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Survey for Placental Disease and Reproductive Pathogens in the Endangered Hawaiian Monk Seal (*Neomonachus schauinslandi*)

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ABSTRACT: There is considerable temporal and spatial variability in the reproductive rates of Hawaiian monk seals (HMS; *Neomonachus schauinslandi*). Poor reproductive performance limits the recovery of this endangered species; however, causal factors are not fully understood. There is serologic evidence that HMS are exposed to pathogens that can impact reproductive success, but the prevalence of placental infections in HMS has not been evaluated. Placental tissues (n=50), including tissues from 25% of known HMS births, were opportunistically collected in 2011 from six Northwestern Hawaiian Islands and three main Hawaiian Islands. Reproductive histories of the sampled females were representative of the breeding population, as determined through comparisons in age of primiparity and mature reproductive rate. Placental tissues were examined histologically and screened by PCR for *Coxiella burnetii*, *Brucella* spp., *Chlamydia* spp., *Leptospira* spp., herpesviruses, and *Toxoplasma gondii*. There was no histologic evidence of placental pathology, and molecular analyses were negative. These negative results can be used to estimate pathogen prevalence in the nonsampled population. For an approximate population size of 1,300 HMS, we can estimate with 99% confidence that the prevalence of each pathogen tested is 9% or less. This is low relative to other pinnipeds and indicates that factors other than reproductive pathology, such as resource limitation, may drive variability in HMS reproductive rates. Further investigation into the cumulative impacts of resource limitation and other stressors on HMS reproduction is warranted.

Key words: Brucella, *Chlamydia*, *Coxiella*, herpesvirus, histology, *Leptospira*, placenta, *Toxoplasma*.

Reproductive performance may limit growth of the endangered Hawaiian monk seal (HMS; *Neomonachus schauinslandi*) population; however, factors such as pathogens, which may impact reproductive success, are not fully understood. Approximately 1,110 of the 1,300 HMS reside in the remote Northwestern Hawaiian Islands (NWHI). Seal abundance in the NWHI underwent a prolonged decline due to poor juvenile survival and recruitment to reproductive age, although recent data are consistent with population growth in this region (Baker et al. 2011, 2016). More than 200 seals inhabit the main Hawaiian Islands (MHI), though seals occasionally move between the NWHI and MHI (Johanos et al. 2014). Threats differ between these regions and include food limitation, shark predation, marine debris entanglement, sea-level rise, intraspecific aggression, disease, and fishery interactions (National Marine Fisheries Service 2007; Baker et al. 2011, 2012; Gobush et al. 2016).

Reproductive success is important for population stability and growth, yet there is considerable range-wide variation in individual HMS reproductive performance and first-year survival (Harting et al. 2007; Baker 2008). Factors affecting reproduction in HMS are not well understood. There is serologic evidence of exposure to multiple pathogens associated with reproductive failure, including *Leptospira*, *Brucella*, *Chlamydia abortus*, herpesvirus, and *Toxoplasma gondii* (Goldstein et al. 2006; Littnan et al. 2006; Aguirre et al. 2007). Unlike serum, placental tissues are noninvasively obtained and screened to evaluate reproductive health and other host, pathogen, and environmental factors that may limit population recovery. We examined placental tissues histologically and molecularly

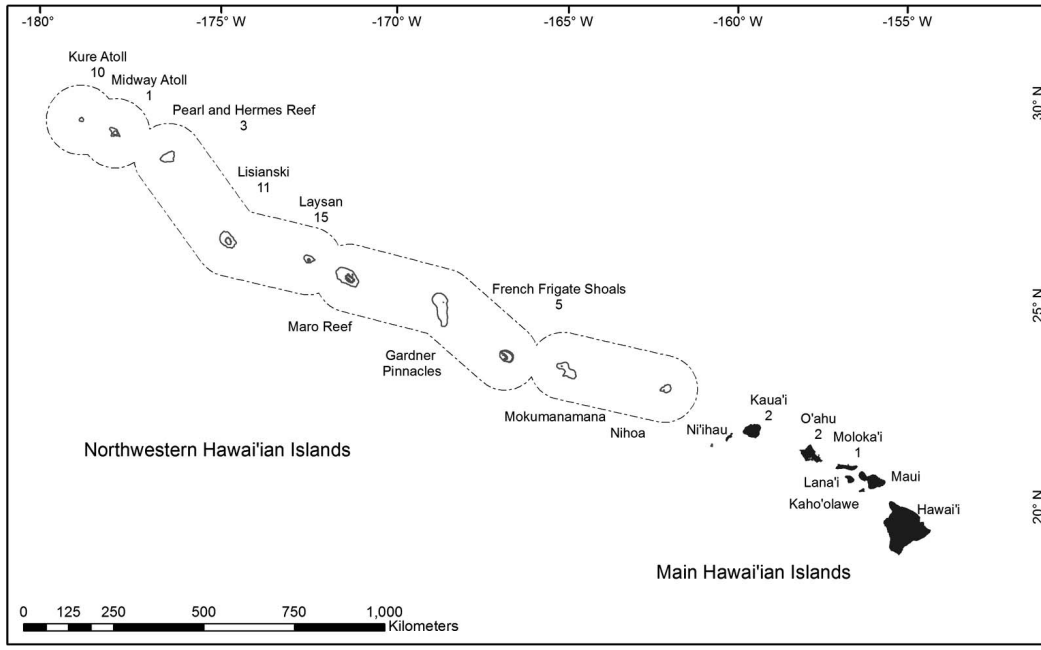

FIGURE 1. Map of the range of the Hawaiian monk seal (*Neomonachus schauinslandi*) from Hawai'i Island to Kure Atoll. Black=main Hawaiian Islands; white=Northwestern Hawaiian Islands; dotted-dashed line=boundary of the Papahānaumokuākea Marine National Monument. The numbers listed below each location are the number of placentas collected at each site in February and August 2011 for testing for disease agents.

for pathogens that may limit reproductive success and recovery of HMS.

Placentas were collected opportunistically from wild HMS as a part of a long-term population-monitoring project. Tissues that were scavenged or decomposed were excluded. Placental tissues (n=50; five from MHI and 45 from NWHI) were collected between February 2011 and August 2011 from six sites in the NWHI and three sites in the MHI (Fig. 1).

Full-thickness subsamples of 50 placentas were screened. Subsamples from 47 placentas were stored in 10% neutral buffered formalin, paraffin embedded, sectioned at 5 µm, stained with H&E, and examined histologically by a veterinary pathologist. Additional subsamples from each placenta (n=50) were frozen in liquid nitrogen and screened by PCR. Samples were pooled (maximum six samples) by location and processed using the BioSpec Mini-BeadBeater (BioSpec Products, Inc., Bartlesville, Oklahoma, USA) and DNA was extracted by using the QIAamp DNA Mini Kit according to manufacturer's instructions (Qiagen, Valencia, California, USA).

Analysis for *Coxiella burnetii* (*COM1* and *IS1111a* gene regions; Kersh et al. 2010) and *Brucella* spp. (IS711 insertion sequence; Hinić et al. 2008) was conducted by real-time PCR. Conventional PCR was conducted for *Chlamydophila* spp. (*OmpA* gene region; Hewinson et al. 1997), *Leptospira* spp. (*23S* gene region; Woo et al 1997; Harkin et al. 2003), and *Toxoplasma gondii* (529-base pair–repeated DNA fragment; Homan et al. 2000). Panherpesvirus primers were used to amplify a 225-base pair fragment of a conserved region of the herpesvirus DNA polymerase gene (VanDevanter et al. 1996). Modifications were made to all PCR-referenced protocols, and assays were validated after modification.

No histologic abnormalities, such as inflammation, were identified within any placental tissues, and molecular analyses of sample pools were negative for all tested pathogens. Hawaiian monk seal reproduction and survival are closely monitored, and these data were

used to relate results from this study to the population. Placental samples consisted of 25% of the 201 total monk seal births in 2011: 26% (47/178) from NWHI; and 13% (3/23) from MHI (Pacific Islands Fisheries Science Center 2017a). Because samples were opportunistically collected, they represented an unbiased random sample of reproductive females. This was verified by using two metrics derived from annual long-term sighting data of females (Pacific Islands Fisheries Science Center 2017b, c): age of primiparity (age first associated with a pup) and mature reproductive rate (for seals older than 5 yr). Two placentas not associated with a specific adult female were excluded, giving a sample size of 48 females for this verification. Using a univariate analysis of variance, we found no significant differences in age of primiparity between sampled and nonsampled parous females, $F(1,502)=0.488$, $P=0.485$, or in the mature reproductive rate when all sites were pooled, $F(1,502)=2.590$, $P=0.108$. Hence, our analyses from 25% of the reproductive population sampled are applicable to the entire breeding female population.

The negative histology and molecular results were used to estimate the prevalence of pathogens tested in the unsampled population. We estimated with 99% confidence that the prevalence of each pathogen tested was 9% or less (or with 90% confidence, a prevalence of 4% or less) for a total population of approximately 1,300 HMS (Putt et al. 1988). Using PCR for gene IS711, Duncan et al. (2014) detected *Brucella* sp. in placentas of 75% of tested northern fur seals (*Callorhinus ursinus*). Using PCR for genes *COM1* and *IS1111a*, *C. burnetii* was detected in placentas of 63% and 75% of harbor seals (*Phoca vitulina*), respectively (Duncan et al. 2012; Kersh et al. 2012). Thus, the minimum prevalence of these pathogens in the HMS population is remarkably low compared with prevalences measured in other marine mammals.

The pathogens for which placentas were screened typically cause midgestational to late gestational impairment; however, any cases of early embryonic death and reabsorption would go undetected in this analysis. This may influence our interpretation. In addition, *Brucella* causes reproductive failure in livestock during the first pregnancy after infection but may go undetected in subsequent pregnancies; it is unclear if this trend holds for HMS.

Our negative results are consistent with a low serologic prevalence to *Brucella*, *Chlamydia*, herpesvirus, *Leptospira*, and *Toxoplasma*, as previously reported in HMS (Littnan et al. 2006; Aguirre et al. 2007). Despite the low serologic prevalence of *Toxoplasma*, detection of this pathogen may have been limited by the small sample size from the MHI, where the definitive feline host was present. Samples were collected from a representative cross section of reproductive females. Despite the absence of lesions and negative molecular results, there is merit in periodic reevaluation to provide longitudinal information about monk seal reproductive health, especially given the noninvasiveness of sampling and lower cost of pooled molecular screening.

Many factors affect maternal health and reproductive success aside from infectious disease. Exposure to environmental contaminants impairs reproduction and offspring fitness; however, contaminant levels in most monk seals are below those thought to have detrimental health impacts (Ylitalo et al. 2008; Lopez et al. 2012). Nutritional condition is important in reproduction and can differ substantially between NWHI sites and the MHI. For many NWHI juveniles, poor condition is a result of food limitation secondary to interspecific competition and prey availability (Baker 2008). In the absence of detectable population impacts from disease or contaminants, the negative results from our study further support the theory that a lack of resources and subsequent poor nutritional condition of females, particularly in the NWHI, may be the primary drivers for low reproductive rates of female monk seals and neonatal mortality.

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