Vol. 102: 73–85, 2012
doi: 10.3354/dao02528

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published December 3

Serologic response in bottlenose dolphins *Tursiops truncatus* infected with *Brucella* sp. using a dolphin-specific indirect ELISA

Jenny Meegan[1,2,*], J. Lawrence Dunn[2], Stephanie K. Venn-Watson[1], Cynthia R. Smith[1], Inga Sidor[2,3], Eric D. Jensen[4], William G. Van Bonn[5], Roberta Pugh[6], Thomas Ficht[6], L. Garry Adams[6], Klaus Nielsen[7,], Tracy A. Romano[2]**

[1]National Marine Mammal Foundation, 2240 Shelter Island Dr. Suite 200, San Diego, California 92106, USA
[2]Mystic Aquarium, A Division of Sea Research Foundation Inc., 55 Cogan Blvd., Mystic, Connecticut 06355, USA
[3]University of New Hampshire, Department of Molecular, Cellular, and Biomedical Sciences, 129 Main Street, Durham, New Hampshire 03824, USA
[4]US Navy Marine Mammal Program, SPAWAR Systems Center Pacific, 49620 Beluga Road, San Diego, California 92152, USA
[5]The Marine Mammal Center, 2000 Bunker Road, Sausalito, California, 94965, USA
[6]Texas A&M University, Department of Pathobiology, Veterinary Research Building, Bldg. 1197, Room 141, College Station, Texas 77843, USA
[7]Canadian Food Inspection Agency, Animal Diseases Research Institute, 3851 Fallowfield Road, Nepean, Ontario, Canada

ABSTRACT: Marine-origin *Brucella* infections and serologic evidence of exposure have been documented in multiple cetacean species. A dolphin-specific indirect enzyme-linked immunosorbent assay (ELISA) was developed to screen bottlenose dolphin sera for anti-*Brucella* antibodies. A total of 131 serum samples collected over a 2 to 18 yr period from 6 bottlenose dolphins *Tursiops truncatus* with confirmed *Brucella* infections were analyzed for the presence and magnitude of antibody titers against marine-origin *Brucella* to compare individual antibody responses to various disease manifestations. Additionally, an epidemiologic serologic survey of a managed population of 64 bottlenose dolphins was performed to evaluate for the presence of antibodies and to determine whether there were any clinical pathology predictors for exposure or infection. The serologic results revealed that the dolphins with *Brucella*-associated abortions were seronegative for 7 to 18 yr until after the abortion and maintained positive titers for several years, with 2 of 3 animals returning to seronegative status. In contrast, the dolphins with *Brucella*-associated pulmonary or bone lesions maintained persistent positive titers for 2 to 18 yr. The population serosurvey revealed no significant differences in antibody levels among males and females, and dolphins between the ages of 17 and 25 yr were 6.8 times more likely to be *Brucella* antibody positive compared to those that were younger or older. Seropositive dolphins did not have significant inflammation compared to seronegative dolphins but were more likely to have higher levels of aspartate aminotransferase and gamma-glutamyl transpeptidase. Among 16 dolphins that tested seropositive, 13 (81.3%) had previously been seropositive for at least 3 to 5 yr.

KEY WORDS: Cetacean · Immunology · Serology · Marine mammal · Antibody · Infectious disease

INTRODUCTION

Brucella sp. infections in cetaceans were first reported in 1994, and serological studies have supported that *Brucella* exposure in cetaceans occurs nearly worldwide (Ewalt et al. 1994, Ross et al. 1994, 1996, Jepson et al. 1997, Patterson et al. 2000, Nielsen et al. 2001, Van Bressem et al. 2001, Ohishi et al. 2003, Tachibana et al. 2006, Davison et al. 2009, Guzmán-Verri et al. 2012). Despite the serologic evidence of global distribution of exposure to *Brucella*, knowledge of the pathogen's impact on cetaceans remains incomplete.

*Email: jenny.meegan@nmmpfoundation.org
**Retired

Marine *Brucella* species have been divided into *B. ceti* and *B. pinnipedialis*; *B. ceti* have been further characterized as *B. ceti* dolphin type, *B. ceti* porpoise type, and distinct *B. ceti* type associated with human infections (Foster et al. 2007, Guzmán-Verri et al. 2012). The different *B. ceti* types have been associated with host preference and variations in pathogenicity. While the overall impact of *Brucella* infections to the health of marine mammal populations is currently incomplete, a variety of disease manifestations associated with *Brucella* infections affecting multiple cetacean species have been reported.

Bottlenose dolphins *Tursiops truncatus* have been among the most commonly reported cetacean species with active brucellosis, and associated diseases have included abortions, placentitis, vertebral osteomyelitis, and pulmonary lesions (Ewalt et al. 1994, Miller et al. 1999, Cassle et al. 2009, Goertz et al. 2011). Other odontocete species have also been affected, including subcutaneous lesions in the common dolphin *Delphinus delphis*; meningoencephalitis, placentitis, osteoarthritis, myocarditis, and blubber abscesses in striped dolphins *Stenella coeruleoalba*; hepatic and splenic necrosis, lymphadenitis, neurobrucellosis associated with an atlanto-occipital joint lesion, and mastitis in Atlantic white-sided dolphins *Lagenorhynchus acutus*; subcutaneous lesions, discospondylitis, splenic necrosis, epididymitis, and isolation of the organism from mammary tissue and testicle in harbor porpoises *Phocoena phocoena*; hydrocephalus associated with non-suppurative meningoencephalitis and vaginitis in the short-beaked common dolphin *D. delphis*; and hydrocephalus associated with non-suppurative meningoencephalitits in the northern right whale dolphin *Lissodelphis borealis* (Foster et al. 1996, 2002, González et al. 2002, Dagleish et al. 2007, 2008, St. Leger et al. 2007, Davison et al. 2009, González-Barrientos et al. 2010, Guzmán-Verri et al. 2012).

Brucellosis has also affected several species in the mysticete family causing hepatic necrosis, granulomatous orchitis, and oophoritis in Minke whales *Balaenoptera acutorostrata,* and granulomatous orchitis and oorphoritis in Bryde's whales *B. edeni* (Tryland et al. 1999, Ohishi et al. 2003, Guzmán-Verri et al. 2012).

The disease manifestations reported in cetaceans are similar to those reported in terrestrial brucellosis. *Brucella* in terrestrial animals most commonly targets the reproductive system, and localization within the female reproductive tract results in an increased rate of spontaneous abortion, stillbirths and weak neonates, retained placentas, mastitis, decreased fertility and sterility, and poor conception rates (Adams 2002; see also www.cfsph.iastate.edu/Factsheets/pdfs/brucellosis.pdf). Brucellosis in the male can lead to epididymitis, seminal vesiculitis, orchitis testicular abscesses, and infertility. *Brucella* can affect the mononuclear phagocyte system leading to disease of the spleen, liver, lung, skin, and bone marrow (as well as the central nervous system, causing meningitis and meningoencephalitis) and the musculoskeletal system, which can lead to arthritis, discospondylitis, and osteomyelitis (Adams 2002).

The diversity of pathologic lesions in terrestrial animals is known to be influenced by the species and strain of *Brucella*, the innate and acquired immunity of the host, the route of exposure, sexual maturity, the stage of pregnancy, and the dose of the inoculum (Adams 2002). The pathogenesis, transmission, and immune response associated with brucellosis in cetaceans are not completely understood; however, as new cases continue to emerge, our knowledge of the disease becomes clearer.

Diagnosing a *Brucella* infection can be challenging due to the survival adaptations of the organism. Microbiological culture is ideal because it provides a definitive diagnosis of an active infection by detecting the presence of the organism. However, the microbiological growth requirements, specialized media, and lengthy incubation times may result in under diagnosis. Further, the best tissues and lesions for *Brucella* isolation are often difficult to access from live, wild marine mammals or stranded, dead marine mammals that may be in an advanced state of decomposition. Therefore, employing multiple testing methods optimizes the chances of obtaining a confident diagnosis. Molecular detection methods, such as polymerase chain reaction (PCR) assays for *Brucella*, which can be completed within days instead of weeks and do not require live *Brucella* isolates, should increase the likelihood of detecting *Brucella* in marine mammals (Cloeckaert et al. 2003, Guzmán-Verri et al. 2012).

Serology is an additional efficient tool for screening animals for the presence of antibodies against *Brucella.* Depending on the age of the animal, the detection of anti-*Brucella* antibodies may indicate presence of maternally derived antibodies, an active infection, a recently cleared infection, or previous exposure without clinical disease. Demonstration of serologic conversion, with a significant rise in antibody titer, is an important tool aiding in the diagnosis of a *Brucella* infection (Al Dahouk et al. 2003, Aliskan 2008). Further, serial serologic testing of infected or exposed animals is beneficial in evaluating the effects of treatment, assessing the duration of an ani-

mal's immunity to the microbe, and identifying animals with possible ongoing infections (Nielsen 2002).

Recently developed assays for testing marine mammal serum have been briefly described, including both indirect and competitive ELISA test methods (Hernández-Mora et al. 2009). The antigens used in these assays range from cell surface lipopolysaccharides to whole-cell antigen. A competitive ELISA (cELISA) has been previously described to test both pinnipeds and cetaceans for antibodies against *Brucella* (Meegan et al. 2010). The benefits of the cELISA include the lack of dependency on marine mammal species, but there can also be value in using a species-specific indirect ELISA.

Previously, results on only single serum samples from dolphins with little or no health history information have been documented. There has been a need to monitor trends in *Brucella* antibodies among culture-confirmed dolphins with clinical disease to better understand and interpret serological change dynamics.

The purpose of this study was to evaluate the serologic responses to *Brucella* infections in bottlenose dolphins and to identify epidemiologic risk factors of seropositivity in bottlenose dolphins. We describe the development of an indirect ELISA (iELISA) developed from a *Brucella* organism isolated from an aborted bottlenose dolphin fetus. This iELISA was designed to screen dolphin sera for specific antibodies to marine-origin *Brucella* and was used to perform the serologic evaluation in this study. The serologic responses of 6 bottlenose dolphins with culture-confirmed *Brucella* sp. infections were evaluated. The serologic data were used to monitor changes in titer over time in an effort to compare individual antibody responses to various disease manifestations. To our knowledge, this is the first report measuring serial serologic titers and documenting seroconversion in active *Brucella* cases in marine mammals.

Additionally, a serologic survey was performed with a group of managed dolphins to evaluate for the presence of *Brucella* antibodies. Age, sex, and clinical pathology values were compared among dolphins that were defined as antibody negative, positive, and suspect.

MATERIALS AND METHODS

ELISA reagents

A methanol-killed, whole-cell suspension of *Brucella* isolated from the placenta of an aborted bottlenose dolphin fetus (National Veterinary Services Laboratory, NVSL, isolate no. 92-1350, accession no. 92-19953) was obtained from the NVSL in Ames, Iowa, USA. (Ewalt et al. 1994, Bricker et al. 2000)

The positive control serum used was collected from bottlenose dolphins with culture positive *Brucella* infections (Miller et al. 1999). Negative control serum was obtained from a naïve bottlenose dolphin calf that had never nursed, indicating a lack of maternal antibodies. The bottlenose dolphin has a diffuse epitheliochorial placenta, similar to equine, swine, and ruminants (http://placentation.ucsd.edu). There is no transplacental transfer of maternal immunoglobulins, and the neonate lacks circulating antibodies until it ingests colustrum (Ruiz et al. 2009).

The secondary antibody used was a rabbit anti-dolphin IgG (h&l) antibody conjugated with horseradish peroxidase (HRP; Bethyl Labs).

ELISA

Antigen coating was performed by diluting whole-cell methanol-killed *Brucella* cells in coating buffer (0.015 M Na_2CO_3 + 0.035 M $NaHCO_3$ + 0.003 M NaN_3, pH 9.6) to a final optical density reading of 0.150 at 420 nm. Diluted antigen (100 µl) was delivered to each well of a 96-well Immulon II plate flat-bottomed plate (Thermo Labsystems no. 3455) and allowed to rotate at moderate speed (75 rpm) for 15 min at room temperature. The plate was then incubated for 12 h at room temperature. Once all of the wells appeared visually dried, the plate was washed 4 times with nanopure water prior to blocking with 100 µl of 1% Tween 20 diluted in wash solution (0.137 M NaCl + 0.003 M KCl + 0.008 M Na_2HPO_4 + 0.001 M KH_2PO_4, 4 l) added to each well, followed by incubation for 15 min at 37°C. The plate was washed 4 times with nanopure water to remove the blocking solution.

A 1:50 dilution of test sera was prepared in wash solution, and 100 µl were delivered to each well. Positive and negative control sera were prepared in the same manner, and 100 µl were delivered to the appropriate wells. The plate was then incubated for 15 min at 37°C. The plate was washed 6 times to remove excess antibody with wash solution using either an automated plate washer (ELx405 Select, BioTek Instruments) or wash bottle. Optimal concentration of the conjugated secondary antibody was determined by a 2-by-2 dilution pattern in a preliminary assay (an optimal dilution of 1:10 000 was chosen).

A 1:10 000 dilution of the HRP-conjugated rabbit anti-dolphin IgG (Bethyl Labs) was added to wash solution, 100 µl delivered to each well, and incubated

for 15 min at 37°C, followed by rinsing the plate 6 times with wash solution.

ABTS 2-component peroxidase substrate system (100 µl, equal volumes of ABTS and hydrogen peroxide; Kirkegaard and Perry Laboratories) was dispensed to each well and incubated at 37°C for 10 min. Nanopure water (200 µl) was then added to each well, and the plate was immediately read using an ELISA plate reader (EL800 Universal Microplate Reader, BioTek Instruments) at a wavelength of 405 nm measuring filter and a reference filter at 490 nm.

The output from the ELISA plate reader was analyzed by using KC4 Analysis software (BioTek Instruments). The ELISA plate reader measures an optical density (OD) value. Samples with an OD reading >1.0 were determined positive, values between 0.99 and 0.5 were considered suspect, and those <0.5 were determined to be negative. The positive (negative) control sera yielded an average OD reading of 1.2 (0.2).

A positive serum sample with an OD reading >1.0 was equivalent to a titer ≥1:50. For every serum sample that tested greater than the 1:50 titer, further dilutions were performed to report a final end-point titer. Additionally, for serum samples just prior to or after seroconversion that tested less than 1:50, smaller dilutions (1:25, 1:12.5, and 1:6.25) were performed to quantify the magnitude of the titer change. Occasionally, due to limited sample volume, the final end-point titer could not be obtained.

Serology test comparison

In total, 106 serum samples collected from 65 bottlenose dolphins from the US Navy Marine Mammal Program were tested in a blind study to compare results obtained from the newly developed cetacean iELISA with existing *Brucella abortus* serologic tests. The samples were shipped blindly to the Canadian Food Inspection Agency, Animal Diseases Research Institute (Nepean, ON, Canada) for testing with a *B. abortus* cELISA and a fluorescence polarization assay (FPA; Nielsen et al. 2000, CFIA 2001).

Case animals

A total of 131 serum samples from 6 bottlenose dolphins with confirmed *Brucella* infections were analyzed for the presence and magnitude of antibodies against *Brucella*. Details of the cases are discussed with the results. Five of the case animals were part of a managed collection of dolphins; 4 animals were originally wild caught in Mississippi, and 1 was wild caught in California. The final case was a wild stranded animal from Texas.

Immunohistochemical staining

Slides of formalin-fixed paraffin-embedded tissues were deparaffinized and rehydrated, then treated with 3% hydrogen peroxide to block endogenous peroxidase staining. After a protein block (Omnitags, Thermo Fisher Scientific) was applied, slides were incubated overnight at 4°C with 2 dilutions (1:4000 or 1:8000) of goat serum containing antibody against a harbor seal *Brucella* isolate (Meegan et al. 2010). After washing, a secondary mouse-anti-goat antibody conjugated to HRP (1:500 dilution; Dako) was applied and incubated for 30 min. After washing, NovaRed chromagen (Vector Laboratories) was added, followed by a 10% Gill's hematoxylin counterstain, dehydration with ethanol and xylene, and coverslipping. Positive and negative controls were run alongside each test tissue. Positive controls were a *B. abortus* culture-positive testicular granuloma from a bison. Negative controls were treatment with pre-immune (negative) goat serum (1:4000 dilution) in place of the positive goat serum.

PCR

Briefly, real-time quantitative PCR (qPCR) analysis was performed on DNA extracted from archived frozen tissues or paraffin blocks (Sidor et al. in press). Formalin-fixed paraffin-embedded tissues were deparaffinized with xylene, precipitated in ethanol, and then purified as for fresh tissues. This assay used primers, probe, and adapted protocols that target the gene for the 31 kDa outer membrane protein *bcsp31*, which is specific to the genus *Brucella.* The assay also included 2 internal controls to assess DNA quality and presence of PCR inhibitors. Samples were run in duplicate. A positive result had at least 1 positive amplification. Positive results were confirmed by a second independent DNA extraction and qPCR test.

Serologic survey

A total of 64 blood samples collected from 64 bottlenose dolphins as part of a managed collection of dolphins from the US Navy Marine Mammal Program were included in the serologic survey. Until the late 1980s, the majority of the population originated from

the northern Gulf of Mexico. For nearly 20 yr, however, dolphins have been born at the Navy facility as part of the managed population. Dolphins are housed in netted enclosures in San Diego Bay, California. This population is fed a diet of high-quality, frozen-thawed fish (capelin, herring, mackerel, and squid), although animals may ingest live flora and fauna in their natural environment. Navy dolphins routinely receive antihelminthics to prevent parasitic infections, including lungworm, a proposed carrier of *Brucella* spp. To date, lungworm has been identified in only 1 dolphin in the Navy program. Blood samples were collected between August 2000 and June 2002. Of the sampled animals, 31 (48.4%) were female, and 33 (51.6%) were male. Median age at the time of sample collection was 21 yr (range 0.6 to 45 yr).

Blood samples were originally collected from dolphins by venipuncture from either the periarterial venous rete in the caudal peduncle or a fluke blade. Animals were trained to present their tail for sampling, or behavioral conditioning was used to collect samples out of the water with animals resting on a foam mat during a routine physical exam. Blood was collected into a Vacutainer® serum separator tube (SST) or a Vacutainer® EDTA (K_3) tube for serum chemistries and complete blood counts (CBC), respectively (Becton Dickinson Systems).

Samples were chilled for 30 min and centrifuged within 2 h. Centrifugation was performed at 1006 × *g* (21°C for 10 min). Fibrin clots were removed and serum was transferred to a 5 ml plastic submission tube. Whole blood was submitted in EDTA Vacutainer® tubes. All samples were sent on ice via courier to a reference laboratory. Automated analyses were used by Quest Diagnostic Laboratories, including the Coulter® LH 1500 Series (Beckman Coulter) for hematology and the Olympus® AU600 (Olympus America) for serum chemistry analysis. Fisherbrand Dispette 2®, correlating with the Westergren method, was used to determine 60 min erythrocyte sedimentation rates from 1 ml EDTA whole blood.

The following hematological and serum biochemical variables were measured and incorporated into the retrospective study: total white blood cell (WBC) count, hematocrit (HCT), hemoglobin (Hb), red blood cell (RBC) count, red blood cell distribution width (RDW), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), mean platelets, absolute neutrophils, absolute lymphocytes, absolute monocytes, absolute eosinophils, glucose, blood urea nitrogen (BUN), creatinine, uric acid, sodium (Na^+), potassium (K^+), chloride (Cl^-), carbon dioxide (CO_2), total protein, globulins, iron, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), creatine phosphokinase (CPK), and 60 min erythrocyte sedimentation rate (ESR).

Among the dolphins that were *Brucella* antibody positive during 2000 to 2002, additional archived serum collected 3 to 5 yr previously was also evaluated for the presence of antibodies.

Epidemiologic data analysis

SAS Release 9.2 was used for all statistical analyses. Comparisons of OD values by age were conducted using simple linear regression. Result categories (negative, positive, and suspect) were compared by sex using chi-squared tests and 2-tailed p-values. Mean ages among result categories (negative, positive, and suspect) were compared using a general linear model (1-way analysis of variance, ANOVA). Due to the association of age with antibody level, subsequent comparisons of clinical pathology values by result categories were conducted using an analysis of covariance (ANCOVA), least squares means, and Type I SS p-value controlling for age. For all statistical tests, significance was defined as $p < 0.05$.

RESULTS

Case animals

Case 1

The first case (C1) was a 30 yr old female collected in the Mississippi Sound (Biloxi, MS). A previously documented *Brucella*-induced late-term abortion occurred in 1992 during the first pregnancy (Miller et al. 1999). Histopathologic evaluation revealed a multifocal suppurative placentitis, but the fetus was not evaluated due to autolysis. *Brucella* was cultured from the placenta.

In total, 24 serum samples collected over a 20 yr period were evaluated (Table 1). Due to limited blood sample availability, the nearest serum samples to the abortion were obtained 7 mo prior to and 5 mo post abortion. The serologic results indicate that the animal was seronegative for 7 yr prior to the onset of the abortion. Seroconversion was demonstrated post abortion, with an 8-fold increase in titer and a final end-point titer of 1:50. The animal reverted to seronegative status 1 yr later.

Table 1. *Tursiops truncatus.* Serologic evaluation for Case 1; antibody titer against *Brucella* sp. Samples with an optical density reading >1.0 were determined to be positive, values between 0.99 and 0.5 were considered suspect, and those <0.5 were determined to be negative

Date (m/d/yr)	Titer	Interpretation
4/3/1985	<1:50	Negative
6/27/1986	<1:50	Negative
12/16/1987	<1:50	Negative
6/19/1991[a]	<1:6.25	Negative
6/23/1992	1:50	Positive
2/9/1993	1:25	Suspect
2/24/1993[b]	1:25	Suspect
9/23/1993	1:25	Suspect
10/23/1993	1:50	Positive
1/12/1994	1:25	Suspect
5/10/1994	<1:50	Suspect
1/9/1995	1:25	Suspect
6/13/1995	1:25	Suspect
4/4/1996	<1:50	Suspect
10/4/1996	1:25	Suspect
4/23/1997[c]	<1:50	Suspect
2/10/2000	<1:50	Suspect
6/13/2000	<1:50	Suspect
11/22/2000[d]	<1:50	Suspect
11/25/2002	<1:50	Negative
6/18/2003	<1:50	Negative
11/5/2003	<1:50	Suspect
1/8/2004[e]	<1:50	Suspect
8/28/2005	<1:50	Negative

[a]*Brucella-positive* abortion 1/27/1992
[b]Live calf born with placentitis 4/29/1993
[c]Live calf born 7/2/1998
[d]Live calf born 6/19/2001
[e]Live calf born 7/30/2004

This animal's second pregnancy in 1993 resulted in a healthy calf, although histopathologic evaluation of the placenta revealed mild, multifocal, suppurative placentitis and omphalitis. Although *Brucella* sp. was suspected, it was not found to be associated with the placentitis using microbiological culture methods. There was a brief elevation in the antibody level 6 mo postpartum, followed by a return to a seronegative status. The animal has remained seronegative, is currently healthy, and has subsequently delivered 6 healthy calves.

Case 2

The second case (C2) was a 21 yr old female, collected off the coast of Cat Island in Mississippi, that had a previously documented *Brucella*-induced late-term abortion in 1997, which occurred during the animal's second pregnancy (Miller et al. 1999). *Brucella* was cultured from multiple fetal tissues, including lung, liver, spleen, kidney, stomach, peritoneal cavity, and fetal blood. The organism was also cultured from the placenta as well as vaginal fluid collected 2 d postpartum. Histopathology of the placenta and fetal tissues revealed acute, multifocal, suppurative placentitis.

We analyzed 36 serum samples collected over a 17 yr period. Results demonstrated the animal to be seronegative for *Brucella* sp. antibodies for 9 yr, 17 d prior to the abortion (Table 2). At the onset of the abortion, a steady rise in the antibody level was demonstrated, and seroconversion occurred 1 mo post abortion, with an 8-fold increase in titer, with a final

Table 2. *Tursiops truncatus.* Serologic evaluation for Case 2; antibody titer against *Brucella* sp. See Table. 1 for explanation of titers

Date (m/d/yr)	Titer	Interpretation
9/26/1988	<1:50	Suspect
3/21/1989	<1:50	Suspect
10/11/1990	<1:50	Suspect
11/29/1990	<1:50	Suspect
1/25/1991	<1:50	Suspect
10/15/1991	<1:50	Negative
6/3/1992	<1:50	Suspect
12/4/1992	<1:50	Negative
1/27/1993[a]	<1:50	Negative
11/16/1994	<1:50	Negative
12/20/1994	<1:50	Negative
1/23/1996	<1:50	Suspect
10/11/1996	<1:6.25	Negative
12/18/1996	<1:6.25	Negative
1/4/1997[b]	1:25	Suspect
1/6/1997	1:25	Negative
1/15/1997	1:25	Suspect
2/4/1997	1:50	Positive
3/4/1997	1:50	Positive
4/9/1997	1:50	Positive
12/2/1997	1:50	Positive
8/19/1998	1:50	Positive
12/15/1998[c]	1:50	Positive
4/1/2002	<1:50	Suspect
6/4/2002[d]	<1:50	Negative
6/10/2002	<1:50	Negative
8/5/2002	<1:50	Negative
2/10/2003	<1:50	Negative
8/20/2003	<1:50	Suspect
12/29/2003	<1:50	Negative
1/29/2004	<1:50	Suspect
5/5/2004	<1:50	Suspect
11/23/2004	<1:50	Suspect
1/27/2005	<1:50	Suspect
5/25/2005	<1:50	Suspect
11/22/2005[e]	<1:50	Suspect

[a]Live calf born 6/1/1993
[b]*Brucella*-positive abortion 1/4/1997
[c]Live calf born 7/28/1999
[d]Weak calf, failure to thrive 6/4/2002
[e]Animal died 1/23/2006

end-point titer of 1:50. C2 remained seropositive for 2 yr following the abortion, then reverted back to seronegative status until its death at age 30, 9 yr later.

C2 gave birth to a weak calf in 2002 that only took a few breaths before expiring. Postmortem examination of the calf and placenta did not reveal the presence of *Brucella* using *Brucella*-specific microbiological and PCR techniques. Vaginal and milk samples from the dam were also found to be culture and PCR negative. Serum samples collected 2 mo prior to parturition, through 2 mo post parturition, revealed the animal to be seronegative before, during, and after the onset of gestation (Table 2).

C2 died in 2006 of unrelated causes. Death was not attributed to brucellosis. A necropsy was performed, and *Brucella* sp. was not identified with culture and PCR methods. Seronegative status was also noted at the time of death (Table 2).

Case 3

The third case (C3) involved a 33 yr old female Pacific bottlenose dolphin *Tursiops truncatus gilli* originally collected in the San Pedro Channel, California. A *Brucella*-induced late-term abortion occurred during the animal's fifth pregnancy. *Brucella* was isolated through microbiological culture from the following fetal tissues: lung, liver, cardiac blood, stomach contents, kidney, feces, and gravid and non-gravid horns of the placenta. PCR was also performed with the fetal tissues, detecting the presence of *Brucella* in the fetal kidney, as well as the placenta. Histopathologic findings revealed focal, mild, neutrophilic, and necrotizing placentitis; mild interstitial pneumonia; splenic sinusoidal congestion; and histiocytosis of the left marginal lymph node. Immunohistochemical staining for *Brucella* was positive in the fetal lung, liver, spleen, and placenta, and negative in thymus, testis, lymph node, and kidney. Staining was most marked in foci of placental necrosis, with positive-staining organisms present in trophoblast cytoplasm (Fig. 1). Only rare, small foci of staining were observed in other positive tissues.

C3 died in 2005 of *Staphylococcus aureus* pneumonia and septicemia. Gross and histopathologic evaluation, microbiological culture, PCR, and immunohistochemical (IHC) tests were performed, revealing a disseminated *S. aureus* infection and associated pneumonia with multifocal pulmonary abscesses. *Brucella* was not cultured from any tissues at the time of necropsy, but was detected in a chronic lung lesion with real-time PCR. Weak single-cell staining was seen in the spleen by IHC staining for *Brucella*.

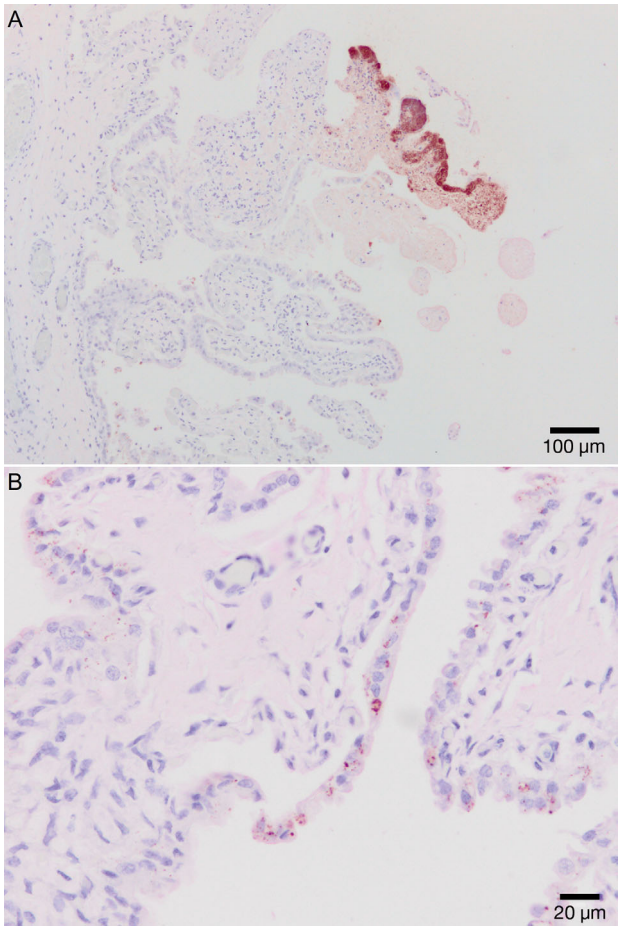

Fig. 1. *Tursiops truncatus.* (A) Immunohistochemical (IHC) staining of placenta from a *Brucella* culture-positive aborted bottlenose dolphin fetus (Case 3). Red-orange staining indicates the presence of *Brucella* in necrotic placental villi. (B) IHC staining of the trophoblast layer in the aborted placenta. Note punctate red-orange staining in cytoplasm of trophoblasts indicating the presence of *Brucella*

We tested 34 serum samples for C3 over a 20 yr period. iELISA test results indicated that the animal was seronegative for 18 yr prior to the abortion, including on the day the abortion occurred (Table 3). Seroconversion occurred 4 d after the abortion with a 256-fold increase and a persistent end-point titer of 1:1600. C3 remained seropositive for 2 yr until the time of death at age 35.

Case 4

The fourth case (C4) was a 21 yr old female collected off the coast of Gulfport, Mississippi. C4 died in 1997 from complications secondary to an acute *Staphylococcus aureus* septicemia. The gross nec-

Table 3. *Tursiops truncatus.* Serologic evaluation for Case 3; antibody titer against *Brucella* sp. See Table 1 for explanation of titers

Date (m/d/yr)	Titer	Interpretation
5/3/1985[a]	<1:50	Negative
2/22/1993	<1:50	Negative
7/22/1993[b]	<1:50	Negative
8/24/1993	<1:50	Suspect
9/28/1993	<1:50	Suspect
6/21/1995	<1:50	Negative
11/14/1995	<1:50	Negative
3/3/1996	<1:50	Negative
7/31/1996	<1:50	Negative
10/25/1996	<1:50	Negative
5/1/1997	<1:50	Negative
10/7/1997	<1:50	Negative
4/3/1998	<1:50	Negative
8/10/1998[c]	<1:50	Negative
2/23/1999	<1:50	Negative
11/30/1999	<1:50	Suspect
2/17/2000[d]	<1:50	Negative
10/25/2001	<1:50	Suspect
11/25/2002	<1:50	Suspect
5/15/2003	<1:50	Negative
7/31/2003	<1:50	Negative
10/22/2003	<1:50	Negative
11/21/2003	<1:50	Negative
12/5/2003[e]	1:6.25	Negative
12/9/2003	1:50	Positive
1/20/2004	1:800	Positive
1/27/2004	1:1600	Positive
5/5/2004	1:1600	Positive
9/29/2004	1:1600	Positive
1/19/2005	1:1600	Positive
3/17/2005	1:1600	Positive
3/29/2005	<1:50	Negative
8/2/2005	1:1600	Positive
8/10/2005[f]	1:1600	Positive

[a]Spontaneous late-term abortion 7/17/1990
[b]Stillborn calf 7/20/1993
[c]Dead calf 8/9/1998
[d]Live calf born 6/16/2001
[e]*Brucella*-positive abortion 12/5/2003
[f]Animal died 8/11/2005

ropsy revealed a previously documented pulmonary granuloma that was noted to be an incidental finding (Miller et al. 1999). *Brucella* sp. was isolated via microbiological culture from this pulmonary lesion. Five serum samples over a 12 yr period were analyzed for the presence of antibodies to *Brucella*. Although there were limited serologic data, C4 demonstrated a persistent titer (1:1600) over a 12 yr period (Table 4).

Case 5

The fifth case (C5) was a 23 yr old female collected off the coast of Gulfport, Mississippi. C5 presented in 2002 with clinical signs of abnormal swimming and a bilateral swelling at the level of the epaxial muscles caudoventral to the dorsal fin. Lumbar vertebral spondylosis was identified based on standard radiography.

Table 4. *Tursiops truncatus.* Serologic evaluation for Case 4; antibody titer against *Brucella* sp. See Table 1 for explanation of titers

Date (m/d/yr)	Titer	Interpretation
5/31/1985	>1:50[b]	Positive
5/30/1991	>1:50[b]	Positive
9/9/1992	1:1600	Positive
12/21/1992	>1:50[b]	Positive
11/6/1997[a]	1:1600	Positive

[a]Animal died 11/7/97, *Brucella* isolated from a pulmonary granuloma found at necropsy. [b]End point titers not obtained due to limited sample availability

Cervical and lumbar computed tomography were performed. There were multiple sites of cervical vertebral ankylosis (left-sided atlanto-occipital and C4–5–6), and all imaged cervical vertebral bodies had abnormally irregular margins. The ventral portion of the vertebral canal at C4–5–6 had an irregular, angular contour, protruding into and narrowing the canal, and there was widening of the intervertebral disc space at C1/2–C3. The cranial lumbar lesion exhibited narrowing of the affected intervertebral disc space, sclerosis of endplate margins, and osseous proliferation of the associated vertebral bodies. The caudal lumbar lesion was at the mid-level of the pelvic vestiges and characterized by complete collapse of the intervertebral disc space, severe mottling and heterogeneity of the affected vertebral endplates with irregular lytic changes, and osseous proliferation of affected adjacent vertebral bodies. The cranial lumbar lesion had an inactive appearance consistent with chronic discospondylitis. The caudal lumbar lesion was typical of active discospondylitis.

C5 died in 2003 due to unrelated causes. Gross necropsy findings revealed multiple vertebral malformations with fused vertebrae and proliferative bone involving the atlanto-occipital joint, lumbar vertebrae L4–5–6 and L11–12. The corresponding intervertebral discs appeared fibrous and inspissated. Microbiological culture was performed from the cervical and lumbar lesions, and *Brucella* sp. was isolated from the L4–6 vertebral lesion. We evaluated 30 serum samples over an 18 yr period. The serologic results revealed a persistent titer ranging from 1:200 to 1:1600 over the entire 18 yr period (Table 5).

Table 5. *Tursiops truncatus.* Serologic evaluation for Case 5; antibody titer against *Brucella* sp. See Table 1 for explanation of titers

Date (m/d/yr)	Titer	Interpretation
5/31/1985	1:800	Positive
12/5/1985	1:1600	Positive
6/27/1986	1:1600	Positive
5/29/1987	1:1600	Positive
4/12/1988	1:1600	Positive
11/16/1989	1:1600	Positive
2/11/1990	1:800	Positive
7/24/1990	1:800	Positive
3/7/1991	1:1600	Positive
7/26/1991	1:400	Positive
2/14/1992	1:1600	Positive
4/22/1993	1:1600	Positive
9/24/1993	1:800	Positive
5/19/1994	1:800	Positive
6/8/1995	1:400	Positive
8/22/1995	1:1600	Positive
4/29/1997	1:1600	Positive
12/31/1998	1:1600	Positive
4/1/1999	1:400	Positive
3/17/2000	1:400	Positive
9/8/2000	1:200	Positive
12/21/2000	1:800	Positive
5/29/2001	1:400	Positive
9/20/2001	1:400	Positive
3/13/2002	1:400	Positive
9/23/2002[a]	1:800	Positive
10/21/2002	1:400	Positive
12/16/2002	1:800	Positive
1/2/2003	1:800	Positive
1/14/2003[b]	1:400	Positive

[a]Lumbar vertebral spondylosis detected
[b]Animal died 1/15/2003. *Brucella* isolated from lumbar vertebral lesion

Case 6

The sixth case (C6) involved a wild juvenile male, estimated to be 1.5 yr of age, that originally stranded on the Texas coast in 1998 and was transferred to Mystic Aquarium (Mystic, Connecticut) for further rehabilitation. The animal developed a previously documented vertebral osteomyelitis at the level of the caudal peduncle (Goertz et al. 2011). Brucellosis was strongly suspected to be the cause of the vertebral disease; however, multiple attempts to isolate the organism antemortem were unsuccessful. Multiple complications developed as a result of the vertebral disease, and ultimately the animal died acutely in 2001 of a *Staphylococcus aureus* septicemia. *Brucella* sp. was isolated via microbiological culture at necropsy from the vertebral lesion (Goertz et al. 2011). Ten serum samples collected over a 2 yr period demonstrated a persistent titer ranging from 1:1200 to 1:1800 (Table 6).

Table 6. *Tursiops truncatus.* Serologic evaluation for Case 6; antibody titer against *Brucella* sp. See Table 1 for explanation of titers

Date (m/d/yr)	Titer	Interpretation
5/10/1999	1:800	Positive
9/22/1999	1:1600	Positive
4/4/2000	1:800	Positive
6/2/2000	1:800	Positive
8/9/2000	1:800	Positive
9/20/2000	1:800	Positive
11/1/2000	1:800	Positive
12/13/2000	1:800	Positive
12/27/2000	1:800	Positive
1/3/2001[a]	1:800	Positive

[a]Animal died 1/16/2001. *Brucella* isolated from vertebral lesion

Serology test comparison and serologic survey

Serologic comparison of the cetacean iELISA results with *Brucella abortus* test methods for the bottlenose dolphin serum samples were performed. The cetacean iELISA, and *B. abortus* cELISA and FPA tests, yielded a total of 52 (49%), 37 (34.9%), and 32 (30.2%) seropositive samples, respectively.

Blood samples collected between August 2000 and June 2002 from 64 bottlenose dolphins were included in the serologic survey, of which 31 (48.4%) were female, and 33 (51.6%) were male. Median age at the time of sample collection was 21 yr (range 0.6 to 45 yr). Among the dolphins in the study, 18 (28.1%) were seronegative for antibodies against *Brucella*, 18 (28.1%) were seropositive, and 28 (43.8%) had results interpreted as suspect. There were no significant differences in female and male prevalence by result category ($p = 0.44$). Dolphins that were seronegative were more likely to be younger than dolphins that were seropositive or suspect (mean age, negative = 15 ± 12 yr, suspect = 23 ± 8 yr, positive = 25 ± 7 yr; $p = 0.002$). When plotting age by OD value, a cluster of positive animals was found between the ages of 17 and 25 yr old (Fig. 2). When comparing this 'middle-age' group to any other age in the study, middle-aged dolphins were 6.8 times more likely to be seropositive ($p = 0.02$).

Among the 40 CBC and chemistry analytes tested, only 3 (7.5%) values had significantly different means by result category (negative, positive, and suspect) when controlling for age (Table 7). Dolphins with positive *Brucella* OD values were more likely to have higher AST and GGT compared to dolphins with negative *Brucella* OD values (least

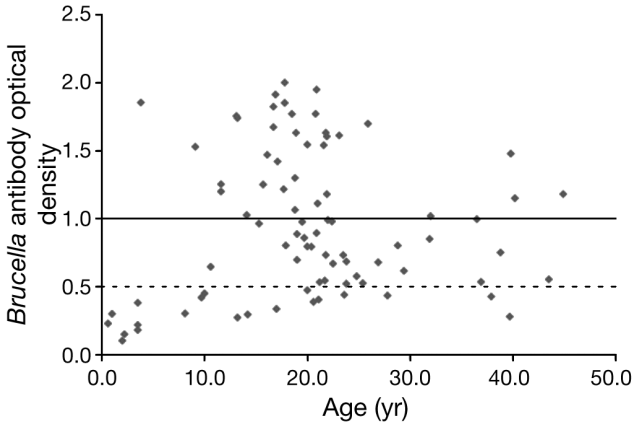

Fig. 2. *Tursiops truncatus. Brucella* antibody optical density values among 64 bottlenose dolphins by age in years. Values above the solid line are positive, values between the solid and dashed line are suspect, and values below the dashed line are negative

squares means for positive dolphins = 310 and 110 U l^{-1}; negative dolphins = 204 and 36 U l^{-1}, respectively; $p < 0.05$).

Retrospective sera, collected 3 to 5 yr prior to the serologic survey, were evaluated for 16 of 18 dolphins that tested seropositive in the initial evaluation. Among those tested, 13 (81.3%) had demonstrated a persistent seropositive status. Two dolphins had a questionable positive and 1 had a negative archived result.

DISCUSSION

The serologic responses of 6 bottlenose dolphins with confirmed *Brucella* infections using a newly developed dolphin-specific iELISA are described. A difference in the antibody response was observed in the animals that developed reproductive infections versus infections involving the musculoskeletal and respiratory systems, which may be based on the nature, site, and duration of the *Brucella* infection.

In the cases that developed *Brucella*-induced abortions (C1, C2, and C3), seroconversion did not occur until after the onset of abortion. These observations are consistent with the ability of *Brucella* in terrestrial animals to evade the host's immune response until proliferation of the organism can occur during an active infection (Gorvel & Moreno 2002). The bacterium is capable of extensive intracellular replication without disrupting basic cellular functions (Adams 2002, Gorvel & Moreno 2002). During pregnancy, the *Brucella* organisms replicate in placental trophoblasts during mid- and late gestation, until further spread can occur via the infected aborted fetus (Gorvel & Moreno 2002). *Brucella* occurring within trophoblasts as seen by IHC in dolphin C3 has been documented previously in cetaceans, supporting a similar pathogenesis (Miller, et al. 1999, González-Barrientos et al. 2010, Guzmán-Verri et al. 2012). It is unknown when these 3 females first became infected. In experimentally infected pregnant cattle and bison, infections resulted in either abortions or delivery of live calves. Seroconversion in the experimentally infected animals occurred prior to the abortions or live births (Davis et al. 1990, Olsen & Johnson 2011). This is in contrast to the dolphin cases reported here, where seroconversion was not demonstrated until after the abortions occurred; however, the serologic response of natural infections versus experimental infections may differ. Interestingly, in both experimentally infected bison and cattle, seroconversion was significantly greater in animals that had abortions versus those with live calves (Davis et al. 1990).

Post-abortion antibody levels in 2 of the 3 females declined over time, and both had subsequent live births. This may suggest clearance of the infection after abortion. However, it is also possible that these animals may have latent infections with the potential to be asymptomatic carriers, with intermittent shedding of the organism (Davis et al. 1990, Rhyan et al. 2001, Tessaro & Forbes 2004). In cattle, subsequent pregnancies following the abortion are typically normal; however, the organism may continue to be shed in milk and vaginal discharges during subsequent pregnancies, emphasizing the importance of evaluating postpartum fluids (Olsen & Johnson 2011; also see www.cfsph.iastate.edu/Factsheets/pdfs/brucellosis.pdf). In addition to the reproductive tract, persistent infections may be maintained in mammary tissue and the lymphoreticular system, such as the supramammary, colorectal, and other lymph nodes as reported in various terrestrial species (Davis et al. 1990, Rhyan et al. 2001, Adams 2002, Tessaro & Forbes 2004, Olsen & Johnson 2011). Evidence of *Brucella* isolates recovered from these tissues have been documented in cetaceans, and when applicable, should be included in postmortem evaluation of females with history of abortions (Foster et al. 1996). Persistent shedding of *Brucella* during subsequent pregnancies has not yet been documented in bottlenose dolphins, and the subsequent pregnancies for C1 and C2 did not reveal the presence of *Brucella* when placental, fetal, or maternal fluids were evaluated, suggesting clearance of the infection. However, C3 did demon-

Table 7. *Tursiops truncatus.* Least squares means of blood values among 64 bottlenose dolphins by *Brucella* antibody category (negative, positive, suspect) using an indirect ELISA, controlling for age. WBC: white blood cell; RBC: red blood cell; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: MCH concentration; RBC: red blood cells distribution width; NRBC: nucleated red blood cells; BUN: blood urea nitrogen; A: albumin; G: globulins; InorgPhos: inorganic phosphate; AlkPhos: alkaline phosphatase; LDH: lactate dehydrogenase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transpeptidase; CPK: creatine phosphokinase; ESR: erythrocyte sedimentation rate

Blood value	Type I SS p-value	Least squares means Negative (n = 18)	Positive (n = 18)	Suspect (n = 28)
WBC count	0.24	10027	8545	8160
RBC count	0.50	3.2	3.4	3.4
Hemoglobin	0.40	14.2	14.4	14.7
Hematocrit	0.32	40.8	41.9	42.7
MCV	0.63	126	125	127
MCH	0.57	43.7	43.0	43.7
MCHC	0.56	34.7	34.4	34.5
RBC	0.87	15.2	15.4	15.2
NRBC	0.67	0.5	0.4	0.9
Platelets	0.58	122285	110465	117839
Neutrophils	0.42	6742	5889	5468
Lymphocytes	0.33	1611	1415	1280
Monocytes	0.07	378	214	218
Eosinophils	0.48	1298	1028	1199
Glucose	0.73	119	113	116
BUN[a]	0.002	45.2	43.2	51.9
Creatinine	0.44	1.4	1.5	1.5
BUN:creatinine	0.07	32.9	29.0	35.9
Uric acid	0.74	0.3	0.3	0.3
Sodium	0.20	155	156	157
Potassium	0.18	3.9	3.7	3.7
Chloride	0.22	122	120	122
CO_2	0.85	23.0	23.5	23.3
Protein	0.44	7.1	7.1	7.0
A	0.41	4.1	4.2	4.2
G	0.24	3.0	2.9	2.7
A:G	0.40	1.5	1.5	1.6
Calcium	0.88	9.2	9.2	9.2
InorgPhos	0.17	5.1	4.9	4.7
AlkPhos	0.45	478	412	364
LDH	0.17	387	438	396
AST[b]	0.01	204	310	245
ALT	0.12	31	48	33
GGT[c]	0.02	36	110	38
Bilirubin	0.31	0.2	0.2	0.2
Cholesterol	0.98	232	229	232
Triglyceride	0.79	102	116	103
Iron	0.28	227	273	217
CPK	0.60	159	134	150
ESR	0.32	16	12	7

Significant group comparisons: [a]Suspect > Neg, Pos; [b]Pos > Neg; [c]Pos > Suspect, Neg

strate a persistent antibody titer post abortion, and *Brucella* was identified postmortem indicating a chronic infection post abortion.

C4, C5, and C6 developed *Brucella* infections of the musculoskeletal and pulmonary systems, including a male and female with vertebral osteomyelitis, and 1 female with a pulmonary granuloma. These disease processes were chronic based on the clinical history and histopathologic lesions, and *Brucella* was isolated from postmortem tissues. All 3 cases demonstrated a persistent elevated antibody titer. It is unknown when these animals first became infected, but the nature of these disease manifestations and the persistent titers are consistent with chronic infections with persistent antibody titers likely due to chronic antigenic stimulation.

Based on the serologic population survey, 28.1% (18 of 64) of the dolphins were seronegative for antibodies against *Brucella*, 28.1% (18 of 64) were seropositive, and 43.8% (28 of 64) had results interpreted as suspect. This degree of seroprevalence is similar to what has been documented in free-ranging species including free-ranging elk and bison, various wild cetacean species, as well as livestock herds in countries where the disease has not been eradicated (Davis et al. 1990, Van Bressem at al. 2001, Meegan et al. 2006, Muma et al. 2006, Tachibana et al. 2006, Scurlock & Edwards 2010, Matope et al. 2011, Olsen & Johnson 2011)

C1 to C5 came from this same population, and despite the presence of *Brucella*, the population has maintained overall low age-specific mortality rates, high annual survival rates, and increasing numbers of dolphins living 30 to 50 yr (Venn-Watson et al. 2011a,b). During the past decade since this study was completed, only 1 additional dolphin has had culture-confirmed brucellosis (Cassle et al. 2009).

When the seropositive dolphins were evaluated retrospectively, 81% of dolphins with positive *Brucella* antibody levels demonstrated chronic positive antibody levels lasting at least 3 to 5 yr. While a seronegative result does not definitively indicate absence of a previous infection or latent disease, conversely, a seropositive result does not distinguish between active infections and sustained immunity to a cleared infection. In experimentally infected cattle and bison, all animals seroconverted post inoculation, but infections did not necessarily occur in all animals (Davis et al. 1990). This suggests that animals may become exposed to the organism and seroconvert without becoming persistently infected.

Age and sex were also evaluated as risk factors. Sex was not determined to be a predictor of the level of *Brucella* antibodies. Dolphins that were seronegative were more likely to be younger than the dolphins that were suspect or seropositive, suggesting that

the risk of exposure increases with age. Dolphins between 17 and 25 yr old were 6.8 times more likely to have positive *Brucella* antibody levels compared to dolphins of all other ages, regardless of sex, indicating that dolphins of the age where sexual activity may be higher were more likely to have positive *Brucella* antibody levels. Sexual maturity in terrestrial animals has also been documented to be associated with *Brucella* infections (Adams 2002, Muma et al. 2006). However, other epidemiologic studies have shown varying results with regard to seroprevalence and age (Davis et al. 1990, Rhyan et al. 2001, Scurlock & Edwards 2010, Matope et al. 2011).

We found no indication of increased inflammation (increased WBC, increased ESR, and decreased iron) when comparing dolphins with positive, questionable, or negative *Brucella* antibody levels. Dolphins with positive *Brucella* antibody levels were more likely to have higher AST and GGT values compared to dolphins that were negative. *Brucella* infections have been shown to cause hepatitis and hepatic necrosis in cetaceans, and given the association in this study of positive *Brucella* antibody levels with increased liver enzymes, further investigation is warranted to determine the significance of these changes (Foster et al. 2002, Ohishi et al. 2003).

As in terrestrial species, our findings suggest that significant increases in antibody titers may be detected in bottlenose dolphins during active *Brucella* infections. This was demonstrated with the abortions, where the animals seroconverted post abortion. Further, animals with chronic lesions associated with *Brucella* may maintain persistent titers. This study emphasizes the importance of longitudinal serologic monitoring in dolphins when diagnosing, treating, and monitoring for *Brucella* sp. infections.

Acknowledgements. This constitutes scientific contribution no. 215 from the Sea Research Foundation. T.A.F. was funded in part by USDA/CSREES-NRICGP 99-35204-7550 and NIH 1RO1 AI48496-01. We thank all veterinary and animal care staff involved with the medical care of the case animals.

LITERATURE CITED

Adams LG (2002) The pathology of brucellosis reflects the outcome of the battle between the host genome and the *Brucella* genome. Vet Microbiol 90:553–561

Al Dahouk S, Tomaso H, Nockler K, Neubauer H, Frangoulidis D (2003) Laboratory-based diagnosis of brucellosis — a review of the literature. Part II. Serologic tests for brucellosis. Clin Lab 49:577–589

Aliskan H (2008) The value of culture and serological methods in the diagnosis of human brucellosis. Mikrobiyol Bul 42:185–195

Bricker BJ, Ewalt DR, MacMillan AP, Foster G, Brew S (2000) Molecular characterization of *Brucella* strains isolated from marine mammals. J Clin Microbiol 38: 1258–1262

Cassle S, Johnson S, Lutmerding B, Jensen E (2009) Pulmonary brucellosis in an Atlantic bottlenose dolphin (*Tursiops truncatus*). Proc Int Assoc Aquat Anim Med 40:129

CFIA (Canadian Food Inspection Agency, Animal Diseases Research Institute) (2001) Protocol for a competitive enzyme immunoassay for detection of serum antibody to *Brucella abortus*. CFIA, Ottawa

Cloeckaert A, Grayon M, Grépinet O, Boumedine KS (2003) Classification of *Brucella* strains isolated from marine mammals by infrequent restriction site-PCR and development of specific PCR identification tests. Microbes Infect 5:593–602

Dagleish MP, Barley J, Howie FE, Reid RJ, Herman J, Foster G (2007) Isolation of *Brucella* species from a diseased atlanto-occipital joint of an Atlantic white-sided dolphin (*Lagenorhynchus acutus*). Vet Rec 160:876–878

Dagleish MP, Barley J, Finlayson J, Reid RJ, Foster G (2008) *Brucella ceti* associated pathology in the testicle of a harbour porpoise (*Phocoena phocoena*). J Comp Pathol 139: 54–59

Davis DS, Templeton JW, Ficht TA, Williams JD, Kopec JD, Adams LG (1990) *Brucella abortus* in captive bison. I. Serology, bacteriology, pathogenesis, and transmission to cattle. J Wildl Dis 26:360–371

Davison NJ, Cranwell MP, Perrett LL, Dawson CE and others (2009) Meningoencephalitis associated with *Brucella* species in a live-stranded striped dolphin (*Stenella coeruleoalba*) in south-west England. Vet Rec 165:86–89

Ewalt DR, Payeur JB, Martin BM, Cummins DR, Miller WG (1994) Characteristics of a *Brucella* species from a bottlenose dolphin (*Tursiops truncatus*). J Vet Diagn Invest 6: 448–452

Foster G, Jahans KL, Reid RJ, Ross HM (1996) Isolation of *Brucella* species from cetaceans, seals and an otter. Vet Rec 138:583–586

Foster G, MacMillan AP, Godfroid J, Howie F and others (2002) A review of *Brucella* sp. infection of sea mammals with particular emphasis on isolates from Scotland. Vet Microbiol 90:563–580

Foster G, Osterman BS, Godfroid J, Jacques I, Cloeckaert A (2007) *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. Int J Syst Evol Microbiol 57: 2688–2693

Goertz CEC, Frasca S Jr, Bohach GA, Cowan DF and others (2011) *Brucella* sp. vertebral osteomyelitis with intercurrent fatal *Staphylococcus aureus* toxigenic enteritis in a bottlenose dolphin (*Tursiops truncatus*). J Vet Diagn Invest 23:845–851

González L, Patterson IA, Reid RJ, Foster G and others (2002) Chronic meningoencephalitis associated with *Brucella* sp. infection in live-stranded striped dolphins (*Stenella coeruleoalba*). J Comp Pathol 126:147–152

González-Barrientos R, Morales JA, Hernández-Mora G, Barquero-Calvo E, Guzmán-Verri C, Chaves-Olarte E, Moreno E (2010) Pathology of striped dolphins (*Stenella coeruleoalba*) infected with *Brucella ceti*. J Comp Pathol 142:347–352

Gorvel JP, Moreno E (2002) *Brucella* intracellular life: from invasion to intracellular replication. Vet Microbiol 90: 281–297

Guzmán-Verri C, González-Barrientos R, Hernández-Mora G, Morales J, Baquero-Calvo E, Chaves-Olarte E, Moreno E (2012) *Brucella ceti* and brucellosis in cetaceans. Front Cell Infect Microbiol 2:1–22

Hernández-Mora G, Manire CA, González-Barrientos R, Barquero-Calvo E and others (2009) Serological diagnosis of *Brucella* infections in odontocetes. Clin Vaccine Immunol 16:906–915

Jepson PD, Brew S, MacMillan AP, Baker JR and others (1997) Antibodies to *Brucella* in marine mammals around the coast of England and Wales. Vet Rec 141:513–515

Matope G, Bhebhe E, Muma JB, Lund A, Skjerve E (2011) Risk factors for *Brucella* spp. infection in smallholder household herds. Epidemiol Infect 139:157–164

Meegan J, Romano TR, Mazzaro L, Sidor I, Dunn JL (2006) Serosurveillance of wild bottlenose dolphins (*Tursiops truncatus*) and evaluation of both newly developed marine-specific and commercially available *Brucella abortus* serologic tests for the detection of antibodies to marine-origin *Brucella*. Proc Int Assoc Aquat Anim Med 37: 65–67 (Abstract)

Meegan J, Field C, Sidor I, Romano T and others (2010) Development, validation, and utilization of a competitive enzyme-linked immunosorbent assay for the detection of antibodies against *Brucella* species in marine mammals. J Vet Diagn Invest 22:856–862

Miller WG, Adams LG, Ficht TA, Cheville NF and others (1999) *Brucella*-induced abortions and infection in bottlenose dolphins (*Tursiops truncatus*). J Zoo Wildl Med 30:100–110

Muma JB, Samui KL, Siamudaala VM, Oloya J and others (2006) Prevalence of antibodies to *Brucella* spp. and individual risk factors of infection in traditional cattle, goats, and sheep reared in livestock-wildlife interface areas of Zambia. Trop Anim Health Prod 38:195–206

Nielsen K (2002) Diagnosis of brucellosis by serology. Vet Microbiol 90:447–459

Nielsen K, Lin M, Gall D, Jolley M (2000) Fluorescence polarization immunoassay: detection of antibody to *Brucella abortus.* Methods 22:71–76

Nielsen O, Stewart REA, Nielsen K, Measures L, Duignan P (2001) Serological survey of *Brucella* spp. antibodies in some marine mammals of North America. J Wildl Dis 37: 89–100

Ohishi K, Zenitani R, Bando T, Goto Y and others (2003) Pathological and serological evidence of *Brucella*-infection in baleen whales (Mysticeti) in the western North Pacific. Comp Immunol Microbiol Infect Dis 26: 125–136

Olsen SC, Johnson C (2011) Comparison of abortion and infection after experimental challenge of pregnant bison and cattle with *Brucella abortus* strain 2308. Clin Vaccine Immunol 18:2075–2078

Patterson IAP, Howie FE, Reid RJ, Ross HM, MacMillan A, Foster G, Buxton D (2000) *Brucella* infection in marine mammals from Scottish waters. Proc Int Assoc Aquat Anim Med 31:396–398

Rhyan JC, Gidlewski CT, Roffe TJ, Aune K, Philo LM, Ewalt D (2001) Pathology of brucellosis in bison from Yellowstone National Park. J Wildl Dis 37:101–109

Ross HM, Foster G, Reid RJ, Jahans KL, Macmillan AP (1994) *Brucella* species infection in sea mammals. Vet Rec 134:359

Ross HM, Jahans KL, MacMillan AP, Reid RJ, Thompson PM, Foster G (1996) *Brucella* species infection in North Sea seal and cetacean populations. Vet Rec 138:647–648

Ruiz CL, Nollens HH, Venn-Watson S, Green LG and others (2009) Baseline circulating immunoglobulin G levels in managed collection and free-ranging bottlenose dolphins (*Tursiops truncatus*). Dev Comp Immunol 33: 449–455

Scurlock BM, Edwards WH (2010) Status of brucellosis in free-ranging elk and bison in Wyoming. J Wildl Dis 46: 442–449

Sidor IF, Dunn JL, Tsongalis GJ, Carlson J Frasca Jr S (in press) A multiplex real-time PCR assay with two internal controls for the detection of *Brucella* spp. in tissues, blood and feces from marine mammals. J Vet Diagn Invest

St. Leger J, Schmitt T, Reidarson T, Scadeng M, Dubowitz D, Danil K (2007) Hydrocephalus and non-suppurative meningoencephalitis associated with *Brucella* sp. infection in two live-stranded dolphins. Proc Int Assoc Aquat Anim Med 38:8

Tachibana M, Watanabe K, Kim S, Omata Y, Murata K, Hammond T, Watarai M (2006) Antibodies to *Brucella* spp. in Pacific bottlenose dolphins from the Solomon Islands. J Wildl Dis 42:412–414

Tessaro SV, Forbes LB (2004) Experimental *Brucella abortus* infection in wolves. J Wildl Dis 40:60–65

Tryland M, Kleivane L, Alfredsson A, Kjeld M, Arnason A, Stuen S, Godfroid J (1999) *Brucella* infection in marine mammals in the North Atlantic Ocean. Vet Rec 144: 588–592

Van Bressem MF, Van Waerebeek K, Raga JA, Godfroid J, Brew SD, MacMillan AP (2001) Serological evidence of *Brucella* species infection in odontocetes from the South Pacific and the Mediterranean. Vet Rec 148:657–661

Venn-Watson SK, Jensen ED, Ridgway SH (2011a) Evaluation of population health among bottlenose dolphins (*Tursiops truncatus*) at the United States Navy Marine Mammal Program. J Am Vet Med Assoc 238:356–360

Venn-Watson S, Smith CR, Gomez F, Jensen ED (2011b) Physiology of aging among healthy, older bottlenose dolphins (*Tursiops truncatus*): comparisons with aging humans. J Comp Physiol B 181:667–680

Editorial responsibility: Michael Moore, Woods Hole, Massachusetts, USA

Submitted: March 22, 2012; Accepted: August 27, 2012
Proofs received from author(s): November 22, 2012