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Protozoal-related mortalities in endangered Hawaiian monk seals *Neomonachus schauinslandi*

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ABSTRACT: Protozoal infections have been widely documented in marine mammals and may cause morbidity and mortality at levels that result in population level effects. The presence and potential impact on the recovery of endangered Hawaiian monk seals *Neomonachus schauinslandi* by protozoal pathogens was first identified in the carcass of a stranded adult male with disseminated toxoplasmosis and a captive monk seal with hepatitis. We report 7 additional cases and 2 suspect cases of protozoal-related mortality in Hawaiian monk seals between 2001 and 2015, including the first record of vertical transmission in this species. This study establishes case definitions for classification of protozoal infections in Hawaiian monk seals. Histopathology and immunohistochemistry were the primary diagnostic modalities used to define cases, given that these analyses establish a direct link between disease and pathogen presence. Findings were supported by serology and molecular data when available. *Toxoplasma gondii* was the predominant apicomplexan parasite identified and was associated with 100% of mortalities (n = 8) and 50% of suspect cases (n = 2). Incidental identification of sarcocysts in the skeletal muscle without tissue inflammation occurred in 4 seals, including one co-infected with *T. gondii*. In 2015, 2 cases of toxoplasmosis were identified ante-mortem and shared similar clinical findings, including hematological abnormalities and histopathology. Protozoal-related mortalities, specifically due to toxoplasmosis, are emerging as a threat to the recovery of this endangered pinniped and other native Hawaiian taxa. By establishing case definitions, this study provides a foundation for measuring the impact of these diseases on Hawaiian monk seals.

KEY WORDS: Protozoa · Mortality · Pathology · Immunohistochemistry · *Toxoplasma gondii* · *Sarcocystis* · Pinniped

INTRODUCTION

Protozoal infections are common and widespread among pinnipeds with many well-adapted protozoan species producing relatively asymptomatic, chronic infections in a variety of pinniped taxa. California sea lions *Zalophus californianus*, for example, are definitive and intermediate hosts to at least 4 coccidian parasites that cause little to no disease (Colegrove et al. 2011, Carlson-Bremer et al. 2012). Exposure to protozoa has been serologically determined in many pinniped taxa (Measures et al. 2004, Littnan et al. 2007,

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Jensen et al. 2010, 2012, Carlson-Bremer et al. 2015) and exposure does not necessarily imply negative effects on the health of the infected host. However symptomatic and life threatening protozoal infections are being detected with increasing frequency in a variety of marine mammals, most notably the southern sea otter *Enhydra lutris* (Miller et al. 2002a, 2008a, 2010, Conrad et al. 2005). In some cases, protozoal disease causes sufficient morbidity and mortality to negatively impact recovery efforts for rare or threatened marine mammal species (Miller et al. 2002a, Dubey et al. 2003, Kreuder et al. 2003, Conrad et al. 2005, Johnson et al. 2009, Roe et al. 2013).

Many of these pathogenic protozoal agents documented in marine mammals are being released into the environment from their terrestrial definitive hosts, which include felids for *Toxoplasma gondii*, canids for *Sarcocystis cruzi* and *Neospora caninum*, and opossum for *S. neurona* (Dubey et al. 2000, Haddad et al. 2005, Hill et al. 2005, Dubey & Jones 2008, Dubey 2009, Xiang et al. 2011). Definitive hosts shed infectious oocysts where they can be flushed into the ocean through runoff (Miller et al. 2002a, 2010, Dubey et al. 2003, Gibson et al. 2011). These oocysts survive well in soil, fresh and salt water, and can encyst in the tissues of other organisms that they infect (Wendte et al. 2010). Sources of infection for marine mammals include direct consumption of oocysts from the water column or consumption of oocysts or tissue cysts present in prey (Conrad et al. 2005, Miller et al. 2008a, Johnson et al. 2009, Massie et al. 2010, Shapiro et al. 2015).

Infections can also be passed transplacentally from mother to fetus. Cases of vertical transmission in marine mammals are rare, but span developmental stages from early gestation to neonates (Van Pelt & Dietrich 1973, Jardine & Dubey 2002, Resendes et al. 2002, Miller et al. 2008b, Barbosa et al. 2015, Carlson-Bremer et al. 2015, Shapiro et al. 2016).

Morbidity and mortality from protozoal disease in pinnipeds are typically diagnosed post-mortem and manifest as abortion, encephalitis, lymphadenitis, meningoencephalitis or myocarditis (Holshuh et al. 1985, Lapointe et al. 1998, 2003, Miller et al. 2001, Colegrove et al. 2005, 2011, Honnold et al. 2005, Barbosa et al. 2015, Carlson-Bremer et al. 2015). Morbidity and mortality from vertically transmitted *T. gondii* infections in marine mammals are characterized by widespread necrosis and inflammation in association with intralesional tachyzoites and tissue cysts (Van Pelt & Dietrich 1973, Jardine & Dubey 2002, Resendes et al. 2002, Miller et al. 2008b, Carlson-Bremer et al. 2015). In contrast, vertically transmitted *S. neurona* infections of marine mammals generally result in severe but localized encephalitis rather than disseminated disease (Barbosa et al. 2015).

Fatal hepatitis associated with a *S. canis*-like protozoan was the first protozoal infection diagnosed in Hawaiian monk seal *Neomonachus schauinslandi*. However, the disease occurred 2 yr after the seal was removed from the wild and it is unclear how and when the animal was exposed (Yantis et al. 2003). Toxoplasmosis was first identified infecting a wild Hawaiian monk seal carcass examined in 2004 with disseminated disease and intra- and extracellular tachyzoites and tissue cysts in affected organs (Honnold et al. 2005).

Since then, several protozoal-related mortalities in Hawaiian monk seals have been detected, despite surveillance for and examination of carcasses by gross necropsy and routine histopathology dating back to the early 1980s. This coincides with growth in the abundance of seals inhabiting the densely human (and felid) populated main Hawaiian Islands (MHI). Although the majority of this endangered population resides in the remote Northwestern Hawaiian Islands (NWHI) where humans and associated pests are relatively absent (NMFS 2007, Baker et al. 2011, Johanos et al. 2014), the abundance of seals frequenting the MHI has risen in recent years. Monk seals were rarely sighted in the MHI prior to the 1990s. The first systematic surveys of the MHI counted 45 and 52 seals in the MHI in 2000 and 2001, respectively (Baker & Johanos 2004). In 2011, the minimum number of known individuals was 113 seals and was expected to increase (Baker et al. 2011). The minimum known number has indeed increased and was 183 individuals in 2015 (PIFSC 2016). Given the co-occurrence of seals and known or suspected protozoal hosts in the MHI and the potential impact of protozoal-related mortalities on the population, the need to provide a systematic framework for classifying cases is paramount. Here, we establish case definitions for protozoal-related mortalities in this species, describe confirmed and suspect mortalities attributable to protozoal disease, and discuss the clinical presentation and pathology of Hawaiian monk seals infected with *T. gondii*.

MATERIALS AND METHODS

Cases

Carcass recovery efforts for Hawaiian monk seals became standard in the early 1980s and were primarily focused on the majority of the population in the

NWHI, although surveillance in this remote segment of the archipelago was limited to seasonal presence of research teams (generally from May to September). In the MHI, sick, injured or dead seals were typically detected by the public and volunteer groups, and stranding responses were then led by the National Marine Fisheries Service (NMFS). Depending on the remoteness of the stranding location and logistical support, post-mortem examinations were conducted in the field or in the laboratory and in some instances carcasses were transported on ice and refrigerated or frozen prior to examination. Post-mortem condition ranged from fair to fresh at the time of gross examination and sample collection.

Pathology

Representative tissue samples from all major organs and lymph nodes (mandibular, prescapular, axillary, tracheobronchial and mediastinal) were fixed in 10% neutral buffered formalin and then examined by board certified veterinary pathologists according to standard methods. If a protozoal organism was suspected based on morphology, tissues were further investigated by immunohistochemistry (IHC). For IHC a rabbit polyclonal *Toxoplasma gondii* antibody, AR125-5R, produced from strain C56 culture derived tachyzoites (Biogenex Laboratories) was used, and testing was conducted at University of Illinois and the California Animal Health and Food Safety Laboratory Systems according to previously established methods (Miller et al. 2001, Suedmeyer et al. 2001, Colegrove et al. 2011). Together, the presence, distribution and severity of parasite-associated pathology and parasite identification by IHC were used to determine whether or not mortality was attributable to *T. gondii*, as defined below. Histopathology was also used to rule out any other (i.e. non-protozoal) causes of morbidity sufficient to cause mortality in these individuals.

Additional diagnostics

Once histopathology and IHC had identified a subset of individuals for assessment of protozoal-related mortality, additional diagnostics (serology and molecular analyses) were evaluated, although these tests were not available for all individuals. When available, serum samples were submitted to diagnostic laboratories to measure antibodies to *T. gondii*, *Sarcocystis* and/or *Neospora*. Titers were measured by a microscopic agglutination test (MAT) (methods described in Dubey et al. 2003) or an indirect fluorescent antibody test (IFAT for protozoal immunoglobin G [IgG]) (methods described in Miller et al. 2002b) (Table 1). Neither test is validated for Hawaiian monk seals, although the IFAT is validated for sea otters and is used for other marine mammal species (Miller et al. 2002b, Colegrove et al. 2011, Carlson-Bremer et al. 2015).

When available, formalin-fixed and frozen tissue samples archived at −80°C were forwarded to the Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, MD for PCR (n = 9). The DNeasy blood and tissue kit from Qiagen (Valencia) was used to extract DNA. Molecular detection for protozoal agents was performed by PCR-DNA sequencing methodology using ApiITS1 primers that target the 110-copy ribosomal SSU rDNA gene array, as described previously (Gibson et al. 2011). Positive (Type I *T. gondii* DNA) and negative (water only) controls were run for each PCR test. PCR products were treated with ExoSAP-IT (USB) prior to DNA sequencing at NIAID Rocky Mountain Laboratories (RML) Genomics, Hamilton, MT.

Clinical evaluation

Two of the seals with case material sufficient for inclusion in this study were sampled ante-mortem during attempts at rehabilitative care in 2015 and later necropsied (seals RN36 and RB24; Table 1). Both individuals were diagnosed post-mortem with disseminated toxoplasmosis. Physical examination and full-body radiographs were conducted within 24 h of admission for each patient. Blood from both patients and a urine sample from 1 patient were submitted to a commercial veterinary diagnostic laboratory (Antech Diagnostics, Kaneohe, Hawaii) for analysis and compared to reference ranges (Sloan 1999, Reif et al. 2004). Methods for histopathology and ancillary diagnostics were consistent with those described above.

Case definitions

Case definitions were developed to systematically classify case material that was examined for evidence of protozoal-related mortality. Confirmed cases of protozoal-related mortality (e.g. toxoplasmosis) were those in which protozoal organisms (i.e.

Table 1. Summary findings of apicomplexan protozoal-associated pathology and mortality in Hawaiian monk seals *Neomonachus schauinslandi*, 1982 to 2015. Confirmed cases: protozoans observed, associated with lesion(s) sufficient to cause mortality, confirmed by IHC; suspect cases: characteristic lesions identified, but protozoans not visible or not confirmed by IHC; Incidental infections: protozoans (cysts) observed, and/or detected by PCR, but no associated inflammation. Serum antibody titers and PCR results (PCR protocol from Gibson et al. 2011) are for *Toxoplasma gondii* unless otherwise specified. IHC: immunohistochemistry; IFAT: indirect fluorescent antibody test; MAT: microscopic agglutination test; NE: not examined

Year	Seal ID/age class/sex	Island/ atoll	Protozoal species identified	Organ(s) affected	Features of organisms	Ante-mortem exami-nation	IgG serology	IHC	PCR
Confirmed mortality due to protozoan infection									
2001	TD09 Juvenile Female	Laysan	*T. gondii*	Heart, lymph node	Not described	No	NE	Positive (heart)	Positive (heart)
2004	RK07 Adult Male	Kauai	*T. gondii* (Honnold et al. 2005)	Adrenal gland, brain, diaphragm, heart, lymph node, spleen	Cysts and tachyzoites, numerous	No	1:100 (MAT); <1:40 (IFAT)	Positive (multiple tissues)	Positive (lymph node)
2006	KA060D03 Juvenile Male	Kauai	*T. gondii*	Adrenal gland, brain, thymus, liver, lung, lymph node, spleen	Cysts & tachyzoites, occasional but widely disseminated	No	1:800 (MAT); 1:320 (IFAT)	Positive (multiple tissues)	Positive (brain)
2010	RTX1 Stillborn pup Female	Molokai	*T. gondii*, *Sarcocystis neurona* (suspected)	Lung, placenta, umbilicus	Cysts, occasional	No	<1:25 (MAT)	Positive (multiple tissues; *T. gondii*)	Positive (brain, lung, umbilicus; *T. gondii*). Suspect positive (brain, heart, lung, placenta; *S. neurona*)
2010	RH40 Adult Male	Kauai	*T. gondii*	Brain	Cysts	No	NE	Positive (brain)	Positive (brain, heart)
2014	R017 Adult Female	Oahu	*T. gondii*	Adipose tissue, brain, spleen, adrenal gland, uterus	Cysts and tachyzoites (occasional to many)	No	NE	Positive (brain)	Positive (brain, heart, liver, muscle)
2015	RB24 Adult Female	Oahu	*T. gondii*	Adipose tissue, brain, heart, liver, lung, lymph nodes, pancreas, uterus	Tachyzoites (many)	Yes	NE	Positive (adipose tissue)	NE
2015	RN36 Juvenile Female	Oahu	*T. gondii*	Adipose tissue, brain, lung, liver, lymph nodes, adrenal gland, stomach	Tachyzoites (occasional to many)	Yes	1:1280 (IFAT)	Positive (adipose tissue)	NE
Suspected mortality due to protozoan infection									
2005	RK29 Adult Male	Oahu	*T. gondii*	Heart, lung (suspect)	Cysts (suspect)	No	<1:25 (MAT); <1:40 (IFAT); 1:1280 (IFAT, *Neospora*)	Negative	Positive (heart, liver, lung)
2007	R011 Adult Female	Lanai	*T. gondii*, *Sarcocystis* spp.	Lung (suspect)	Not described	No	NE	NE	Positive (brain, heart, liver, lung)
Incidental protozoan infection									
2000	T38F Adult Female	Laysan	*Sarcocystis* spp.	Skeletal muscle (no inflammation)	Cysts	No	NE	NE	NE
2002	GM04 Juvenile Female	Lisianski	*Sarcocystis* spp.	Skeletal muscle (no inflammation)	Cysts	No	NE	NE	NE
2002	BZ20 Adult Female	Midway	*Sarcocystis* spp.	Skeletal muscle (no inflammation)	Cysts	No	NE	NE	NE
2012	KA120399 Juvenile Male	Kauai	*T. gondii*, *Sarcocystis* spp.	Skeletal muscle (no inflammation)	Cysts	No	NE	NE	Positive (lymph node; *T. gondii*). Positive (muscle, heart; *Sarcocystis* spp.)
Total seals examined	14				12	2	5	9	9

tachyzoites, cysts) were observed microscopically in association with a lesion(s) sufficient to cause mortality and positive reactivity to *T. gondii* antibodies was demonstrated via IHC. Suspect cases of protozoal-related mortality were those in which microscopic lesions or inflammation typically associated with protozoal disease were identified but suspect organisms were either not visible or were not confirmed by IHC. Suspect cases may have had evidence of protozoal exposure by serology or by molecular polymerase chain reaction (PCR). Carcasses identified with incidental infections had protozoal cysts in skeletal muscle and/or protozoal DNA detected by PCR, but exhibited no associated inflammation.

RESULTS

Cases

Surveillance for dead monk seals has been a routine component of seasonal field research efforts in the NWHI for 3 to 12 mo annually since the early 1980s. As seal abundance has increased in the MHI, carcass surveillance has been accomplished through public and volunteer reports to an established stranding hotline. Between 1982 and 2015, 306 stranded Hawaiian monk seals in fresh to fair postmortem condition were necropsied and tissues were examined by routine histopathology. Case material from 14 individuals in which organisms with protozoal morphology were identified was further evaluated in this study (Table 1). No protozoal organisms were observed in tissue sections of carcasses examined by histopathology prior to 2001; therefore case material reported in this study encompasses a 15-yr range (2001 to 2015), during which 183 seals were necropsied and examined by histopathology.

Application of case definitions to these 14 individuals yielded 8 confirmed cases of protozoal-related mortality, including that described in Honnold et al. 2005 (Table 1). Specifically, 7 of these cases were attributed to toxoplasmosis and an eighth case (RTX1) was attributed to vertical transmission of *Toxoplasma gondii* in which co-infection with *Sarcocystis neurona* was suspected. Two cases were classified as suspect protozoal-related mortalities (*T. gondii* and *Sarcocystis* spp.). Sarcocysts were found incidentally in 4 seals, including one that was infected with both *T. gondii* and *Sarcocystis* spp., based on molecular findings. There was no evidence of tissue inflammation associated with any protozoa in these 4 seals. No consistent age or sex predilections were detected.

Pathology

T. gondii infections were often disseminated and affected multiple organs (n = 6; Table 1), leading to mortality. Among these, gross lymphadenopathy was noted in 3 cases. Striking gross lesions of multifocal to coalescing firm, yellow nodules were noted in the blubber and adipose tissue along the subcutaneous fat, superficial and visceral fascia, epicardium, and mesentery of 2 seals (RB24, RN36; Fig. 1). Histologically, the lesions corresponded to areas of severe multifocal necrotizing and histiocytic steatitis. Steatitis was also noted in 2 other seals (R017 and RK07; Honnold et al. 2005). Similarly, and across multiple other affected tissues, protozoal infection was associated with severe necrosis and histiocytic inflammation. In the most recent 2 cases, protozoal organisms (cysts, free tachyzoites, and intracellular tachyzoites) were noted in extremely high numbers within lesions, especially in affected adipose tissue (Fig. 2).

In 2010, a full-term stillborn Hawaiian monk seal pup (RTX1) was born to a presumptive primiparous dam at Kalaupapa, Molokai. The classification of the dam as primiparous was based on her age at parturition (4.5 yr); in the MHI, the earliest known age of parturition in this species is 4 yr, however most seals do not reach adulthood until at least age 5 (Baker & Johanos 2004, Baker et al. 2011). Extensive necrotizing and granulomatous inflammation was noted in multiple tissues and intralesional protozoal cysts were observed in the lungs and umbilicus of the pup as well as the placenta. Multiple tissues (heart, unspecified lymph node, lung, umbilicus) were positive for *T. gondii* by IHC and PCR, fulfilling the case definition as a confirmed case of toxoplasmosis. PCR screening also identified *S. neurona* in the brain, heart, lung and placenta; IHC could not be conducted retrospectively, so the significance of the *S. neurona* infection remains suspect for this individual. The primiparous dam was never observed after parturition and is presumed dead, though a carcass was never detected.

Additional diagnostics

While serum was not available from all 14 seals, *T. gondii* serum IgG antibody titers were above the positive threshold used in other species (>1:25 MAT or >1:40 IFAT; Miller et al. 2002b, Dubey et al. 2003) for at least 1 serum test in 3 of the confirmed cases of protozoal-related mortality (Table 1).

In combination with findings from histopathology and IHC, molecular analyses support case mortality

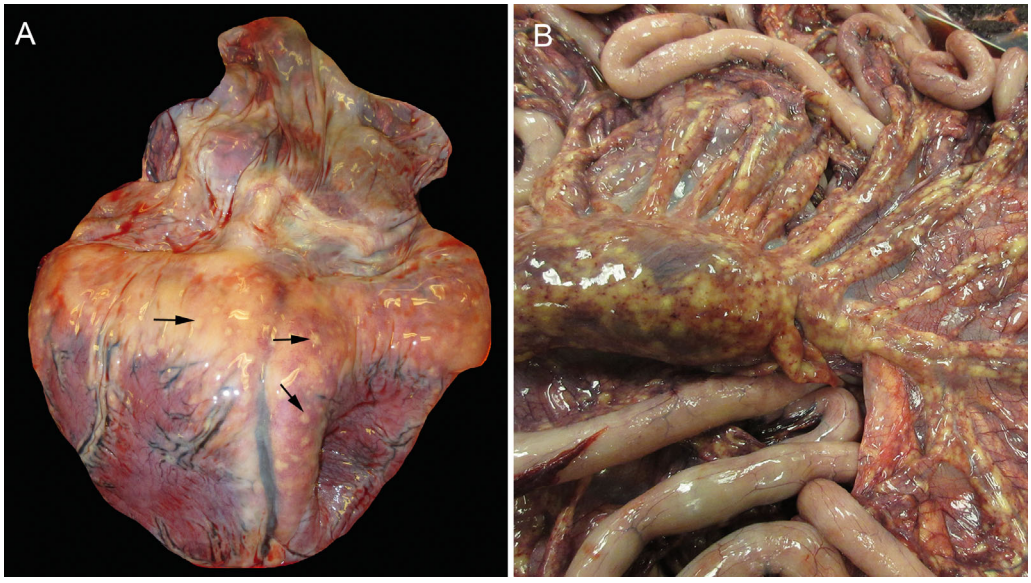

Fig. 1. Gross lesions of affected adipose tissue from a Hawaiian monk seal *Neomonachus schauinslandi* (ID: RB24) with disseminated toxoplasmosis. (A) Heart with multiple nodular areas of necrotizing steatitis throughout epicardial adipose tissue (arrows). (B) Mesentery and mesenteric lymph node with numerous nodular yellow areas of necrotizing steatitis

designations of toxoplasmosis as the confirmed cause of death for 6 individuals. PCR was also positive for the 2 suspect cases, RK29 (*T. gondii*) and R011 (*T. gondii*, *Sarcocystis* spp.), but because these findings were either not fully supported by IHC, or only suspect cysts were noted by histopathology, they remain conservatively classified as suspect.

Clinical evaluation

Two of 14 seals, 1 adult (RB24) and 1 juvenile (RN36), were clinically evaluated ante-mortem during care at a rehabilitation facility run by the NMFS Pacific Islands Fisheries Science Center in March and November 2015, respectively, but succumbed to infection between 24 and 96 h after rescue. Both were in good nutritional condition despite anorexia during treatment. Elevated respiratory rates (40 to 60 breaths per minute, compared to a normal rate of approximately 2 to 10 breaths per minute) and elevated respiratory effort were observed. Both individuals were alert and responsive. They were reluctant to ambulate on land or in the water, though strong stimuli could elicit coordinated movement with an appropriate range of motion for all limbs, head and neck. No neurologic deficits were appreciated.

Upon admission to rehabilitation, hematology and serum chemistry analyses revealed multiple abnormalities that were consistent between both cases (Table 2). Abnormalities included moderate to marked leukopenia, thrombocytopenia, hypoalbuminemia, hyperbilirubinemia, hyperglobulinemia, hyperphosphatemia and elevations in alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine kinase (CK) enzymes compared to reference ranges (Reif et al. 2004). Mild elevations in blood urea nitrogen (BUN) were also observed; creatinine was within normal limits. Elevations in CK, and to some extent ALT, are associated with muscle damage (cardiac and skeletal), which are explained by protozoal myositis observed histologically after death. Taken together, elevations in bilirubin, ALT and AST also indicate cholestasis, which were likely secondary to hepatic infiltration with protozoa and inflammatory cells.

Serum from RN36 was positive for *T. gondii* antibodies (1:1280, IFAT) and less than the minimum dilution tested for antibodies to *Sarcocystis* (<1:40, IFAT) and *Neospora* (1:160, IFAT). RB24 was not tested.

DISCUSSION

Prior to this study, only a single case of *Toxoplasma gondii* causing mortality in a wild Hawaiian monk seal had been documented (Honnold et al. 2005). We present here the detection of additional protozoal-related mortalities in Hawaiian monk seals since

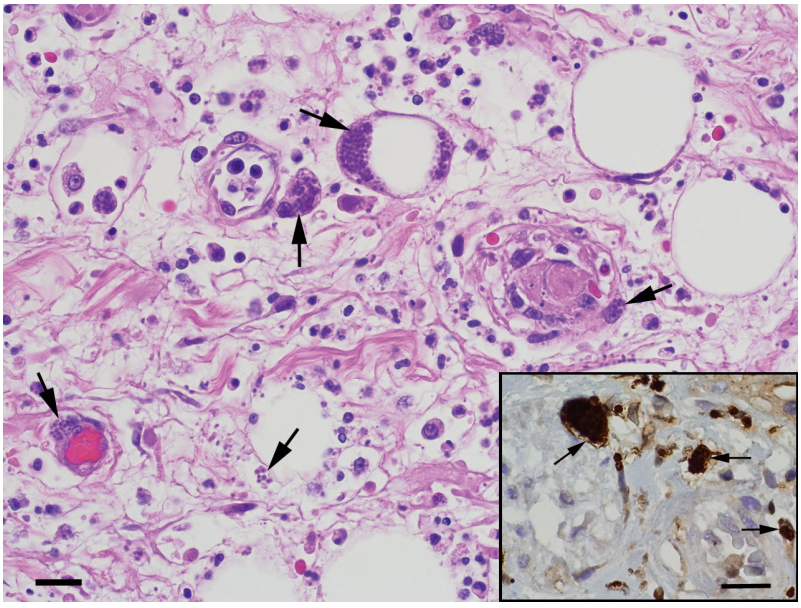

Fig. 2. Hematoxylin and eosin stained section of mesenteric adipose tissue from a Hawaiian monk seal *Neomonachus schauinslandi* (ID: RB24) with disseminated toxoplasmosis. There is necrosis and histiocytic inflammation throughout the adipose tissue associated with numerous free and intracellular tachyzoites (arrows). Scale bar = 20 µm. Inset: immunohistochemistry for *Toxoplasma gondii* using a polyclonal antibody. Tachyzoites exhibit positive staining (arrows). Scale bar = 20 µm

2001; this increase appears to coincide with the increasing abundance of seals in the MHI, where seal and human populations coexist (Baker & Johanos 2004, Baker et al. 2011). We establish a standardized case definition to classify Hawaiian monk seal mortalities and identify toxoplasmosis as the primary cause of protozoal-related morbidity and mortality in this endangered species.

Pathology in most cases was severe and associated with numerous protozoal organisms, similar to observed cases in other mammalian species such as macropods and new world primates deemed highly susceptible to toxoplasmosis (Innes 1997, Parameswaran et al. 2009, 2010). Steatitis may be emerging as a primary feature of infection in some monk seals given the severity of the inflammation in the adipose tissue and abundance of associated protozoal organisms in recent cases (RB24, RN36), relative to many of the adjacent parenchymal tissues. Steatitis has been identified in sporadic cases of toxoplasmosis in other species, such as from a red kangaroo in Australia and several aborted Angon goat kids in New Zealand (Chen & Alley 1987, Dubey & Hartley 1992). Some seals with disseminated toxoplasmosis lacked evidence of seroconversion, suggesting that there may not be a latent period in these infections. In some cases however findings were suspect or incidental and were not associated with tissue pathology or did not appear to be related to mortality.

This is supported by a serosurvey of Hawaiian monk seals carried out in 2004 to 2005 that identified positive titers in a few apparently healthy, wild seals for the protozoa *Neospora caninum*, *Sarcocystis neurona*, and *T. gondii* using the available tests at that

Table 2. Selected hematology and serum chemistry values from 2 Hawaiian monk seals *Neomonachus schauinslandi* with disseminated toxoplasmosis. Physical examination revealed that both patients were in good nutritional condition, anorexic, and tachypneic with increased respiratory effort. Reference values (±SD) for live-captured, healthy animals from Reif et al. (2004)

Parameter	RB24 (adult female)	RN36 (juvenile female)		Reference value
		Day 1	Day 3	
White blood cells (ml^{-1})	4900	5200	4700	7956.0 (±2003.0)
Neutrophils (μl^{-1})	4116	3068	2444	4014.0 (±1239.1)
Bands (μl^{-1}) (%)	49 (1%)	0 (0%)	329 (7%)	
Lymphocytes	637	2080	1457	2270.0 (±884.5)
Platelets (10^3 μl^{-1})	92	63	16	
Albumin (g dl^{-1})	2.1	2.3	2.1	3.1 (±0.3)
Globulins (g dl^{-1})	4.7	4.9	4.6	5.2 (±0.6)
Total bilirubin (mg dl^{-1})	0.6	0.9	2.4	
Phosphorus (mg dl^{-1})	6.8	8.0	7.9	6.9 (±1.4)
Alanine aminotransferase (IU l^{-1})	126	265	373	99.0 (±49.7)
Aspartate aminotransferase (IU l^{-1})	733	974	1778	105.0 (±44.7)
Creatinine kinase (IU l^{-1})	4080	1101	2996	655.0 (±352.9)
Blood urea nitrogen (mg dl^{-1})	30	32	41	34.8 (±15.2)
Creatinine (mg dl^{-1})	1.2	1.0	1.1	1.2 (±0.3)

time (Littnan et al. 2007). While risk factors for the development of fulminant disease are not yet well understood, the increasing number of mortalities and severity of *T. gondii* infections in affected seals suggest that terrestrially-sourced protozoal parasites could pose a substantial impediment to population recovery, in particular in the MHI, where the definitive host of this parasite is abundant.

Suspect protozoal organisms were identified morphologically in association with myocarditis in RK29, but IHC was negative. This individual was seronegative (IFAT) to *T. gondii* and *Sarcocystis* (<1:40) yet suspect positive for *Neospora* (1:1280). However PCR of heart, liver and lung tissue was positive for *T. gondii*. Together, these discordant findings may suggest that this seal was acutely infected and that mortality occurred prior to development of detectable serum antibodies, though serum electrophoresis was not specifically investigated. These serologic tests and their positive cutoffs have not been validated in Hawaiian monk seals; thus serology data were not used for making case determinations.

Tissues of seal R011, diagnosed with interstitial pneumonia, were positive for both *T. gondii* and a *Sarcocsytis* spp. by PCR. However protozoal organisms were not definitively identified histologically and tissues were not tested by IHC. The pneumonia had a bacterial component, which likely had a significant role in disease. The post-mortem condition of this individual also made histological interpretation difficult.

In this study, most cases of toxoplasmosis were systemic and affected multiple organs. The 2 seals that were examined both ante- and post-mortem offer clues to the pathogenesis and severity of some *T. gondii* infections in this species. The reluctance of both patients to ambulate, both in water and on land, may be explained by pain associated with diffuse systemic inflammation, especially in the adipose tissue, which is emerging as a unique feature of toxoplasmosis in this species. Hematology and serum chemistry analyses of these 2 individuals revealed multiple abnormalities that, while non-specific, were consistent with the protozoal myositis, hepatitis and systemic infiltration of tissues with protozoa identified histologically. The clinical pathology abnormalities noted in these 2 individuals were highly unusual in Hawaiian monk seals, where common causes of stranding are malnutrition (NWHI) or fish hook ingestion (MHI) and stranding is only rarely associated with broad or severe departures from normal hematology and serum chemistry values (Reif et al. 2004). Although many of the confirmed cases of protozoal mortality described in this study were detected post-mortem, the clinical features of RB24 and RN36 provide further support that the systemic inflammatory response to *T. gondii* led to mortality. Together, diffuse whole body inflammation, a high burden of *T. gondii* organisms in association with lesions and average nutritional condition suggest rapidly progressing fulminant disease.

These findings have implications for mitigation efforts and may aid animal caretakers in diagnosing and treating future cases of this disease more rapidly. Because they are unlikely to be confused with other causes of stranding in this species, the observed patterns in clinical presentation and hematology can be used to make difficult but necessary empirical treatment decisions rather than waiting for serum antibody titers, PCR or blubber biopsies, which may take several days. Pharmaceutical options for treatment of toxoplasmosis are limited and largely untested in Hawaiian monk seals and in pinnipeds in general. Despite the possible side effects of available options, aggressive multi-drug therapy at the first onset of this clinical presentation may be the only way to contain the rapid progression of systemic inflammation and multiple organ failure. The pathogenesis of these infections is worthy of further study as additional case material across pinniped species is acquired. While useful to rule out other differential diagnoses such as foreign body ingestion, radiographs did not add to the clinical picture for these 2 patients. The stress imposed on highly compromised patients should be considered when electing to pursue this diagnostic modality in the future.

The full-term stillborn female pup identified on Molokai in 2010 (RTX1) was transplacentally infected with *T. gondii.* In contrast to the disseminated protozoal disease confirmed in RTX1, a second fetus was aborted in early to mid-gestation from a *T. gondii*-infected dam 12 d prior to the dam's death. The fetus (RGX2), placenta and dam (RB24) were examined by histopathology. Histopathology did not identify organisms or inflammation in the tissues of the aborted fetus or placenta. However, histopathology of RB24 revealed severe, disseminated toxoplasmosis which likely led to fetal hypoxia, protozoa-related necrosis in the uterus, and ultimately placental detachment. Hence, while RB24 was included in the 8 confirmed cases enumerated in this study, RGX2 did not meet all criteria for case definition, though this mortality was a secondary result of toxoplasmosis in the dam.

The observed difference in pathology between RTX1 and RGX2 may be explained by differences in gestational timing of infection, the genotype of *T.*

gondii, or by co-infection status. Some strains of *T. gondii* are known to be avirulent in certain host species, yet others highly pathogenic (Parameswaran et al. 2010, Pinheiro et al. 2015). Pathogenicity appears to be increased among marine mammals with co-infections of *T. gondii* and *S. neurona* (Gibson et al. 2011). RTX1 was transplacentally infected with *T. gondii.* Whether this pup was also co-infected with *S. neurona* is less clear. A molecular signature for *S. neurona* was detected years later, but IHC was not completed. In the absence of further confirmation, molecular detection alone is insufficient to make this claim at this time and the differences between these 2 cases highlight the need for genotyping and continued molecular surveillance to evaluate future mortalities.

In contrast, the minimal reaction of tissues to *Sarcocystis* spp. found incidentally infecting 4 seals suggests that there may be some differences in the stage of infection, pathogenicity of certain protozoal species or genotypes circulating in Hawaii, in the degree of co-infection, or in the duration of time that monk seals have been exposed to different protozoal genera. IHC for *S. neurona* is less useful for confirmation, in contrast to the reliability of IHC for *T. gondii*.

Autolysis hindered definitive interpretation of results from the 2 cases categorized as suspect and may preclude diagnosis of protozoal disease in decomposed seal carcasses. Further, discordant histopathology, IHC, serum antibody titer and PCR results may result from differences in the distribution of lesions, whether the infection is acute or chronic, and if organisms are present in the tissue samples analyzed. Thus, the definition of a confirmed case established in this study was made conservatively and cases may be underrepresented by use of these stringent criteria.

CONCLUSIONS

Our comprehensive case-based data provide evidence that connects the land-to-sea flow of terrestrial parasites, notably *Toxoplasma gondii*, to mortalities in Hawaiian monk seals. We document for the first time transplacental transmission of *T. gondii* and abortion of a Hawaiian monk seal fetus, thus further highlighting the potential of this parasite to negatively impact the reproductive potential of Hawaiian monk seals. Morbidity and mortality, especially associated with protozoal parasites such as *T. gondii*, are a function of the immune competence of the host, the pathogenicity of the parasite, and the route and degree of exposure. All of these features can vary temporally, spatially and individually, and are particularly challenging to investigate for protozoa, which can vary in virulence, can remain latent for long periods and can be transmitted vertically. Our investigation suggests that protozoal-related mortalities in Hawaiian monk seals are dominated by *T. gondii* and that cases of toxoplasmosis have similar features. This study provides clinical and histopathological support among cases examined to date and describes important and perhaps pathognomonic features of toxoplasmosis in Hawaiian monk seals, which is often disseminated, aggressive and severe. Even though recognition of these patterns may enhance diagnosis, curative treatment options in this species have not been tested and mitigation of this threat should not rely on rehabilitative care at this time.

Broader molecular screening of tissues collected systematically from carcasses could permit detection of more infections prospectively and retrospectively. Such investigation, regardless of clinical suspicion or mortality, is important given that this parasite has a latent stage that can remain undetected for life or recrudesce in times of immunosuppression. Serologic assessment of exposure in wild, healthy animals will also augment post-mortem investigations to more broadly explore the prevalence of exposure and the cumulative impacts of these infections on monk seal survival.

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