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# *Brucella ceti* sequence type 23, 26, and 27 infections in North American cetaceans

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ABSTRACT: *Brucella ceti* infection is associated with a variety of disease outcomes in cetaceans globally. Multiple genotypes of *B. ceti* have been identified. This retrospective aimed to determine if specific lesions were associated with different *B. ceti* DNA sequence types (STs). Characterization of ST was performed on 163 samples from 88 free-ranging cetaceans, including common bottlenose dolphin *Tursiops truncatus* (*T.t.*; n = 73), common short-beaked dolphin *Delphinus delphis* (*D.d.*; n = 7), striped dolphin *Stenella coeruleoalba* (n = 3), Pacific white-sided dolphin *Lagenorhynchus obliquidens* (n = 2), sperm whale *Physeter macrocephalus* (n = 2), and harbour porpoise *Phocoena phocoena* (n = 1), that stranded along the coast of the US mainland and Hawaii. ST was determined using a previously described insertion sequence 711 quantitative PCR. Concordance with 9-locus multi-locus sequence typing was assessed in a subset of samples (n = 18). ST 26 was most commonly identified in adult dolphins along the US east coast with non-suppurative meningoencephalitis (p = 0.009). Animals infected with ST 27 were predominately perinates that were aborted or died shortly after birth with evidence of *in utero* pneumonia (p = 0.035). Reproductive tract inflammation and meningoencephalitis were also observed in adult *T.t.* and *D.d.* with ST 27, though low sample size limited interpretation. ST 23 infections can cause disease in cetacean families other than porpoises (Phocoenidae), including neurobrucellosis in *D.d.* In total, 11 animals were potentially infected with multiple STs. These data indicate differences in pathogenesis among *B. ceti* STs in free-ranging cetaceans, and infection with multiple STs is possible.

KEY WORDS: *Brucella ceti* · Brucellosis · Bottlenose dolphin · Cetacean · IS711 · *Tursiops truncatus* · *Delphinus delphis* · Pathology · Dolphins

## 1. INTRODUCTION

*Brucella ceti*, a zoonotic pathogen, has been isolated from dolphins, whales, porpoises, and some pinnipeds and phocids (Foster et al. 2007, Whatmore et al. 2008, Nymo et al. 2011, Guzmán-Verri et al. 2012, Hernández-Mora et al. 2013, Whatmore et al. 2017). *B. ceti* has been detected in numerous cetacean species in the majority of oceans across the world (Guzmán-Verri et al. 2012). Pathologic lesions associated with *Brucella* infection in cetaceans are well documented and include meningoencephalitis, arthritis, multi-organ abscessation, reproductive tract inflammation, abortions, placentitis, and *in utero* pneumonia

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(González-Barrientos et al. 2010, Nymo et al. 2011, Guzmán-Verri et al. 2012, Colegrove et al. 2016a,b, Buckle et al. 2017, Sánchez-Sarmiento et al. 2019). *Brucella* sp. can cause sub-clinical to severe chronic infections in multiple organ systems through its ability to survive and spread in the lymphoreticular system (de Figueiredo et al. 2015). Multiple genotypes of *B. ceti* have been described; however, the relationship between sequence type (ST) and pathogenicity or organ tropism is less clear (Whatmore et al. 2007, 2017, Zygmunt et al. 2010, Cloeckaert et al. 2011).

The majority of strain analysis of cetacean *Brucella* sp. has been completed from animals stranded in Europe or the eastern Atlantic Ocean, and this analysis has primarily been performed using cultured isolates (Whatmore et al. 2017). Marine *Brucella* strains have been placed into ST groups first based on 9-locus multi-locus sequence typing (MLST; Whatmore et al. 2007) and more recently by an extended 21 locus multi-locus sequence analysis (MLSA; Whatmore et al. 2017). These PCR tests examine multiple housekeeping genes, and surveys have grouped marine strains accordingly into hooded seal *Cystophora cristata*/harp seal *Pagophilus groenlandicus* (STs 53 and 54), pinniped (STs 24, 25, and 52), dolphin (ST 26), porpoise (ST 23), and ST 27 clusters. Strains are not family- or even order-specific; as an example, ST 27 has been isolated in California sea lions (Whatmore et al. 2017), and ST 23 and ST 26 have been detected in both cetacean sub-orders Mysticeti and Odontoceti (Davison et al. 2017, 2021). Strains within the ST 27 cluster have been identified in multiple bottlenose dolphins *Tursiops truncatus* stranded along the east and west coast of the USA, in a single bottlenose dolphin in the northern Adriatic sea, in Hector's dolphins *Cephalorhynchus hectori* on the coast of New Zealand, and most recently in an aborted captive bottlenose dolphin on the east coast of Australia and a stranded dwarf sperm whale cow/aborted calf along the central Pacific coast (Whatmore et al. 2008, Wu et al. 2014, 2017, Cvetnić et al. 2016, Buckle et al. 2017, Mackie et al. 2020, Hernández-Mora et al. 2021). ST 26 has been identified in dolphins and whales from the Mediterranean, North American, Central Pacific, and Scottish coasts (Maquart et al. 2009, Alba et al. 2013, Whatmore et al. 2017, Davison et al. 2021, Hernández-Mora et al. 2021). ST 23 has predominately been detected in porpoises, but also has been identified in dolphins and baleen whales off the coast of Norway, France, and Scotland (Maquart et al. 2009, Whatmore et al. 2017). Culture and isolation with full sequence typing (21-locus MLSA) is considered the gold standard to confirm infection when full genome sequencing is not practical or available (Whatmore et al. 2017). While MLST and MLSA protocols are ideal for sequence typing, they require high quality and adequate quantity of DNA for up to 21 PCR reactions and sequence analysis. Such DNA is not always available in stranded cetaceans that have varying carcass quality often with extensive bacterial overgrowth or where extraction substrate is limited to formalin fixed paraffin-embedded (FFPE) tissues. Though the larger assays have great utility for molecular characterization of *B. ceti* in a research setting, the number of reactions is impractical for routine diagnostic screening. Furthermore, culture and isolation techniques that are preferred for full MLST and MLSA assays lengthen the interval between sample collection and typing, and require equipment and safety practices not available in all laboratories. Though the risk of zoonotic transmission appears low, questions remain as to the mode of transmission in community acquired cases (Whatmore et al. 2008).

The single mobile genetic element, insertion sequence 711 (IS711), is widely used for characterization of many different *Brucella* species (Halling et al. 1993, Bricker & Halling 1994). This insertion sequence is unique to the *Brucella* genus and highly conserved among different *Brucella* species and strains. It has distinctive, species-specific locations within the chromosome (fingerprinting) and often has many copies, as is true for marine *Brucella* strains (Cloeckaert et al. 2003, 2011, Wu et al. 2014, 2017). These attributes make the IS711 genetic element an ideal candidate for differentiation of *Brucella* species among both marine and non-marine species. Wu et al. (2017) used the mobile genetic element to develop a quantitative PCR (qPCR) SYBR assay for discerning among 3 *B. ceti* cetacean STs (23, 26, 27) based on the 9-locus MLST assay. This study aimed to confirm previously reported data that the *Brucella*-specific mobile genetic element, IS711, is an accurate marker for the 9-locus MLST for *B. ceti* STs 23, 26, and 27 in porpoises, dolphins, and whales (Whatmore et al. 2007, Zygmunt et al. 2010, Cloeckaert et al. 2011, Wu et al. 2014, 2017) and to determine if different *B. ceti* STs are associated with specific lesions in affected cetaceans.

## 2. MATERIALS AND METHODS

### 2.1. Initial *Brucella* molecular testing

*Brucella*-positive cetaceans (n = 88) were selected from animals received by the Zoological Pathology

Program Molecular Diagnostic Laboratory (MDL) at the University of Illinois between 2011 and 2017 for *Brucella* sp. PCR diagnostic testing. Selected animals were those with a known PCR-positive *Brucella* sp. test result on at least one tissue or fluid sample. Initial molecular testing for the presence or absence of *Brucella* within submitted samples was previously performed using the 16S rRNA gene semi-quantitative real-time PCR as described by Colegrove et al. (2016a). Samples from stranded cetaceans along the East Coast (EC; n = 30), Gulf of Mexico (GoM; n = 51), and Hawaii/West Coast (WC; n = 7) of the USA represented 6 species from the Odontocete suborder: *Tursiops truncatus* (*T.t.*; n = 73 animals), *Delphinus delphis* (*D.d.*; n = 7 animals), *Stenella coeruleoalba* (*S.c.*; n = 3 animals), *Lagenorhynchus obliquidens* (*L.o.*; n = 2 animals), *Phocoena phocoena* (*P.p.*; n = 1 animal), and *Physeter macrocephalus* (*P.m.*; n = 2 animals). In all animals, postmortem examination and sample collection were performed by members of the US Marine Mammal Stranding Network (MMSN) in the field or at participating institution laboratories. For each stranded cetacean, the authorized responding agency completed a Marine Mammal Stranding Report — Level A Data form (NOAA Form 89-864; OMB No. 0648-0178; https://www.fisheries.noaa.gov/national/marine-life-distress/level-data-collection-marine-mammal-stranding-events), which includes details such as demographics, stranding location, carcass condition, and signs of human interaction. Samples collected during necropsy were frozen at −80°C or shipped fresh to the MDL for testing. Available archived tissues and fluids (n = 163) from the selected animals included adrenal gland, bone, brain, cerebrospinal fluid (CSF), colostrum, epididymis, heart, joint fluid, kidney, liver, lung, lymph nodes, skin lesions, spinal cord, spleen, testes, umbilical cord, urine, uterus, and abscesses from various tissues. DNA extractions were performed using a DNeasy blood and tissue extraction kit (QIAGEN). The same DNA extract aliquot was used for all assays.

### 2.2. MLST typing

To allow for comparison between IS711 type and the 9-locus MLST, both assays were first completed on a subset of 18 samples from 18 *T.t.* selected based on adequate quality DNA. For each sample, a total of 9 reactions were performed using previously described methods (Whatmore et al. 2007). DNA extracts (2.5 µl) were added to a reaction volume (22.5 µl) containing standard amounts of GeneAmp reagents (Applied Biosystems), 25 pmol of each primer, and 1.5 mM of $MgCl_2$. Amplification cycling parameters were an initial stage at 95°C for 10 min followed by denature, anneal, and extension at 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and 72°C for 5 min (GeneAmp™ PCR System 9700 thermocycler). No template control (NTC; $dH_2O$ substituted for DNA extract) and positive controls (*B. canis* DNA extract, generously provided by National Veterinary Services Laboratories) were included in each set of reactions. PCR amplicons were separated via 1.5% agarose gel electrophoresis and purified using ExoSAP-IT (USB Corporation). Amplicons were sent for bi-directional Sanger sequencing performed on an Applied Biosystems 3730XL 96-capillary sequencer (University of Chicago Comprehensive Cancer Center DNA Sequencing and Genotyping Facility). Forward and reverse sequences were aligned using Geneious v.11.1.5 software (Biomatters) and compared with available published sequences for *B. ceti* ST 23, ST 26, and ST 27 and to each other to determine genotype.

### 2.3. IS711 typing

Sequence typing for *Brucella* samples (n = 163) was then performed on the IS711 SYBR qPCR, including the 18 samples for which the 9-locus MLST was performed. The IS711 SYBR qPCR was performed using previously described protocols (Wu et al. 2017). DNA extracts (2.5 µl) were added to a total PCR volume (25 µl) that contained 1 µM of forward primer (ST23F, ST26F, or ST27F) and 1 µM of STGenR primer reaction$^{-1}$ along with a standard volume (12.5 µl) of Power-SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific). Amplification cycling parameters were 50°C for 2 min and 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min, and a dissociation stage cycle at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s (Applied Biosystems Model 7300 real-time PCR system). Negative NTC (sterile $dH_2O$) and positive controls were tested in each assay. A sample was considered positive if the amplification curve cycle threshold ($C_t$) was ≤38.0 in at least 2 of 3 replicates and dissociation melting temperature was <1°C from respective positive controls (ST 23: 84°C; ST 26: 79°C; ST 27: 82.6°C). The IS711 ST of positive controls were verified using *B. ceti* 9-locus MLST and confirmed via Sanger sequencing from aligned IS711 PCR amplicons (Zygmunt et al. 2010, Cloeckaert et al. 2011, Wu et al. 2017).

### 2.4. Histologic evaluation

To compare ST with specific lesions, histopathology findings were reviewed in a subset of animals (n = 62), including *T.t.* (n = 56) and *D.d.* (n = 6). Animals were included for review if there was a histopathology report or Hematoxylin & Eosin (H&E)-stained slides available for analysis, there was suspicion of *Brucella* sp. infection by the initial reporting pathologist based on characteristic lesions, and if *B. ceti* ST could be determined in at least one tissue. Animals were excluded due to lack of histopathology available for review, lack of confirmatory ancillary diagnostics, or low numbers of species (<5 animals; *S.c.*, *L.o.*, *P.p.*, and *P.m.*) represented in the group for adequate statistical comparison. Age groups were classified as perinatal (P; n = 36) and non-perinatal (NP; n = 26) based on previous guidelines by Colegrove et al. (2016a); perinatal *T.t.* were <115 cm in total body length and had one or more perinatal histological and/or gross characteristics (fetal atelectasis, fetal folds, rostral hairs, hair follicles, un-erupted teeth, prominent lingual papillae, nuchal dent, dorsal fin folded and/or creased, caudal blow hole dent, patent ductus arteriosus, presence of an umbilicus). *T.t.* perinatal total body lengths ranged from 40.75–114.3 cm. Subadults and adults were classified as NP based on total length and lack of any perinatal characteristics. All cases included in the histopathology portion were re-evaluated by the same American College of Veterinary Pathologists board-certified veterinary pathologist (J.B.C.). During routine histopathologic evaluation, the type of inflammation, chronicity, severity, and etiology (bacterial, viral, protozoal, parasitic, fungal, trauma, toxic, human interaction, unknown) were re-assessed. In data analysis, a lesion was categorized as *B. ceti*-associated (primary pathogen or co-pathogen) if there was presence of the bacteria in cultured isolates, a positive result in at least one of the PCR assays, and/or lesion characteristics were consistent with *B. ceti* pathology as previously described in the literature (González-Barrientos et al. 2010, Nymo et al. 2011, Guzmán-Verri et al. 2012, Colegrove et al. 2016a, Buckle et al. 2017, Sánchez-Sarmiento et al. 2019). In perinates, *in utero* pneumonia was classified based on the presence of inflammation, partial or complete atelectasis, presence of at least alveolar squamous cells or meconium, and absence of lungworms (Colegrove et al. 2016a). Stranding and medical histories, demographic and morphometric data, postmortem images, and postmortem diagnostics were reviewed where available and recorded for each case. In the majority of animals, cause of death was determined by gross and histological examination, with confirmatory bacteriology and/or molecular testing, and special histochemical staining (Gram, acid-fast, Periodic Acid-Schiff, Grocott-Gomori's Methenamine Silver) incorporated where indicated. Immunohistochemistry using a rabbit polyclonal antibody to *B. abortus* was performed on lung tissue of 3 perinates, as described in Colegrove et al. (2016a). Cetacean morbillivirus PCR was completed on 26 of 62 animals, using methods previously published. Animals that were diagnosed with morbillivirus infections had PCR-positive tissue(s) for cetacean morbillivirus and histologic lesions consistent with the viral infection per previously utilized criteria (Fauquier et al. 2017). Other viral infections (herpesvirus, influenza) in a small number of animals (n = 5) were ruled less likely based on negative PCR testing. Bacteriology was performed on the majority of animals with isolation of *Brucella* sp. from 27 of 62 animals. In the remainder of the animals (the majority recently aborted perinates), either carcass condition or poor sampling protocols prohibited meaningful bacteriology.

### 2.5. Statistical analyses

Results of the IS*711* qPCR were compared to sequence typing from the 9-locus MLST, considered the gold standard for purposes of this study. For comparison among independent variables, bivariate and multivariate logistic regression analyses were utilized for ST-specific *B. ceti* infection lesion associations. All data points had binomial categorization according to Field ID (*y*-axis) and variables of ST, age group, location, species, and organ inflammation (*x*-axis) (Table S1 in the Supplement at www.int-res.com/articles/suppl/d148p057_supp.pdf). Morbillivirus infection status was also compared. Data analysis was performed using R v.3.6.0 (R Core Team 2019). Logistic regressions were performed to evaluate for any significance of association between the variables at a cutoff of $p < 0.05$. A manual backward stepwise regression was used to sequentially eliminate variables using information-theoretic model selection and the Akaike information criterion (AIC). Meaningful pairwise and 3-way interactions were assessed between ST and the model variables. In multiple different iterations of the model, age group, sex, species group, location groups, and lesion types were controlled. Co-linearities existed in the data between the ST 27 type and age groups (perinates). ST 23 positives were in low numbers

(n = 3); thus, comparisons were limited due to small sample size.

## 3. RESULTS

### 3.1. IS711 SYBR qPCR

There was 100% concordance between the 9-locus MLST results and IS711 SYBR qPCR identification of ST; however, the IS711 qPCR for 3 samples were positive for both ST 26 and ST 27 (Tables 1 & S1). The single ST 23 sample on the 9-locus MLST did not amplify on the IS711 qPCR. Using serial dilutions, the IS711 SYBR qPCR test had an analytical efficiency (for assay ≥ 92%), correlation coefficient (0.99 for all 3 STs), and minimum detection rate (0.23 fg genomic DNA per PCR reaction) similar to published data.

On the IS711 PCR, ST 26 (n = 20) and ST 27 (n = 33) were identified in *T.t.* Dual ST 26–ST 27 infections were detected in 9 *T.t.* (Table 2). No *T.t.* were identified with ST 23, and 11 *T.t.* were negative for all 3 STs tested. Among *D.d.*, 1 was ST 23-, 2 were ST 26-, and 1 was ST 27-positive. One *D.d.* was negative for all 3 STs tested in all tissues tested. The 2 *D.d.* dual infections had STs in separate tissues (ST 27 in brain and testes; ST 23 in spleen). Of the 3 *D.d.* from the EC with histologic neurobrucellosis, one was PCR-positive for ST 23 in the brain, though ST 23 was detected in the spleen of all 3 animals. All *S.c.* (n = 3) and *L.o.* (n = 2) animals were negative for the 3 STs despite being positive on the 16S qPCR. One *P.p.* animal was positive for ST 23, and *P.m.* perinates were positive for ST 27.

In total, 11 animals had multiple STs according to IS711 testing, either within a single tissue or in multiple tissues (11 of 88; 12.5%) (Table 2). In both dual-infected *D.d.* adults, ST 23 was detected in spleen, while brain and testicle were ST 27-positive. Nine *T.t.* tested positive for ST 26 and ST 27 in one or more tissues. Of these individuals, 6 perinate *T.t.* had both ST 26 and ST 27 identified in lung or lung lymph node. The remaining 3 *T.t.* animals were adults, with both ST 26 and ST 27 identified in neural tissues or CSF. The 9-locus MLST was only successful in one perinate (lung) and 2 adult (spinal cord) *T.t.* cases; the perinate lung typed to ST 27 and the spinal cord samples typed to ST 26 (Table 1). In these 3 samples with multiple ST-positive results on the IS711 assay, the MLST sequence data had multiple peaks (double ladders) in specific genetic regions, predominately in *gap* and *trpE* housekeeping genes, suggesting possible mixed sequences.

Table 1. Concordance of the insertion sequence 711 (IS711) quantitative PCR to the 9-locus multi-locus sequence typing (MLST) PCR for *Brucella ceti* sequence types (STs) 26 and 27 (n = 18). Note 3 animals in parentheses which had double positive results on the IS711 despite being recognized on the 9-locus MLST as a single type

| 9-gene MLST type | IS711 PCR type | |
|---|---|---|
| | ST 26 | ST 27 |
| ST 26 | 8 | (2) |
| ST 27 | (1) | 10 |

### 3.2. Lesions according to *B. ceti* ST

#### 3.2.1. Pathology in adults/subadults (NPs)

In NP dolphins with *B. ceti* amplification by PCR (n = 26), there were no significant statistical associations between lesion type and species groups, location, or morbillivirus infection status. The majority of NP animals had meningoencephalitis (18 of 24, 75%), and close to half had myocarditis (11 of 25,

Table 2. Species included in molecular characterization of *Brucella ceti* according to insertion sequence 711 (IS711) sequence types (STs) 23, 26, and 27

| Species | IS711 PCR type | | | Dual | Dual | no ST | Total |
|---|---|---|---|---|---|---|---|
| | ST 23 | ST 26 | ST 27 | ST 23 and 27 | ST 26 and 27 | identified | n |
| *Tursiops truncatus* | 0 | 20 | 33 | 0 | 9 | 11 | 73 |
| *Delphinus delphis* | 1 | 2 | 1 | 2 | 0 | 1 | 7 |
| *Stenella coeruleoalba* | 0 | 0 | 0 | 0 | 0 | 3 | 3 |
| *Lagenorhynchus obliquidens* | 0 | 0 | 0 | 0 | 0 | 2 | 2 |
| *Phocoena phocoena* | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Physeter macrocephalus* | 0 | 0 | 2 | 0 | 0 | 0 | 2 |
| | | | | | | | 88 |

44%) and reproductive tract inflammation (12 of 25, 48%) (Table 3). A minority of NP animals had various other lesions including lymphadenitis (7 of 26, 27%), pneumonia (5 of 26, 19%), arthritis (7 of 25, 28%), vasculitis (6 of 25, 24%), and nephritis (4 of 25, 16%). All *B. ceti*-positive lesions in NP dolphins had some degree of histologic chronicity (Table S2). *B. ceti* was determined to be the primary cause or contributor to death in 12 animals (12 of 26, 46%), the majority having meningoencephalitis (11 of 12, 92%). In the remaining NP animals (14 of 26, 54%), *B. ceti* infection was considered a co-pathogen, with morbillivirus infection also likely playing a role in death in many of these cases (11 of 26, 42%). Parasitic infections (especially lungworms) were very common; however, parasitic infection was indicated as a contributor to death in only 2 cases.

The majority of NPs with *B. ceti*-associated meningoencephalitis (neurobrucellosis) tested positive for ST 26 (13 of 18, 72%). Dual ST infections were detected in 4 animals with meningoencephalitis: 2 *T.t.* with ST 26 and ST 27 and 2 *D.d.* with ST 23 and ST 27. The 2 *T.t.* had both STs in neural tissue (spinal cord, brain, CSF); the third *T.t.* had dual ST 26–ST 27 in spinal cord tissue but no spinal cord was saved for histopathology. In the multivariate logistic regression, meningoencephalitis had the strongest association with a specific ST (ST 26) when controlling for species ($p = 0.009$) (Table S1). Meningoencephalitis remained significant regardless of species, age, location, or morbillivirus infection in ST 26 animals ($p = 0.02$). The majority of *B. ceti*-associated neurologic lesions were primarily lymphoplasmacytic meningitis (15 of 18, 83%), ranging from minimal to severe (Fig. 1A,B). Fewer animals (12 of 18, 67%) had lymphoplasmacytic encephalitis; 10 of which also had spinal cord inflammation (myelitis). Inflammation involving the ependyma and/or choroid plexus (ventriculitis) was present in fewer cases (3 of 18, 17%) (Fig. 1A,B), though sections including this portion of the brain were not always available for evaluation. Most meningoencephalitis was moderate to severe. Of the total animals with meningoencephalitis, 6 of 18 were co-infected with morbillivirus (33%). Morbillivirus infection status was not significant when added to the ST 26 and ST 27 analyses ($p = 0.993$ and $p = 0.996$, respectively).

Close to half (12 of 25, 48%) of the stranded adult (i.e. NP) animals with *B. ceti* had some degree of reproductive tract inflammation (Table 3). In animals with reproductive tract inflammation and reproductive tissue available for testing, 4 animals had ST 26 (4 of 12, 33%), and 2 cases were ST 27-positive (2 of 12, 17%) within the tissue. In the 6 other cases, ST 26 was detected in joint fluid or neural tissue; reproductive tissue was not available for testing by PCR. In one *D.d.* with a co-infection, ST 27 was detected in the testis and ST 23 detected in the spleen. One *T.t.* male with chronic *B. ceti* orchitis had detection of both ST 26 and ST 27 in the brain and CSF tissues. Of the adults with reproductive tract inflammation, 4 were females (4 of 12, 33%), all of which typed to ST 26, including 2 *T.t.* and 2 *D.d.* All 4 females were from the US EC. Three of 4 had mild to moderate lymphoplasmacytic or granulomatous endometritis (Fig. 1C). One female *D.d.*, pregnant with a 45 cm long mid-gestation fetus, had a mild lymphoplasmacytic oophoritis but no evidence of endometritis. Chronic orchitis was noted in males with all 3 STs. Testicular lesions in half the *B. ceti*-positive males (8 of 16, 50%) were characterized as mild to severe lymphoplasmacytic orchitis. The more chronic tes-

Table 3. Lesions identified in *Brucella ceti*-positive dolphins. Note that *B. ceti* pneumonia is largely represented by perinatal infections. The majority of adults with *B. ceti* had neurobrucellosis. Close to half of infected adults also had myocarditis and/or reproductive tract inflammation

| Lesion | All dolphins | Perinates | Adults |
|---|---|---|---|
| Pneumonia | 38 of 62 (61%) | 33 of 36 (92%) | 5 of 26 (19%) |
| Bacterial pneumonia | 5 of 62 (8%) | 0 of 36 (0%) | 5 of 26 (19%) |
| *In utero* pneumonia | 33 of 62 (53%) | 33 of 36 (92%) | 0 of 26 (0%) |
| Encephalitis (±meningitis/myelitis) | 18 of 38 (47%) | 0 of 14 (0%) | 18 of 24 (75%) |
| Myocarditis (±epi-/endocarditis) | 12 of 53 (23%) | 1 of 28 (4%) | 11 of 25 (44%) |
| Vasculitis | 6 of 61 (10%) | 0 of 36 (0%) | 6 of 25 (24%) |
| Reproductive inflammation | 12 of 47 (26%) | 0 of 22 (0%) | 12 of 25 (48%) |
| Arthritis | 7 of 47 (15%) | 0 of 22 (0%) | 7 of 25 (28%) |
| Lymphadenitis | 8 of 46 (17%) | 1 of 20 (5%) | 7 of 26 (27%) |
| Nephritis | 4 of 49 (8%) | 0 of 24 (0%) | 4 of 25 (16%) |
| Chronic inflammation | 26 of 62 (42%) | 0 of 36 (0%) | 26 of 26 (100%) |

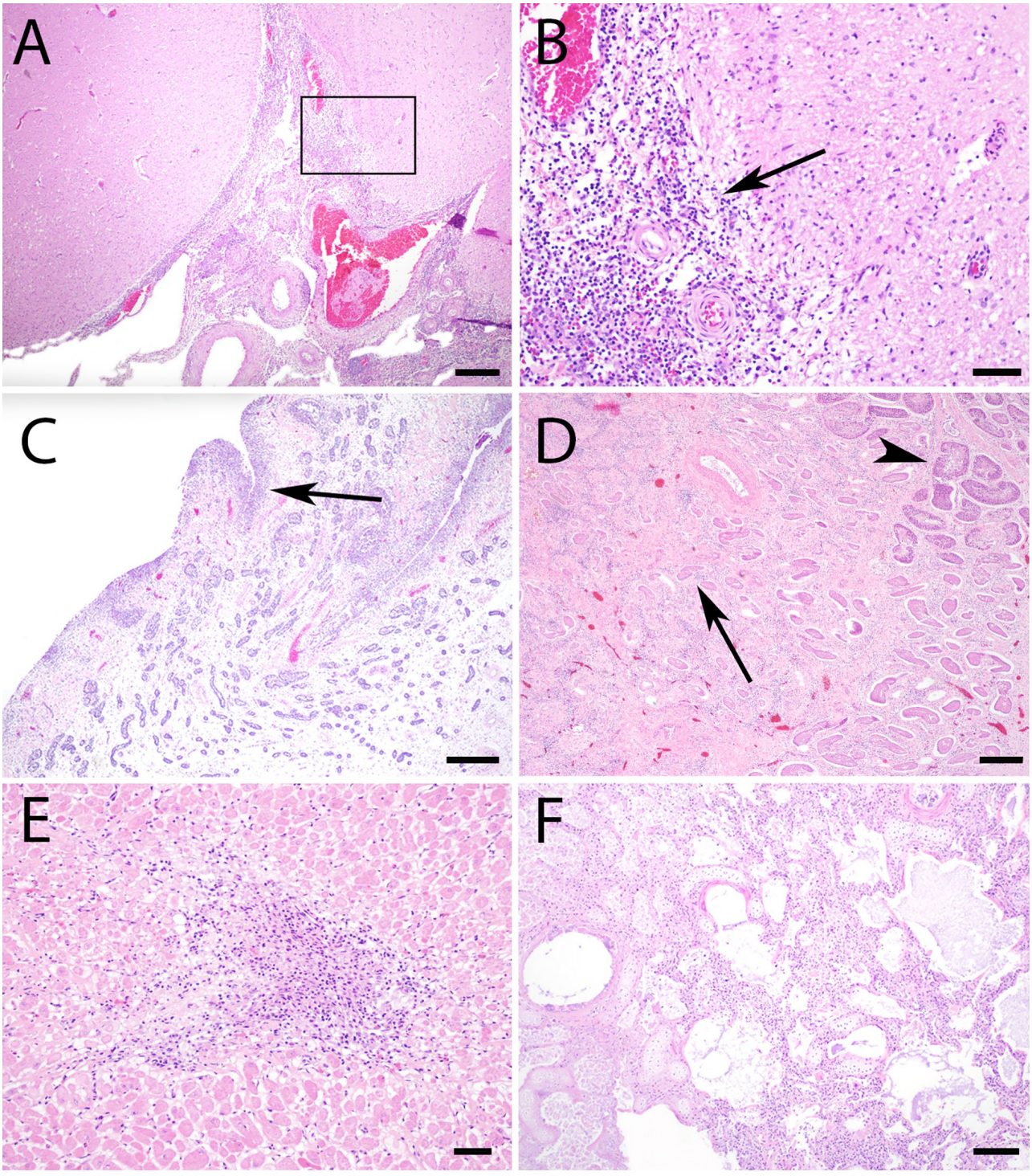


Fig. 1. Photomicrographs of *Brucella ceti*-associated lesions in dolphins, Hematoxylin & Eosin (H&E). (A) Cerebrum and lateral ventricle in an adult dolphin with severe nonsuppurative meningitis and ventriculitis (box), ST 26 infection. Scale bar = 200 µm. (B) Higher magnification of the area within the box in (A). Severe lymphocytic inflammation (arrow) of the ependymal lining (ventriculitis). Scale bar = 50 µm. (C) Female with lymphocytic endometritis in superficial mucosa (arrow), ST 26 infection. Scale bar = 200 µm. (D) Chronic lymphocytic orchitis with fibrosis, and seminiferous tubular loss and atrophy (arrow) and remnant normal tubules (arrowhead), animal with dual ST 26–ST 27 infection. Scale bar = 200 µm. (E) Suppurative and histiocytic myocarditis with myocardial loss and fibrosis. Scale bar = 50 µm. (F) Perinatal lung with severe suppurative and histiocytic, necrotizing interstitial pneumonia, ST 27 infection. Scale bar = 100 µm

ticular lesions had moderate to severe lymphocytic orchitis with fibrosis and seminiferous tubule loss and atrophy (Fig. 1D). Epididymitis, urethritis, and vasitis were noted only in ST 26 males (4 of 8).

For adults with heart tissue available for evaluation, 11 of 25 (44%) had myocarditis, though no heart or pericardial fluid was available for PCR testing. Among the myocarditis cases, the majority were positive for ST 26 in other tissues (9 of 11, 82%). This lesion had statistical association with *B. ceti* infection, with significance ($p = 0.02$) in the age class and location-controlled model (Tables 3 & S1). Two *T.t.* with myocarditis that were positive for ST 26 also were positive for ST 27. One *D.d.* with myocarditis had a dual infection with ST 23 and ST 27, and one *D.d.* had only ST 27 identified in other tissues. The degree of myocarditis ranged from mild to severe, with the majority having evidence of chronic degenerative changes (10 of 11, 91%), including myocardial and valvular degeneration, myocardial fibrosis, or some combination of these lesions. Myocarditis and pericarditis lesions were most commonly chronic and lymphoplasmacytic (9 of 11, 81%). Two animals with chronic inflammation also had a histiocytic and suppurative component with vasculitis or abscessation suggestive of an active infection/sepsis (Fig. 1E). Half of the animals with heart lesions had focal to multifocal vasculitis, which ranged from lymphoplasmacytic in large-caliber arteries (aorta) to suppurative and histiocytic inflammation with fibrinoid arteritis in smaller arteries (adrenal gland/spinal cord).

Joint lesions were common in adults (10 of 25, 40%), with PCR confirmation on joint fluid in most of these cases (7 of 25, 28%). All were *T.t.* and ST 26-positive. Chronic fibrinosuppurative to granulomatous arthritis was most often in the scapulohumeral joint (6 of 7), followed by the occipital joint (1 of 7) in PCR-confirmed cases. A single *D.d.* adult with severe fibrinosuppurative arthritis and synovitis was ST 23-positive in brain, spleen, and skin lesions, though no joint tissue or fluid was available for testing. Small intralesional coccobacilli were detected in this joint lesion, suggestive of an active infection.

Other chronic lesions in *B. ceti*-positive cetaceans included lymphadenitis, pneumonia, dermatitis, nephritis, and mastitis. Over one-quarter of the adult *T.t.* and *D.d.* dolphins with *B. ceti* (7 of 26, 27%) had lymphadenitis (Table 3), most of which were granulomatous lesions (6 of 7). Nearly all non-perinate dolphins had some degree of pneumonia on postmortem examination (24 of 26, 92%), but in the majority of animals, morbillivirus infection, verminous pneumonia, and/or aspiration pneumonia was considered to be the primary cause of the inflammation. Over half of lungs had the presence of lungworms or trematode ova (14 of 26, 54%). Of the 5 NP *T.t.* with PCR-positive *B. ceti* lung tissue, all were ST 26, and the majority had lungworms (4 of 5, 80%), though the inflammation type was mixed and the primary cause of pneumonia was multifactorial. Most of these animals had moderate, chronic, suppurative and/or histiocytic bronchopneumonia with interstitial and sometimes pleural fibrosis. Dermatitis was reported in 17 of 26 animals (65%), the majority of which were diagnosed as primary lesions of trauma, viral, parasitic, or protozoal infection. A single *D.d.* with suspect proliferative poxviral skin lesions also had an ulcerative, pustular, bacterial dermatitis with dermal vasculitis containing fibrin thrombi, intralesional cocci, *Brucella* sp. isolated on culture, and detection of *B. ceti* ST 23. Though no other cases had skin available for testing, other suspect bacterial dermatitis lesions had one or more characteristics: intralesional small cocci/coccobacilli, suppurative and/or granulomatous inflammation with abscessation, ulceration, or evidence of chronicity including dermal fibrosis. Other lesions in *B. ceti*-positive animals lacked bacteriology or PCR detection to make any meaningful comparisons.

#### 3.2.2. Perinate bottlenose dolphins

In the P dolphins (n = 36), the majority (33 of 36, 92%) had *in utero* pneumonia (Table 3) and all P tissues were positive for ST 27, despite most being culture-negative. Perinates stranded in both the GoM (n = 34) and EC (n = 2). Six of the 36 perinates had dual infections, with amplification for ST 26 in lung or lung lymph node in addition to the amplification of ST 27 (Table S2). ST 27 was significantly associated with *in utero* pneumonia ($p = 0.035$) according to multivariate logistic regression analysis. Most *in utero* pneumonias were characterized as necro-suppurative, histiocytic interstitial pneumonia (Fig. 1F) with inflammatory cells and amniotic squamous cells within alveolar spaces consistent with a fetal distress reaction and similar to lesions previously described in perinatal dolphins (Colegrove et al. 2016a). Other lesions noted in *Brucella*-positive perinates included a necro-suppurative lymphadenitis (n = 1), omphalitis (n = 2), perivascular dermatitis (n = 3), and suppurative myocarditis (n = 1). No perinates had central nervous system (CNS) or joint inflammation.

## 4. DISCUSSION

### 4.1. Sequence types and disease

*Brucella ceti*-associated lesions in this retrospective study were overall typical of those previously reported in cetacean brucellosis (Foster et al. 2002, Nymo et al. 2011, Guzmán-Verri et al. 2012); however, there were multiple significant findings obtained using a multivariate analysis according to *B. ceti* STs. In stranded perinatal dolphins, the majority of *B. ceti*-positive animals had *in utero* pneumonia and infection with ST 27. This further supports the strong abortive potential of *B. ceti* ST 27. While ST 27 was less frequently identified in adults, the finding of ST 27 in the testes of 2 males provides further evidence for reproductive tract tropism, as observed with other STs. Thus, ST 27 *B. ceti* strains may impact recruitment in addition to the potential for zoonosis (Sohn et al. 2003, McDonald et al. 2006, Whatmore et al. 2008). In contrast, most non-perinatal dolphins had chronic, frequently multi-organ infections with either ST 26 or ST 23. Neurobrucellosis in *D.d.* from an ST 23 infection indicated that this ST can cause disease in cetacean families other than Phocoenidae. Most non-perinate *T.t.* were typed to ST 26 and had neurobrucellosis. These and other case reports are further suggestive of a CNS tropism for certain *B. ceti* STs in multiple members of Delphinidae (Alba et al. 2013, Davison et al. 2013, Sierra et al. 2019). Cetaceans have at least 2 distinct manifestations of *B. ceti* infection: a chronic form of infection that can affect multiple tissues resulting from non-ST 27 infections and an abortigenic form caused primarily by ST 27.

#### 4.1.1. ST 27-associated lesions

For over 3 decades, *Brucella* has been detected in captive and free-ranging dolphin populations across the globe, with its abortive potential widely known (Ross et al. 1994, Ewalt et al. 1994, Miller et al. 1999, Guzmán-Verri et al. 2012, Colegrove et al. 2016a, Buckle et al. 2017, Mackie et al. 2020). This is not dissimilar to other *Brucella* species, where abortions and reproductive tract tropism are common (Olsen & Palmer 2014). In past case reports, marine strains with abortive potential were attributed with increased virulence and the ability to spread to fetal tissues, though typing was not available at the time of those initial reports (Miller et al. 1999). Buckle et al. (2017) found ST 27 from the uterus of a pregnant female Hector's dolphin *Cephalorhynchus hectori* with necrosuppurative metritis and placentitis. More recently, Mackie et al. (2020) found ST 27 in a stillborn *T.t.* calf with placentitis and encephalitis, and Hernández-Mora et al. (2021) found ST 27 in a stranded pregnant female dwarf sperm whale *Kogia sima*. The dwarf sperm whale aborted a late-term fetus and subsequently died; necrotizing placentitis and encephalitis in the cow were observed. Given the abortigenic potential of ST 27, it was interesting to find this same ST in the testes of 2 *D.d.* males and in neural tissues of a *T.t.* male, all 3 of which had orchitis. These and other cases contribute to the growing evidence of reproductive tropism of this ST. Information on female ST 27 infections could not be extrapolated from this data set and is an area for further study.

Based on these data, ST 27 was not frequently associated with chronic infections in adults, though rarely was identified as the cause of neurobrucellosis and reproductive lesions. Certain *Brucella* types, potentially including ST 27 strains in cetaceans, may have the ability to remain latent within macrophages and/or dormant within the lympho-reticular system until activation during late pregnancy with trophism for the pregnant uterus, placenta, and even to cerebral tissues in more severe cases (Meegan et al. 2012, Olsen & Palmer 2014, Mackie et al. 2020, Hernández-Mora et al. 2021). Infected females may be able to carry ST 27 with a high rate of abortion but low morbidity in most cases, as noted in *B. abortus* infections in terrestrial species. The high *Brucella* sp. seropositivity rates in free-ranging cetaceans further indicate that certain strains (i.e. ST 27) may lead to subclinical or mild infections in the dam and intra-cellular survival until late in gestation followed by abortion, similar to what is noted with *B. abortus* in hoof stock (Samartino & Enright 1993, Meegan et al. 2012, Baldi & Giambartolomei 2013). It is also possible that abortions due to ST 27 do not result in longstanding uterine damage or inflammation in pregnant females, which would support the finding that some dolphins have had successful pregnancies following *Brucella* abortions (Meegan et al. 2012). A single pregnant female dolphin was available for analysis in this study. That female *D.d.* died while in mid-gestation, and while uterine and other tissues were positive for ST 26, there was no evidence of endometritis or placentitis. Reproductive tract tissues and regional lymph nodes are not frequently sampled for *Brucella* sp. PCR screening; thus, more consistent and frequent sampling of reproductive tract tissues and associated lymph nodes will be needed to better understand tissue tro-

pism, transmission, and latency of ST 27 and its abortigenic role.

Fetal pneumonia caused by *Brucella* is consistently reported as the most common lesion in bovine abortions from *B. abortus* (Sözmen et al. 2004). In the present study, most aborted dolphins had *in utero* pneumonia with ST 27, which is supportive for gestational brucellosis. *Brucella in utero* pneumonia is thought to occur from either bacterial sepsis and consequent pneumonia or from a primary pneumonia due to aspiration of infected amniotic fluid and debris (Colegrove et al. 2016a, Buckle et al. 2017). Placental infection leading to *Brucella* pneumonia and sepsis seems most likely in these animals, though placental tissue was not available for examination. The general tropism of *Brucella* to bovine and human fetal trophoblasts has been observed in terrestrial *Brucella* species (Samartino & Enright 1993, García-Méndez et al. 2019), and likely a similar pathogenesis exists for cetaceans (González-Barrientos et al. 2010, Mackie et al. 2020, Hernández-Mora et al. 2021). Milk was seldom available for PCR testing, though a female with a positive *Brucella* PCR on milk lacked evidence of mastitis. Still, lactational transmission could be possible for young dolphins (Palmer et al. 1996, Foster et al. 2002, Davison et al. 2011). Moreover, ST 27 may be able to cause neonatal death and abortions across all odontocete species given the positive PCR results in 2 sperm whale calves in this study and a recent report of gestational death in a dwarf sperm whale cow/calf (Hernández-Mora et al. 2021). Efforts to collect and test placentas and milk from future stranded animals could increase our knowledge of these pathways.

#### 4.1.2. ST 26-associated lesions

The most common finding in dolphins with ST 26 brucellosis was meningoencephalitis. Under certain environmental stressors and immunosuppressed statuses, ST 26 appears to have the ability to cause a wide range of lesions, yet with a tropism for neural tissues, largely the brain. Predilection for the CNS is not common to other human and animal *Brucella* infections but is commonly reported in cetacean species (González-Barrientos et al. 2010). Non-suppurative meningoencephalitis with predominately meningeal inflammation was typical of positive lesions, similar to previous reports (González et al. 2002, Hernández-Mora et al. 2008, Guzmán-Verri et al. 2012, Alba et al. 2013, Sierra et al. 2019, Davison et al. 2021). The mononuclear nature of marine brucellosis may be from a cell-mediated predominate response, as *Brucella* sp. do not have the ability to secrete proteases, lytic enzymes, or toxins leading to cell death (Baldi & Giambartolomei 2013). Chronic cell-mediated inflammation and intracellular persistence typifies lymphoplasmacytic (mononuclear) and sometimes granulomatous inflammation. In 3 of the animals with moderate to severe non-suppurative meningoencephalitis, there was also severe choroiditis and ventriculitis (González et al. 2002, Dagleish et al. 2007, Hernández-Mora et al. 2008, González-Barrientos et al. 2010, Davison et al. 2015, 2021). While speculative, the caudo-ventral- and ventricle-oriented inflammation may lead to decreased CSF uptake, blockage, or potentially a combination of both, and consequent hydrocephalus; a lesion which has been sporadically reported in Cetacea (and rarely in humans) with neurobrucellosis (Guney et al. 2008, Davison et al. 2015, 2021). While *Brucella*-associated CNS inflammation in most cases was associated with significant meningeal inflammation, it should be noted that some animals with less severe inflammation in the brain parenchyma had non-specific multifocal lymphocytic encephalitis. In these animals, it would be difficult to differentiate between *Brucella* sp., viral (i.e. morbillivirus or herpesvirus infection), and protozoal infections based on the lesions alone, emphasizing the need for ancillary pathogen testing.

Although ST 27 was associated with fetal *in utero* pneumonia and abortion, female dolphins with reproductive tract inflammation had detection of ST 26. As animals with ST 26 infections had multi-organ infections, reproductive tissues may be affected with this ST more chronically as part of systemic spread of the bacterium. The single pregnant *D.d.* that died from *Brucella*-related complications had a mid-gestation fetus. The fetus had no significant histological lesions, yet *B. ceti* ST 26 was detected in the uterus, brain, and other tissues, indicating a difference between manifestations of abortogenic ST 27 infections and chronic systemic ST 26 infections sometimes affecting reproductive tissues. Whether ST 26 infection of the uterus alone can cause abortion in pregnant dolphins is unknown, though the chronic inflammation noted could potentially lead to decreased fertility. Some STs, specifically ST 26 and ST 23, may be associated with fetal loss as a bystander effect in sick dams with systemic infections. The majority of male animals with reproductive tract inflammation also had *B. ceti* detected via PCR in submitted brain or spinal cord tissues. Animals with orchitis had no ST specificity; however, epididymitis was only associated with ST 26 infection. ST 26 epi-

didymitis may serve as an example of a unique tissue tropism similar to that observed in some terrestrial species, such as *B. ovis* epididymitis in rams (Foster 2016b). Males with chronic lesions noted in this and other studies in many cetacean species are likely serving as *Brucella* carriers and vectors for horizontal transfer to females during coitus (Ohishi et al. 2003, Dagleish et al. 2008, Davison et al. 2011). More research is needed to understand the importance of ST 26 infection in reproductive tissue damage and potential infertility.

#### 4.1.3. ST 23-associated lesions

All ST 23 animals were adult male *D.d.* that stranded along the US northeast coast. Though associations were difficult to evaluate due to the small sample size and location bias, this group of affected animals also had neurobrucellosis. All 3 males had non-suppurative inflammation in the brain mostly affecting the meninges. This ST may also have a predilection or endemic characteristics for this species along the US northeastern coast. This ST also likely has the ability to cause chronic multi-organ infections in cetaceans other than porpoises, as one of the more chronically infected animals had meningitis, fibrinosuppurative synovitis, and moderate lymphoplasmacytic orchitis. *B. ceti* ST 23 was also detected from the epididymis in a single *P.p.* male in the molecular portion of this project. ST 23 strains and those clustered with ST 23 have previously been reported in other harbor porpoises (i.e. *P.p.*) and in a harp seal *Pagophilus groenlandicus* from New England (Groussaud et al. 2007, Jauniaux et al. 2010, Whatmore et al. 2017). Short-beaked common dolphins have a large range in the Northern Hemisphere and share this range with the smaller harbor porpoise. Cross-species infections may occur in *B. ceti*, with open-ocean species more likely to come into contact with new strains (Davison et al. 2017). Based on the findings in this and other studies regarding ST 23, this ST may cause brain and reproductive tract lesions in both dolphins and porpoises.

#### 4.1.4. Additional lesions and *B. ceti*

Heart was not among the tissues routinely submitted for *Brucella* sp. culture and PCR testing; thus, no associations could be made. Regardless, cardiovascular lesions were noted in multiple cetaceans with brucellosis and there was statistical significance. Most commonly, there was multifocal lymphoplasmacytic inflammation in the myocardium with regions of replacement by fibrosis. In more severe, active infections, there was both endocarditis and epicarditis with histiocytic and suppurative inflammation. *Brucella* myocarditis in dolphins could be similar to humans with *Brucella* infections in which endocarditis has been implicated in death (Jeroudi et al. 1987, Abid et al. 2012, Lagadinou et al. 2019). *B. ceti* has been isolated from a dolphin with a vegetative endocarditis lesion (González-Barrientos et al. 2010). This lesion and other reported heart lesions in animals with *Brucella*-related cardiomyopathy presumably contribute to decreased cardiac function in a species specially adapted for rapid hemodynamic changes (Sánchez-Sarmiento et al. 2019). In this study, half of the animals with myocarditis also had some degree of vasculitis. The ability of *Brucella* to infect endothelial cells and effect a pro-inflammatory state may lead to the vasculo-centrism often noted of inflammation in chronic marine *Brucella* lesions (Baldi & Giambartolomei 2013). In the future, complete postmortem exams should include sampling of heart for *Brucella* testing to confirm infection.

Arthritis is often associated with brucellosis in cetaceans, and in this study, 28% of adults with *B. ceti* infection had arthritis with affected joint fluid commonly PCR-positive. The majority of these animals were infected with ST 26. *Brucella*-associated arthritis demonstrates the high likelihood of *B. ceti*'s capability to infect multiple cell lineages. Infection of osteoblasts with *B. abortus* can cause a cytokine-monocytic storm leading to osteolysis and joint remodeling in chronic cases, which is a similar lesion noted in marine brucellosis (Dagleish et al. 2007, Baldi & Giambartolomei 2013, Sánchez-Sarmiento et al. 2019). Whether arthritic lesions are caused solely by *B. ceti* or the bacterium potentiates pre-existing lesions remains to be determined.

*Brucella*-associated pneumonia in non-perinates was minimal compared to *B. ceti* pneumonia in perinates, and both ST 26 and ST 27 were detected. This lesion in subadults/adults is sporadically reported, and *B. ceti* is often difficult to distinguish as a primary vs. secondary pulmonary pathogen (Guzmán-Verri et al. 2012, Cassle et al. 2013, Sánchez-Sarmiento et al. 2018). As noted in other studies, verminous pneumonia is a common and often incidental postmortem finding in cetaceans that has been described in multiple animals with concurrent *Brucella*-associated bronchopneumonia (Dawson et al. 2008, Jauniaux et al. 2010, Rhyan et al. 2018, Sánchez-Sarmiento et al. 2018). Most *Brucella*-posi-

tive lung samples had nematode parasitism, although in dolphins with verminous pneumonia in this study, no discernable difference was noted in lesions between positive and negative *Brucella* PCR status. Lungworms, *Parafilaroides* sp. and *Pseudalius inflexus*, with convincing *Brucella* organisms have caused granulomatous verminous pneumonia in a Pacific harbor seal and harbor porpoise, respectively (Garner et al. 1997, Dawson et al. 2008, Rhyan et al. 2018). Lungworms certainly may exist as vectors for the bacteria in cetaceans; there was a general lack of specific *Brucella*-associated pulmonary lesions in examined culture and PCR-positive tissues in this study. There is good evidence to suggest that *Parafilaroides* sp. lungworms are the primary host for *B. pinnipedialis* in Pacific harbor seals (Rhyan et al. 2018). If cetacean lungworms were indeed primary hosts of *B. ceti* and not simply vectors for the disease, lesion specificity for lung and lack of lesions in other organs would be expected in cetaceans as it is in pinnipeds, though this supposition is not supported by the findings in this study. *B. ceti*-specific immunohistochemistry and immunofluorescent studies in nematode-infected lung tissue and distant tissue with corresponding bacterial strain or sequence typing would likely aid with this investigation.

Lymphadenitis was not significantly associated with any specific *Brucella* ST, though was frequently noted in *B. ceti*-positive animals, with multiple animals having lymph nodes testing positive on PCR. While lymph node inflammation and necrosis has been reported to be associated with *Brucella* infections, interpretation of these lesions should be done with caution, as a multitude of infectious etiologies can be associated with these lesions. Additional lesions, including most skin lesions and organ abscesses, were presumed due to primary parasitism, viral infection (dolphin morbillivirus, poxvirus, or herpesvirus), or from trauma (skin abscesses) with possible secondary bacterial infections. Though *Brucella* infection and ST could not be statistically correlated to any dermatitis or organ abscesses, leukocyte trafficking of *B. ceti* to pre-existing lesions was considered possible. Despite other possible etiologies, skin lesions were fairly common in animals presumed infected with *B. ceti*. There was frequently evidence of chronic suppurative dermatitis and/or abscessation with regional vasculitis and fibrin thrombi suggestive of a hematogenous origin for inflammation (Foster et al. 2002, González et al. 2002). Future collection and testing of skin lesions could aid in understanding if these lesions are related to *Brucella* infection.

### 4.2. Co-infections

The utility of the IS711 qPCR as a rapid diagnostic assay for these 3 STs may also have benefits in the detection of *B. ceti* type co-infections. Dual strain infections have been reported in other species, including elk *Cervus canadensis*, where *B. abortus* biovar 1 and *B. abortus* biovar 4 were isolated from both cows and calves in a herd with known abortions (Etter & Drew 2006). Dual infections could not be confirmed on the 9-locus MLST, though the findings in this study warrant further investigation. Most potential dual infections were associated with ST 27 and were noted in both perinates and adults. Adults with potential co-infections had ST 27 detected in neural or reproductive tissues and ST 23 or ST 26 detected in spleen or in the same neural tissues (brain, spinal cord, CSF). These adult co-infections suggest that some neurobrucellosis cases in dolphins and porpoises are either potentiated by multi-ST infections, or more likely, lesions are caused by a dominant ST with the second ST detected as a subclinical infection. The presence of multiple STs of *Brucella* within a single individual suggests that cetaceans can be exposed to *Brucella* multiple times throughout life and likely through several different pathways.

### 4.3. Sampling methods

Multiple animals had a positive *B. ceti* qPCR with no corresponding lesions in that tissue. Spleen was most often PCR-positive in the absence of specific lesions. While this may be due to selected sampling for histopathology, *Brucella* can survive within host cells (macrophages, endothelial cells, others) for long periods without causing microscopic lesions (de Figueiredo et al. 2015). Therefore, sampling of the spleen may be warranted when testing for subclinical infection and *Brucella* carriers (Buckle et al. 2017). Comprehensive sampling of reproductive tissues is needed to understand where ST 27 strains reside in adult animals and what role (if any) ST 26 plays in reproductive lesions and fetal loss. Thorough sampling protocols will help pathogenesis investigations of *Brucella* abortion in cetaceans. Routine sampling and PCR testing of heart and skin lesions and testing of lungworms may also provide insight into the involvement of these organs or parasites in *B. ceti* infections. Minimum protocol samples should include CSF, brain, spinal cord, and joint

fluid in adults, and lung, lung lymph nodes, and placenta (where available) in perinates, as these had high positive detection rates among *B. ceti*-positive animals.

### 4.4. Molecular utility of the IS711 SYBR

Concurrent with Wu et al. (2014, 2017), the findings in this study demonstrate that the IS711 ST-specific PCR assays provide a rapid, sensitive method for distinguishing among *B. ceti* STs 23, 26, and 27. However, it should be noted that other STs have been diagnosed in cetaceans and thus could be missed if using this assay alone for molecular diagnosis (Maquart et al. 2009, Whatmore et al. 2017). In this study, 2 species (*S.c.* and *L.o.*) tested negative for the IS711 ST assay despite being positive for *Brucella* on other tests. This finding suggests the current assay may not detect all *B. ceti* STs; thus, future studies could pair culture with other PCR assays to identify optimal diagnostics for different sample types and different species.

One advantage of this assay is the potential to identify co-infections with multiple STs, which was noted in 11 animals in this study. This test also functioned well in samples with poor DNA quality and was able to be used as a surrogate for sequence typing in samples from which culture or MLST were unsuccessful. A recent report suggests this test can also detect *B. ceti* ST 27 DNA in FFPE tissues (Mackie et al. 2020), providing additional options for retrospective studies. While bacterial culture and whole-genome sequencing remain the gold standard for typing *Brucella* within appropriately collected and stored samples, postmortem contamination and/or over-growth are common. In this study, it was not unusual to have negative *Brucella* sp. culture in PCR-positive perinates. Culturing marine *Brucella* strains requires selective media, special incubation parameters, and potentially long culture times (Guzmán-Verri et al. 2012).

### 4.5. Study limitations

The animals included in this project appeared to cluster such that subadult/adult dolphins were more likely to be infected with ST 23 and ST 26 *B. ceti*, and most perinates were infected with ST 27 *B. ceti*. However, the majority of the perinates in this study (34 of 36) stranded in the GoM; thus, age and location were co-linear statistical variables. Additional data from perinates outside the GoM are needed to confirm that ST 27-associated abortions are common in other geographic regions. While there was strong co-linearity between ST 27 and perinatal pneumonia, these perinates were infected from their dam *in utero*. The low numbers of reproductive tissues available for ST typing in this study limited evaluation of potential ST 27 infections in adult reproductive tract tissues. This study took advantage of opportunistic sampling and thus may have missed associations, especially among reproductive and heart tissues and ST. Animals were classified by ST based on the available tissues for molecular testing, which was not the same in all animals. Future designed studies following a standardized and complete sampling protocol for culture, molecular, and histologic evaluation are needed so that comparisons with lesions can be made to STs within affected tissues. Alternatively, the use of *in situ* hybridization to localize bacteria and STs to lesions will be of value to make definitive associations. Underrepresentation of ST 27 in adults limited the conclusions that could be drawn for this age class. Location bias and small sample size also limited conclusions that could be drawn regarding lesions associated with ST 23, and this is an area for needed future study. Another limitation was the over-representation of *T.t.* among the sample groups, although the high numbers of this species in both the perinates and non-perinates gave insight into which STs affect this species. When *T.t.* species type was added to the analysis, certain models were stronger according to the AIC index, including those with pneumonia, myocarditis, and reproductive tract inflammation. This finding suggests that *Brucella* infections in this species statistically favor specific organ systems or are at least have associations to the tissue types affected.

In many cases, pathologists attributed *Brucella* as a secondary infection or a co-morbidity. Underlying immunosuppression due to morbillivirus infection was the most commonly reported co-morbidity; 12 adults in this study were sampled during a known morbillivirus associated mortality event. This did complicate interpretation of some lesions, though sample size was large enough to compare lesions of presumed co-morbidities and primary infections. Furthermore, morbillivirus infection status was included in the statistical analysis. Close to half (42%) of the included adults in this study were positive for morbillivirus via PCR and/or had lesions corresponding to morbillivirus infection (viral syncytial cells, characteristic inclusion bodies ± lymphoid depletion). The wide range of chronic lesions reported in

this study and others, as well as serologic investigations indicative of long-lasting infections, all suggest that many cetacean populations may be persistently infected with *Brucella*. With conditions that cause decreased immune surveillance or function, whether from viral-induced immune-dysfunction (morbillivirus) or localized immune suppression (during pregnancy or other environmental factors), the bacteria is able to spread (Meegan et al. 2012, Venn-Watson et al. 2015, West et al. 2015, Colegrove et al. 2016a, Foster 2016a, Sánchez-Sarmiento et al. 2019, Sierra et al. 2019). Poor cell-mediated immunity and *B. ceti* leukocyte trafficking to susceptible tissues may help explain this seemingly large proportion of morbillivirus and *B. ceti* co-infected animals.

Presumably, the largest limitation was due to the retrospective nature of this study. Carcass condition and lack of comprehensive sample collection at the time of necropsy prohibited meaningful bacteriology as well as molecular and histopathologic characterization in some cases. While PCR was able to be used as a surrogate for detection, some lesions (largely myocarditis, reproductive tract inflammation, and pneumonia) could only be listed as possible associations due to lack of ancillary testing. Furthermore, lack of *in situ* detection methods made eliminating cross-contamination from other organs systems difficult, especially where there were no histological lesions (i.e. splenic tissue).

### 4.6. Summary

ST 26 was most frequently identified in adult dolphins along the US EC with non-suppurative meningoencephalitis, while ST 27 infections were predominately found in perinatal animals from the GoM with evidence of *in utero* pneumonia. Reproductive tract inflammation was detected in both ST 26 and ST 27 adults. Possible organ system tropism points to ST differences in pathogenesis of infection, which has implications for individual and population health assessments. Future investigations should include culture, assays with broader ST detection, *in situ* detection methods, and more thorough sampling and testing protocols, all of which will aid investigation into pathogenesis and potential co-infectivity of different *B. ceti* STs.

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