status. Significance of region and sex effects on seroprevalence were determined by effect likelihood ratio tests and those variables that significantly influenced titers were retained in the final assessment.

Reproduction and seroprevalence. Reproduction and seroprevalence of females was assessed using nominal logistic fits of the seroprevalence of females (pregnant or lactating) relative to their respective regional SA–AD *Brucella* seroprevalence (PWS–GB, TAFT), to test the likelihood that *Brucella* seroprevalence rates were equivalent among females known to have been reproductively viable and the remainder of the SA–AD population. Comparisons were made separately for each reproductive stage.

Statistical analysis. Quantitative comparisons were conducted using JMP version 11.2 statistical software (SAS Institute) with a significance level of $\alpha = 0.05$.

RESULTS

Serum samples

Serum samples analyzed for *Brucella* antibodies (n = 554) were obtained from harbor seals captured in PWS (n = 202; 108 males, 94 females), GB (n = 292; 156 males, 136 females) and TAFT (n = 60; 3 males, 57 females) (Tables 1 & 2). Nine seals were recaptured 3 to 12 mo after initial capture. Among AD female harbor seals (n = 68) captured from April to July, 12 were pregnant, 32 had lactated, 4 were not pregnant, and 20 did not show evidence of pregnancy or lactation (Table 3).

Table 1. Age and sex distribution of harbor seals *Phoca vitulina* tested for seroprevalence of *Brucella* antibodies. Values in parentheses denote the proportion of samples that tested seropositive based on cELISA assays

	Female n (%)	Male n (%)	Total n (%)
Pup (near-weaning)	27 (22)	41 (39)	68 (32)
Pup (post-weaning)	55 (11)	54 (33)	109 (22)
Pup (spring)	16 (75)	26 (73)	42 (74)
Yearling	55 (75)	38 (82)	93 (77)
Subadult	56 (46)	54 (59)	110 (53)
Adult	78 (54)	54 (50)	132 (52)
Total	287	267	554

Table 2. Temporal and regional frequency distribution of harbor seal *Phoca vitulina* serum samples analyzed by cELISA for *Brucella* antibodies by the seal's sex, location, and year of capture. Regions include Glacier Bay (GB), Prince William Sound (PWS), and Tracy Arm Fords Terror (TAFT) Wilderness Area

Region	2003	2004	2005	2006	2008	2009	2010
Males							
GB	0	41	60	55	0	0	0
PWS	31	38	39	0	0	0	0
TAFT	0	0	0	0	1	2	0
Females							
GB	0	38	48	50	0	0	0
PWS	19	39	36	0	0	0	0
TAFT	0	0	0	0	26	20	11

Seroprevalence among region, age, and sex

An initial GLM of seroprevalence (positive, negative) with covariates age (AD, SA, YRL, pup), region (PWS, GB, TAFT), and sex (M, F) was used to assess regional, age, and sex influences on seroprevalence. Sex differences or age differences between AD and SA were not significant. AD and SA categories were therefore combined and sex was removed from the model. The resulting model included the covariates age (SA–AD, YRL, pup) and region (PWS, GB, TAFT). The proportion of seals testing positive for *Brucella* differed by age and region (Table 1, Figs. 2 & 3). Based on effects-test comparisons (Table 4), seroprevalence in pups differed significantly from SA–AD and YRL; YRL differed significantly from SA–AD. Although PWS and GB did not differ significantly from each other, positive titers were more prevalent in PWS and in GB than in TAFT (Table 4).

Female reproductive status

Brucella seroprevalence was contrasted among 82 females of different reproductive categories (Table 3) relative to other SA–AD seals.

Among pregnant females, 67% of 12 tested seropositive compared to 53% of 218 other SA–AD. A nominal logistic fit of the seroprevalence among pregnant females with other SA–AD and region identified regional differences (Wald effect test, df = 2, χ^2 = 15.594, $p < 0.001$), but no difference with respect to pregnancy status.

Lactating females exhibited a lower likelihood of positive titers than other SA–AD, where 57% of 210 SA–AD seals tested positive in contrast to 25% of 32 lactating females (Fisher's exact test, 2-tail, p = 0.001). When lactating females were contrasted with other SA–AD by regions, significant regional (Wald effect test, df = 2, χ^2 = 7.771, p = 0.02) and lactation status (Wald effect test, df = 1, χ^2 = 4.428, p = 0.035) effects were identified, with lactating females less likely to be seropositive than other SA–AD seals (Fig. 4). Among 7 females that were captured in late-June and July in PWS and TAFT and that did not show evidence of lactating or weaning a pup, 43% were seropositive.

Table 3. Seasonal distribution of the reproductive status of adult female harbor seals *Phoca vitulina* plus morphologically categorized subadults that showed evidence of reproduction. Reproductive categories Yes (reproductive) and No (not known reproductive) only include seals sampled from April to July. Values in parentheses denote the proportion of samples that tested seropositive based on cELISA assays

Reproductive status	Reproductive category	April–May n (%)	June–July n (%)	Sept–Feb n (%)	Total
Not pregnant	No	4 (50)			4
Pregnant	Yes	12 (67)			12
Lactating	Yes		32 (25)		32
Not lactating	No		7 (43)		7
Unknown	No		13 (62)	14 (71)	27
Total		16	52	14	82

Seroprevalence among pups

Positive *Brucella* titers were detected in 32% of the 68 near-weaning pups and 22% of the 109 post-weaning pups sampled in PWS, GB, and TAFT (Table 1).

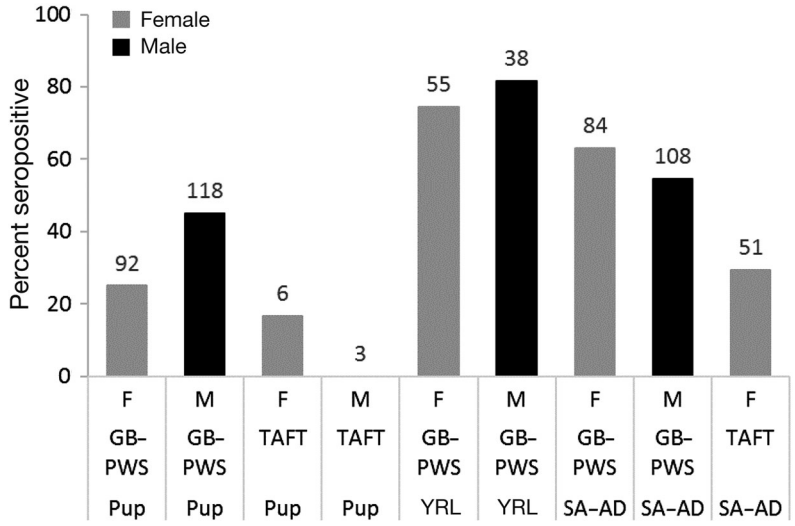

Fig. 2. Percent of harbor seals *Phoca vitulina* sampled from 2002 to 2010 that tested seropositive to *Brucella* based on cELISA assay. Results are summarized by age, capture location, and sex. GB–PWS: Glacier Bay and Prince William Sound region; TAFT: Tracy Arm Fords Terror Wilderness Area; SA–AD: subadult and adult age classes combined; YRL: yearlings. Sample sizes are indicated above bars

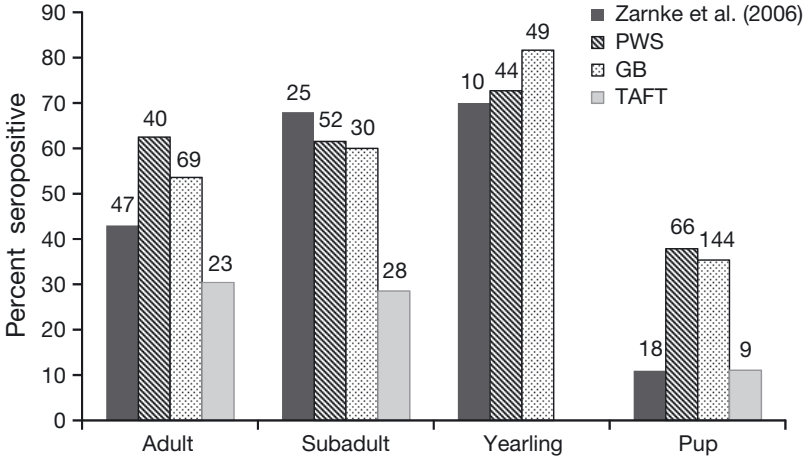

Fig. 3. Comparison of the percent of seals *Phoca vitulina* in 4 age categories that tested seropositive for *Brucella* antibodies based on cELISA assays. Prince William Sound (PWS) and Glacier Bay (GB) samples were from depressed or declining populations sampled from 2002 to 2006, while the population status for seals from Tracy Arm Fords Terror (TAFT) Wilderness Area, sampled from 2008 to 2010, is unknown. Historical samples from declining populations in southcentral Alaska from 1976 to 1999 are based on Zarnke et al. (2006). Sample sizes are indicated above bars

Sampling bias precluded most regional comparisons as near-weaning pups were primarily sampled in PWS and all post-weaning pups were sampled in GB. Of the near-weaning pups in TAFT (n = 9), 11% were seropositive, 22% suspect, and 67% were seronegative. In PWS (n = 36), 36% were seropositive, 3% suspect, and 61% seronegative. Seroprevalence in near-weaning pups did not differ significantly between regions or by sex. Among all pups, seropositive titers were more frequent in males (29%, n = 59) than females (14%, n = 64) (likelihood ratio test, df = 1, $\chi^2 = 4.046$, $p < 0.05$), but that tendency was only apparent in PWS.

During their first year of life, the proportion of pups testing positive for *Brucella* antibodies increased over time (Table 1). The proportion of near-weaning and post-weaning pups testing positive was relatively low (32 and 22%, respectively). High proportions of spring pups tested positive (74%), similar to the high seroprevalence detected in yearlings (77%). Effects of region or sex were not significant.

Mother–pup comparison

Brucella seroprevalence was contrasted between 8 mother seals and their near-weaning pups captured in TAFT. All 5 mothers testing seronegative had seronegative pups. Of the 2 pairs in which the mothers tested suspect, 1 pup was seropositive and the other was suspect. One mother tested positive and her pup tested suspect. These results are consistent with pups receiving passive immunity from their mothers, including mothers suspect for positive titers.

Persistence of response

Nine seals were recaptured during the study. Six seals were recaptured approximately 1 yr later, the remaining 3 were recaptured 3 to 8 mo after their initial capture date (Table 5). The proportional change among seals averaged 0.5% mo^{-1} (range 3.3 to –4.2%). Only 1 of the 9 seals (PV04PWS45) changed antibody status (from positive to negative) during a 1 yr span, a change that was also reflected in a University of Connecticut *B. abortus* card test (A. Hoover-Miller unpubl. data).

Table 4. Summary of covariates that significantly contributed to a generalized linear model (GLM) of the seroprevalence of *Brucella* antibodies based on cELISA in Alaska harbor seals *Phoca vitulina*. Groups summarize covariate category associations based on GLM effects tests. Within covariates categories, different letters indicate categories that significantly differed from each other. Age categories include pups, yearlings (YRL), subadults (SA), and adults (AD); regions include Prince William Sound (PWS), Glacier Bay (GB), and Tracy Arm Fords Terror (TAFT) Wilderness Area; NLL: negative log likelihood

Covariate	Category	Group	Effects tests
Age	Pup	a	Pup vs. SA–AD (NLL = 361.028, df = 1, χ^2 = 20.78, p < 0.0001)
	YRL	b	Pup vs. YRL (NLL = 373.142, df = 1, χ^2 = 45.01, p < 0.0001)
	SA	c	YRL vs. SA–AD (NLL = 355.65, df = 1, χ^2 = 10.025, p < 0.005).
	AD	c	SA vs. AD (p > 0.05)
Region	PWS	a	PWS vs. GB (p > 0.05)
	GB	a	TAFT vs. PWS (NLL = 358.62, df = 1, χ^2 = 15.97, p < 0.0001)
	TAFT	b	TAFT vs. GB (NLL = 357.89, df = 1, χ^2 = 14.50, p < 0.001)
Sex	M	a	M vs. F (p > 0.05)
	F	a	

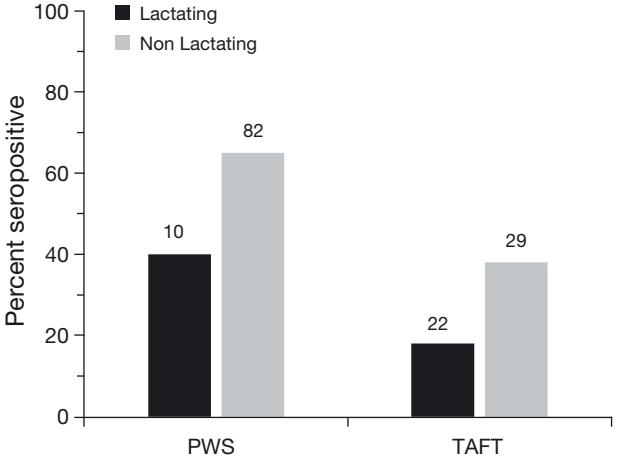

Fig. 4. Comparison of percent of lactating female harbor seals *Phoca vitulina* testing seropositive for *Brucella* antibodies with other subadult–adult seals in Prince William Sound (PWS) and in the Tracy Arm Fords Terror (TAFT) Wilderness Area. Numbers above bars represent sample size

DISCUSSION

Research conducted on harbor seals sampled throughout Alaska since 1976 has determined that high proportions of seals have been exposed to *Brucella* (Fig. 3). Similar to the present study, based on a sample of 100 seals, Zarnke et al. (2006) detected antibodies to *Brucella* in 46% of samples (43% AD, 68% SA, 70% YRL, and 11% pups) with statistically insignificant differences in antibody prevalence between AD, SA, and YRL cohorts, regions, or sex, but a significant difference in seroprevalence between non-pups (54%) and pups (11%). In comparison, our study detected 53% positive titers (52% AD, 53%, SA, 77% YRL, and 35% pups). Differences observed may be confounded by the sensitivity and specificity of the cELISA analysis used in each study, as Zarnke et al. (2006) used microtiter plates coated with lipopolysaccharide extracted from *B. melitensis*, in contrast to our use of a harbor seal origin *Brucella* cELISA. The similarity in seroprevalence levels in the subadult and yearling categories in PWS and GB compared to results obtained by Zarnke et al. (2006) suggest similar test sensitivity; however, unlike Zarnke et al. (2006), we did not detect declining tendencies in seropositive titers between SA and AD age categories, and the proportion of pups testing positive was considerably higher in our measurements in PWS and GB than that measured by Zarnke et al. (2006). These comparisons suggest elevated *Brucella* exposures in adult and pups sampled from 2002 to 2010 in GB and PWS relative to those sampled from 1976 to 1999. In this study, we also detected regional differences in that titers were significantly higher in PWS and GB than in TAFT, suggesting seals in TAFT may be relying on prey less burdened by *Brucella*.

Hueffer et al. (2013) used the same protocols and laboratory as our study to assess seroprevalence of *Brucella* antibodies for harbor seals captured in 2007

Table 5. Change in *Brucella* cELISA percent inhibition for 9 harbor seals *Phoca vitulina* recaptured within a 1 yr period. Months represent number of months between samples. Proportional monthly change = (monthly change)/(1st sample % inhibition)

Animal ID	Months	Inhibition (%) 1st sample	Inhibition (%) 2nd sample	Difference	Monthly change	Proportional monthly change
PV03PWS12	3	63.66	66.39	2.73	0.91	1.4%
PV03PWS62	8	55.88	70.59	14.71	1.84	3.3%
PV03PWS67	8	57.16	60.35	3.19	0.40	0.7%
PV03PWS60	12	67.73	67.9	0.17	0.01	0.0%
PV04GB29	12	13.83	19.59	5.76	0.48	3.5%
PV04PWS31	12	56.77	54.77	–2.00	–0.17	–0.3%
PV04PWS45	12	48.07	24.07	–24.00	–2.00	–4.2%
PV05GB11	12	40.37	45.03	4.66	0.39	1.0%
PV05GB55	12	25.33	22.65	–2.68	–0.22	–0.9%

to 2008 in Glacier Bay. They determined that 37% of their samples were seropositive, which is considerably lower than the results we obtained from 2004 to 2006 in Glacier Bay (50% positive, 3.4% suspect, and 46.6% negative). The seals sampled by Hueffer et al. (2013) included higher proportions of younger seals (49% pups, 20% YRL, 11% SA, and 19% AD), compared to our study (39% pups, 17% YRL, 20% SA, and 24% AD). Differences suggest interannual variation in *Brucella* seroprevalence in Glacier Bay, but without the provision of age-specific results, it is unclear how differences were manifested.

Our results were consistent with general age-dependent patterns of *Brucella* antibodies reported by Zarnke et al. (2006) and Lambourn et al. (2013), who identified highest seroprevalence rates in yearlings. We obtained a high correlation between the seroprevalence status between mothers and their pups. Seropositive near-weaning and post-weaning pups most likely received passive immunity from seropositive mothers. By 8 mo of age, however, the proportion of seropositive pups was high, comparable to yearlings. Our results indicate that seroconversion occurs between 4 and 8 mo of age and that high proportions of pups in Alaska are exposed to *Brucella* during their first year of life.

The high seroprevalence of yearlings relative to other age groups has been attributed to exposure as seals become proficient in foraging (Lambourn et al. 2013). *Brucella* exposure may be influenced by multiple dietary pathways including the consumption of *Brucella*-infected prey (Nymo et al. 2016) and by parasites that use fish as intermediate hosts. In Washington State, for instance, *Brucella*-infected lungworms *Parafilaroides* sp. were found concentrated in the lungs of a *Brucella*-infected harbor seal (Garner et al. 1997). Lungworms commonly afflict harbor seals in Alaska. In another study, adult or larval lungworms were detected with tracheal swabs in 46% of seals sampled in Prince William Sound and 73% of those in Glacier Bay (Herreman et al. 2011). The presence of *Brucella* in those lungworms, however, was not assessed.

All but 1 of the 9 seals recaptured within a year of their initial capture showed similar % inhibition titers at recapture, indicating persistence in seroprevalence during that time period. Lambourn et al. (2013) resampled 4 harbor seals in Washington State. One that was positive as a yearling remained serologically positive 4 yr later, 3 other recaptured seals had diminished *Brucella* antibodies when retested 2 (n = 2) and 5 (n = 1) yr later. Across all seals tested by Lambourn et al. (2013), peak seropositive results were detected for seals as yearlings, with diminishing proportions of seropositive seals with age (0% pups, 17% weaned pups, 38% YRL, 22% SA, and 2% AD). Although testing methods differed between the studies, which impedes direct comparisons of seroprevalence, the results from Lambourn et al. (2013) indicate that seropositive titers can, but do not always, persist over multiple years and that in Washington State exposure to *Brucella* markedly diminished with age. Conversely, in our study, roughly half of SA and AD seals tested positive, with no diminishment with age.

Our results show an association between *Brucella* seroprevalence and reproductive status in that lactating seals were less likely to be seropositive for *Brucella* antibodies than the other SA and AD seals. Pregnant seals did not differ in seroprevalence from the wider regional population, and many pregnant females were seropositive, indicating that pregnancy persists in seropositive seals. Similarly, the seroprevalence of a small sample of reproductive-age females not determined to be pregnant or lactating in late-June and July was similar to the broader population (Table 3). Roughly 46% (38% TAFT and 52% PWS) fewer lactating females tested positive for *Brucella* antibodies relative to their respective regional SA–AD populations, including pregnant females. Why lactating seals were less likely to be seropositive is not understood. Potentially, it could be associated with depression of immune function late in lactation and during estrus (Ross et al. 1993), or the survival of neonatal pups infected with *Brucella.* However, in Scotland, necropsies of stranded common seal and grey seal *Halichoerus grypus* pups infected with *Brucella* did not show evidence of *Brucella*-caused disease (Foster et al. 2002, 2007), nor has *Brucella* infection been described in stranded neonatal pups in Alaska (Bauer et al. 2016).

Harbor seal pups are born with competent immune function but low levels of circulating immunoglobulins. Temporary, passive immunity in harbor seals is acquired by consuming high concentrations of immunoglobins from colostrum and milk early in lactation, prior to late-lactation maternal immune depression (P. S. Ross et al. 1993, 1994). Regional similarities in the seroprevalence of near-weaning pups (36% PWS and 11% TAFT) and lactating females (40% PWS and 18% TAFT) (Fig. 4), and the similarities between mothers and their respective pups measured in this study are consistent with expectations of antibody titers in nursing pups approaching maternal levels by 2 wk of age (P. S. Ross et al. 1994).

If antibody differences between lactating seals and the broader population reflect diminished reproduc-

tive success of seropositive seals, then those differences may be substantial in Alaska and may have contributed to the persistent, widespread population declines that have been observed. Currently, however, evidence of disease or compromised neonatal viability resulting from *Brucella* is lacking. Although our results show harbor seals in Alaska have experienced persistently high exposure to *Brucella* across decades, they do not provide direct evidence of effects of *Brucella* infection on seals because the presence of *Brucella* at the time of sampling has not been confirmed. The high proportions of harbor seals exposed to *Brucella* in regions affected by substantial harbor seal population declines (PWS and GB) invite further investigation. *Brucella* bacterial isolation attempts, the gold standard, complemented by molecular and serologic methods, are needed to identify and describe local Gulf of Alaska strains of marine-origin *Brucella*. Stranded and subsistence-harvested seals are good sources for the numerous tissue types in which *Brucella* can be detected by microbial culture and PCR analysis, including lung, lymph nodes, placenta, testis, liver, and spleen (Thakur et al. 2012, Sidor et al. 2013). Among live-captured seals, collection of vaginal and preputial swabs, feces, milk samples, and plasma samples (excluding heparin) also support microbial culture and PCR analysis (Thakur et al. 2012, Sidor et al. 2013). Isolation, immunohistopathologic, and molecular diagnostic methods could then be used to further assess potential influences of *Brucella* infection on harbor seal population declines and recovery.

Brucella is also known for its zoonotic potential associated with the handling and consumption of domestic species and their products (cattle, pigs, goats, and sheep) (Olsen & Palmer 2014). In recent years, marine *Brucella* has been diagnosed in 3 cases of naturally acquired human infections (not caused by exposure to marine mammals) and 1 case of laboratory-acquired infection, which caused diverse diseases including bacteremia, neurobrucellosis, and spinal osteomyelitis (Sohn et al. 2003, McDonald et al. 2006) thereby demonstrating a zoonotic potential for marine *Brucella* (Whatmore et al. 2008, Hernández-Mora et al. 2013). Harbor seals are an important food resource for Alaska Natives, whose food security relies on robust seal populations and the safety of foods they consume. Although subsistence harvesters may be exposed to *Brucella* from multiple sources, both marine and terrestrial (Brubaker et al. 2010), the high seroprevalence of *Brucella* antibodies in harbor seals in the Gulf of Alaska spurs the need for human exposure assessments and a better understanding of any infection consequences for those who hunt or handle marine mammals in the Gulf of Alaska.

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