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NOTE

Congenital defects and herpesvirus infection in beluga whale *Delphinapterus leucas* calves from the Critically Endangered Cook Inlet population

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ABSTRACT: Cook Inlet beluga whales (CIBs) *Delphinapterus leucas* are Critically Endangered and genetically distinct from other beluga populations in Alaska. CIBs are exposed to numerous natural and anthropogenic sources of mortality and morbidity. This study describes congenital defects observed in 2 CIB calves. The first case, an aborted fetus, was characterized by lack of a peduncle and flukes, anorectal and genitourinary dysgenesis, and probable biliary dysplasia. The second case, a male calf, had a perineal groove defect and suspected secondary peritonitis; it also had a systemic herpesvirus infection. Further studies are needed to determine if such defects are due to genetic mutation, infectious diseases, nutritional imbalances, or contaminant exposure.

KEY WORDS: Beluga · Congenital defect · Caudal regression syndrome · Perineal groove

1. INTRODUCTION

Beluga whales *Delphinapterus leucas* in Cook Inlet, Alaska (CIBs), are genetically distinct, geographically isolated, and Critically Endangered (Lowry et al. 2019). CIBs reside in the inlet year-round and are exposed to numerous natural and anthropogenic sources of mortality and morbidity such as killer whale *Orcinus orca* predation, live stranding during extreme tides, malnutrition, disease, pollutants, ship strikes, and entanglements (Burek-Huntington et al. 2015, McGuire et al. 2020). The 2 case studies herein pertain to an aborted fetus with severe congenital deformities including agenesis of flukes, biliary dysplasia, and anorectal and genitourinary dysgenesis, and a male calf with a perineal groove defect and suspected secondary peritonitis. This is the first report of congenital defects in free-ranging CIBs.

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2. MATERIALS AND METHODS

The first case, National Marine Fisheries Service (NMFS) ID 2016333, was an aborted fetus found stranded on July 14, 2016, south of Anchorage, Alaska, USA (61.11° N, 148.93° W). The second case, NMFS-ID 2017217, was a male calf found October 3, 2017, near Anchorage (61.21° N, 149.91° W). Necropsies followed standard protocols per Geraci & Lounsbury (2005). Due to the advanced state of decomposition of 2016333, no ancillary testing of tissues or histopathology was pursued. For 2017217, swabs and tissue samples were collected and most stored at −80°C. Subsamples of all major tissues were fixed in 10% neutral buffered formalin.

Chilled samples of lung, peritoneal fluid, whole blood, and feces for aerobic culture and feces for fecal pathogen screening were sent to the University of California, Davis School of Veterinary Medical Teaching Hospital Microbiology Laboratory. For polymerase chain reaction (PCR), total DNA was extracted (Smolarek Benson et al. 2006) from blowhole and genital swabs, tonsil and brain, and a PCR amplification was performed with primers specific for beluga whale alphaherpesvirus-1 (BWHV-1) (FP-5′-GAC TTT GCC AGC TTA TAC CCC AGC AT-3′ and RP-5′-TTG CGC ACG AGG TCG ACG CCC TTC AT-3′) that target the DNA polymerase gene and amplify a DNA fragment of 741 bp. DNA from these swabs was further used for amplification of the complete glycoprotein B (gB) gene with primers (FP-5′-ATG YCC CYT GGT GGC GGT GYT MAA CA-3′ and RP-5′-TCA GGG CCC CAG CGT CCC GTA CTT-3′) designed after a multiple sequence alignment with the ClustalW module of Lasergene DNA Star using homologous sequences derived from the BWHV viral repository. Gaps in sequences were filled with internal primers and assembled DNA was sequenced using the Sanger approach.

Fixed tissues were paraffin-embedded, sectioned at 4–5 µm and stained with hematoxylin and eosin (HE) at Histology Consultation Services (Everton, Washington, USA). Based on findings from histopathology, transmission electron microscopy (TEM) on thymus was performed at University of Minnesota Veterinary Diagnostic Laboratory (St. Paul, Minnesota). Briefly, tissue retrieved from formalin-fixed paraffin-embedded block was deparaffinized in a decreasing alcohol gradient followed by post-fixations, first in 2.5% glutaraldehyde, followed by osmium tetroxide in 0.1 M sodium cacodylate buffer. Samples were processed per Armién et al. (2020).

3. RESULTS

Case 2016333 was a 91.4 cm long aborted fetus whose posterior end was smooth and rounded, lacking a peduncle, flukes, anus, and genital slit (Fig. 1). On internal examination, the lungs were atelectatic, and the colon was distended with thick brown/orange-tinged meconium. No urinary bladder or reproductive tract was present. Biliary system dysplasia was characterized by large, multilobulated cystic structures filled with abundant clear serosanguineous fluid and lined by bright orange-yellow tissue (Fig. 2).

The spinal column from the thoracolumbar junction distally was collected and cleaned using dermestid beetles. One vertebra showed a globular ver-

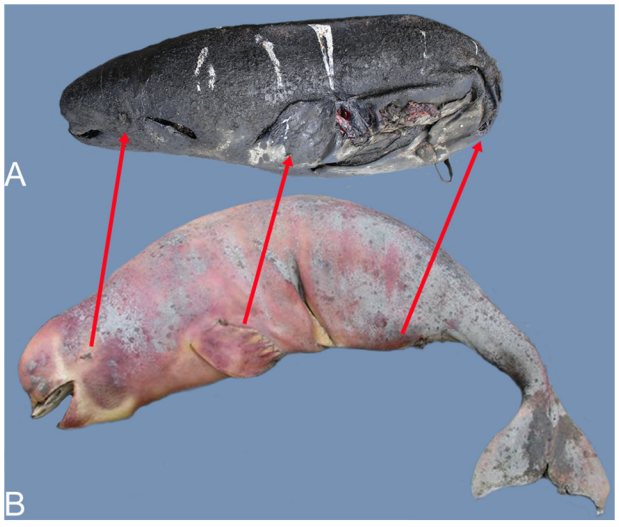

Fig. 1. (A) Aborted beluga fetus (2016333) with caudal regression compared to a (B) fully formed 136 cm female fetus (arrows: eye, pectoral flipper, end of body cavity)

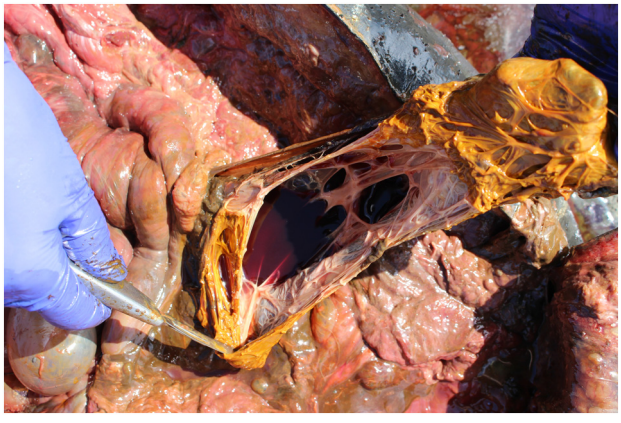

Fig. 2. Aborted beluga fetus (2016333) liver with large, multiloculated cysts suggestive of biliary dysplasia

tebral body or centrum lacking epiphyses, with a neural arch (vertebral arch) attached only along the left side (Galbusera & Bassani 2019). The spinous process was absent, and the part of the neural arch to which it attaches was not fused in the median plane. Two additional globular, poorly defined centra were recovered with no other boney attachments; one had a flat surface that could be an epiphysis. Parts of 8 vertebral arch fragments were collected, consisting of fragments of the arch that connect the articular processes to the centrum or the spinous process (Fig. 3). The entirety of bones collected from the thoracolumbar junction back are presented in Fig. 3.

Case 2017217 was a mildly autolyzed 188 cm long male calf; the estimated age based on length and time of year was ~36 d old per Shelden et al. (2020). The calf was in poor body condition as indicated by atrophy of the nuchal fat pad and epaxial muscles. A 4 cm long linear area of missing tissue was present on the ventral abdominal body wall on the midline between the genital slit and the anus (Fig. 4A,B). The edges of the epithelium lining this cavity were reddened and exfoliated. The skin and blubber were missing within this defect, the deep aspect of which was a pink to tan, irregular, granulation tissue-like material of about 4 mm thick providing a thin barrier between the defect and the parietal peritoneum. On histopathology, only the edges of the lesion were examined, of which there was erosion of the epithelium and chronic lymphoplasmacytic dermatitis. The deep aspect of the defect was not examined histologically. The peritoneal cavity was filled with odiferous, pink, flocculent exudate indicative of fibrinosuppurative peritonitis. There was also fibrinous pleuritis (Fig. 4C). There was a volvulus of the small intestine at the mesenteric root with no associated change in color to the serosa or mucosal surface and no associated fibrin on the serosal surface of the intestine, indicating a postmortem volvulus. Small intestinal lacteals were massively distended and filled with cloudy white chyle while the stomach was empty (Fig. 4D).

Multiple bacteria were cultured from the lung tissue, including *Shewanella putrefaciens,* coagulase-negative *Staphylococcus*, *Streptococcus* sp. non-hemolytic group G, and *Vibrio cholerae. Escherichia coli* was isolated from the peritoneal cavity and umbilicus and non-hemolytic *Staphylococcus* from the blood. *E. coli* was the presumed cause of the purulent peritonitis and likely the pleuritis, since it was the only bacteria isolated from the peritoneal fluid. The mixed cul-

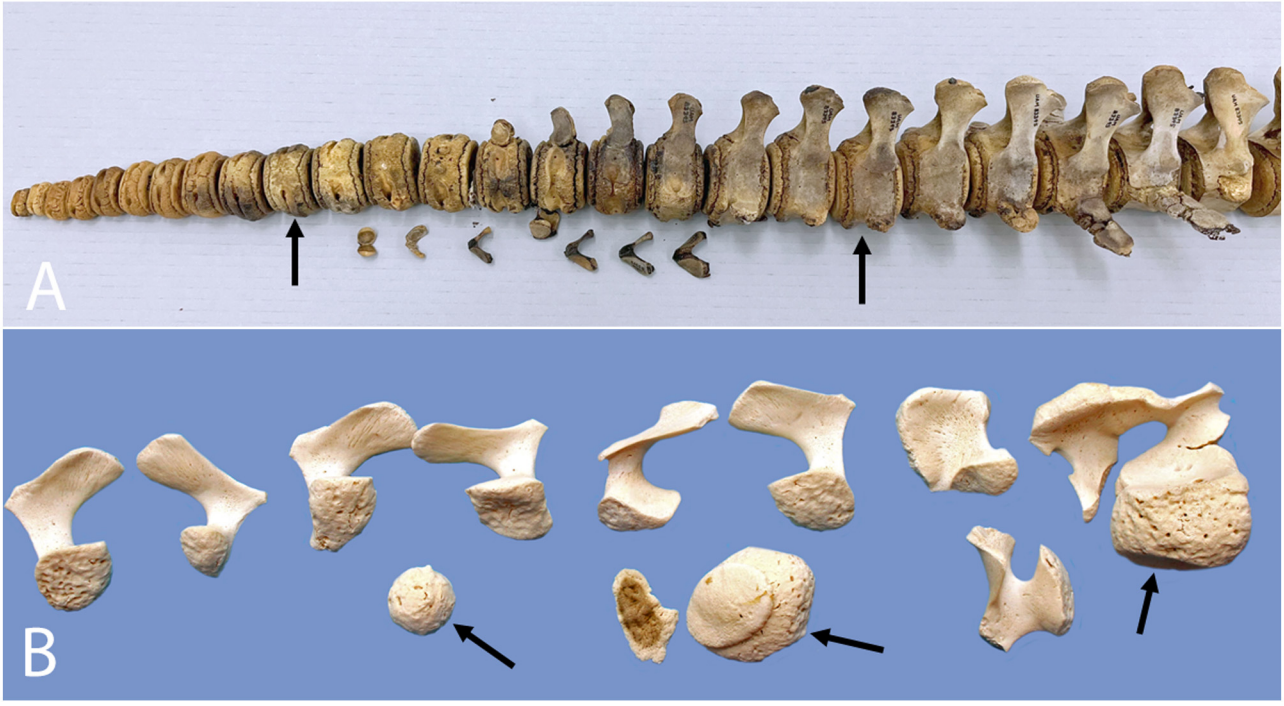

Fig. 3. Comparison between a normal thoracolumbar spinal column and 2016333. Thoracolumbar junction is to the right of the photo and caudal limit to the left. (A) Normal spinal column from a 180 cm long, neonatal beluga whale calf NMFS ID 2000121 (UAM:Mamm:123585). Normal centra indicated by arrows. Image provided by Aren Gunderson, University of Alaska Museum Mammal Collection. (B) Entire set of vertebrae 2016333 from the thoracolumbar junction to the terminus with multiple deformities. Poorly defined centra are indicated by arrows

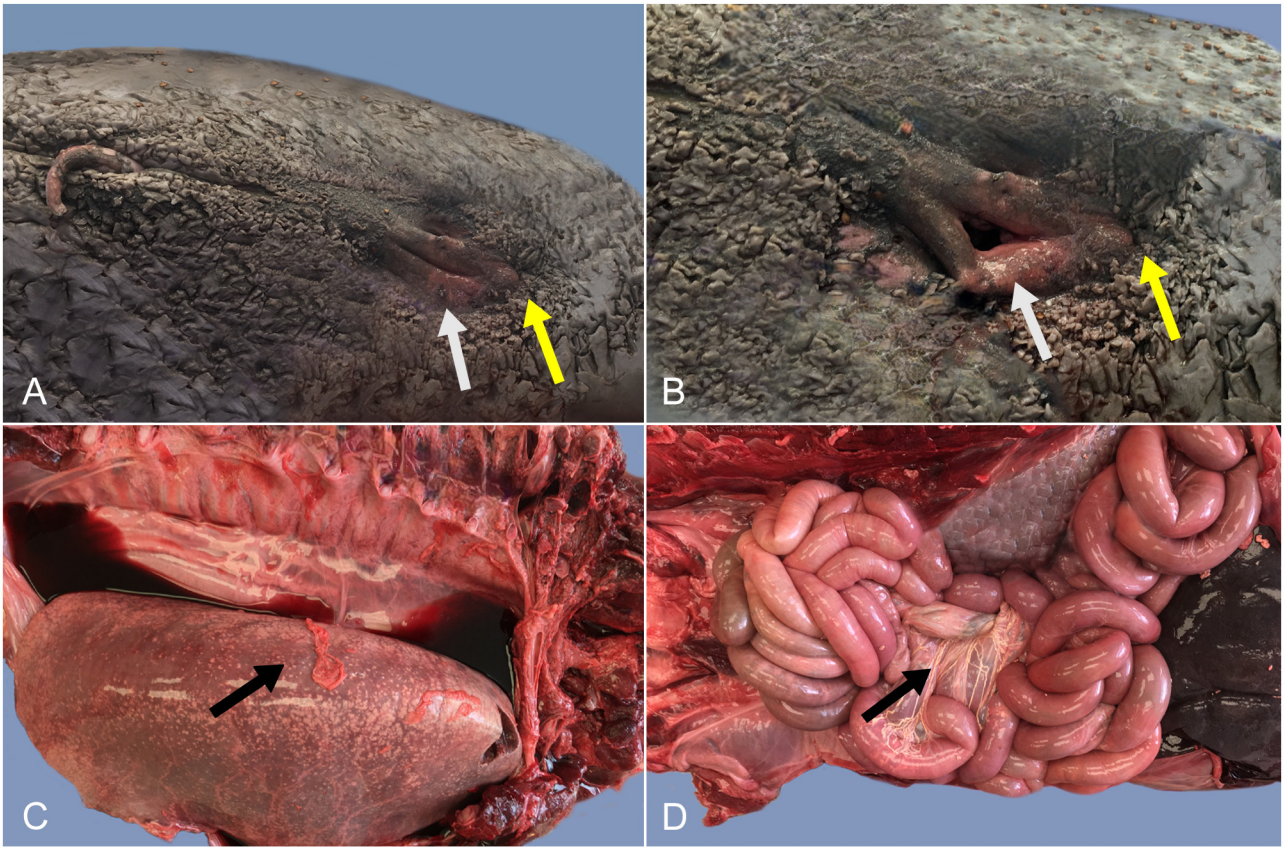

Fig. 4. Male beluga calf (2017217) ventral body wall defect (white arrows) between genital slit and anus (yellow arrows). (A) Overview. (B) Close-up. (C) Fibrinous effusion in pleural cavity (black arrow). (D) Volvulus and mesenteric lymphangiectasia (black arrow). Volvulus was postmortem due to the lack of color differential and presence of fibrosis around areas of lymphangiectasia which would not develop in a peracute ante-mortem event

ture from the lung likely represents terminal aspiration. Mixed bacteria were present in the fecal culture, with negative results for *E. coli* 0157, *Salmonella* sp., *Campylobacter* sp. and pathogenic streptococci. *V. cholerae* was isolated from enrichment broth, along with small numbers of *Clostridium perfringens* and *C. difficile* on anaerobic culture.

On histopathology, there was multifocal random necrotizing hepatitis, pancreatitis and adrenalitis, the cause of which could not be determined with routine HE stain. Differentials for these lesions include sepsis, systemic viral infection, or parasite migration. Additionally, there was an ulcerative colitis with lymphoid necrosis of the Peyer's patches. The lymphatics on the peritoneal serosal surfaces were massively dilated and surrounded by increased fibrous tissue and scattered foamy macrophages. Because of the chronic fibrosing reaction, this was interpreted as secondary lymphangiectasia due to the peritonitis.

Pulmonary histopathology revealed moderate to marked pulmonary edema and atelectasis and many alveolar squamous cells and rare meconium aggregates associated with scattered intraalveolar neutrophils and histiocytes. The presence of alveolar meconium and large numbers of squamous cells suggested fetal distress and aspiration in utero. Since this calf was estimated to be 36 d old but still had the squamous cells and meconium, it is possible this was a larger than expected neonate and/or was weak at birth. Microscopic examination of the brain revealed polioencephalomalacia characterized by laminar neuronal necrosis in a single section of cerebrum not further identified to site. Examination of skeletal muscle from multiple sites demonstrated hypereosinophilia and contraction bands consistent with acute degenerative myopathy. We suspect these last 2 lesions are likely related to stranding alive on the mudflats prior to death and the resultant hypoxia and acute stress.

The thymus was markedly depleted, with thinning of the cortex due to reduction of lymphocytes with eosinophilic intranuclear inclusion bodies within the thymic epithelial cells (Fig. 5A). TEM confirmed

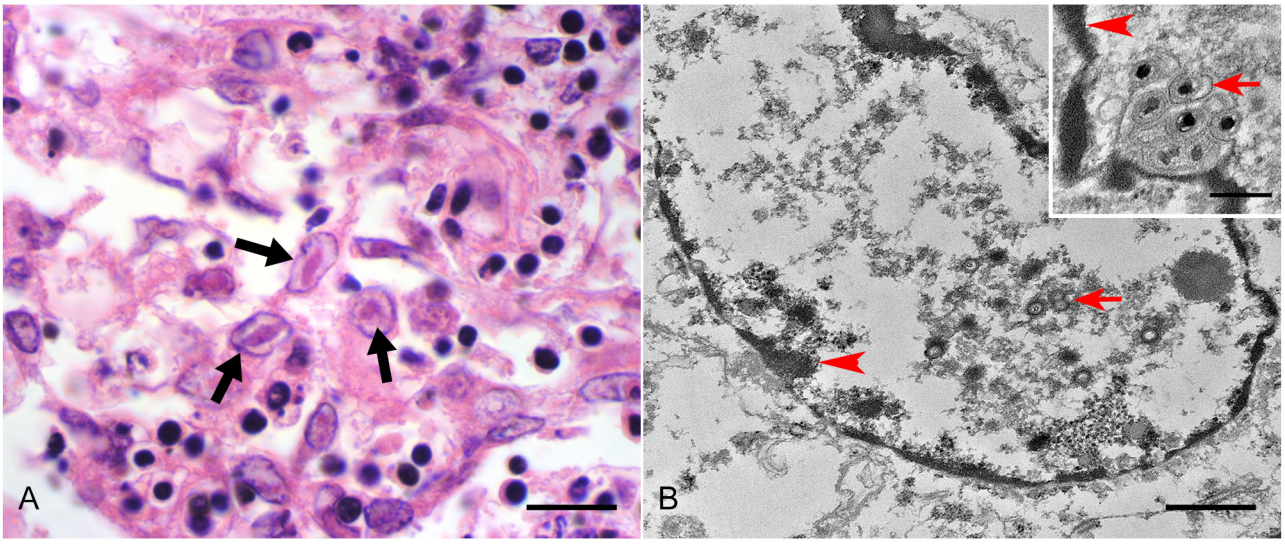

Fig. 5. Male beluga calf (2017217) thymic histopathology and transmission electron microscopy (TEM). (A) Eosinophilic intranuclear inclusion bodies (arrows) with margination of the chromatin. Scale bar = 20 µm. (B) On TEM examination, the inclusion bodies consisted of herpesvirus replication and assembly complexes (arrow) that marginalized degenerated heterochromatin (arrowheads). Particles were randomly scattered within the nucleus (average size 05.37 nm (SD = 6.67 nm). Scale bar = 0.6 µm. Inset: particles wrapped with the first envelope within the nuclear membrane (arrow). Scale bar = 200 nm

that the inclusion bodies were consistent with herpesvirus replication and assembly complexes that marginalized a degenerated heterochromatin (Fig. 5B). The particles were randomly scattered within the nuclei (average size 105.37 nm, SD = 6.67 nm). Occasionally, particles wrapped with the first envelope were present within the nuclear membrane (inset, Fig. 5B).

Blowhole and genital swabs, positive when targeting the DNA polymerase gene of beluga whale alphaherpesvirus, were further PCR-tested targeting the complete gB gene open reading frame (ORF). The sequences obtained were found to be, respectively, 100 and 99.5% identical at the amino acid and nucleotide levels, to the sequences of a BWAHV-1 (accession numbers OM105894 and OM105895) of the GenBank database (Davison et al. 2017).

4. DISCUSSION

To our knowledge, the only congenital defect reported in free-ranging belugas was a case of true hermaphroditism in a St. Lawrence beluga (De Guise et al. 1994), and none have been reported in CIBs. An atrial septal defect was reported in an aquarium-bred beluga (Robeck et al. 2005). Congenital aberrant coloration, many cardiac anomalies, congenital hyperplastic goiter, and skeletal defects including craniofacial abnormalities, scoliosis and spinal deformities, and spina bifida have been reported in other cetaceans (St. Leger et al. 2018).

The defect in 2016333 is like the congenital defect in humans, caudal regression syndrome (CRS). CRS was initially described in children that exhibit anomalies of the lumbar and sacral spine and may include an imperforate anus, agenesis of the kidneys, urinary tract, and/or internal genital organs (Duhamel 1961). A possible mutation in *HLBX9* homeobox gene on chromosome 7 has been described in humans with this defect (Jadav et al. 2012). Other studies suggest the teratogenic role of chemicals such as minoxidil and trimethoprim-sulfamethoxazole in development of CRS. The active derivative of vitamin A, retinoic acid (RA), is essential for normal embryonic development, and both RA deficiency and excess can result in congenital defects including CRS (Chan et al. 2002, Singh et al. 2005). Further study of chemical exposure including pharmaceutical products and vitamin A levels for CIBs is needed.

Case 2017217 had a perineal body wall defect, which is like the perineal groove defect in humans (Cheng et al. 2018). The presence of only a thin membrane between the aquatic environment and the peritoneal cavity in this calf likely resulted in septic peritonitis. The pathogenesis of this defect in humans is unknown. Abnormal expression of genes *SHH*, *Gli2*, *Gli3*, *Hoxa-13*/*Hoxd-13*, *Fgf10*, and *BMP4* may be associated with this defect (Cheng et al. 2018).

The systemic herpesvirus infection found in this calf could have contributed to its death. Although the observed adrenalitis, hepatitis and pancreatitis could be viral lesions, PCR confirmation was not possible, and no bacteria or viral inclusion bodies were detected in these tissues. The gB sequences were consistent with BWHV-1, which has been previously described in CIBs (Davison et al. 2017, Nielsen et al. 2018). The herpesvirus could have been acquired through uterine infection or during birth, like human newborns infected with alphaherpesviruses (Pinninti & Kimberlin 2018). In many cetaceans, including belugas, herpesviruses are associated with skin or mucosal lesions (Nielsen et al. 2018, St. Leger et al. 2018). However, some systemic infections, including in one CIB case, were characterized primarily by systemic vasculitis (Burek-Huntington et al. 2015). Alphaherpesvirus associated lesions in a beaked whale, striped dolphin and bottlenose dolphins were like those observed in 2017217, with lymphoid necrosis and multifocal random necrosis in multiple organs (St. Leger et al. 2018). Whether viral infection is related to the birth defect is unknown; however, other herpesviruses are known to cause diverse birth defects in humans (Feldman & Tibbetts 2015).

Epidemiological studies suggest a relationship between environmental contaminant exposure and congenital anomalies in humans and wildlife, including urogenital defects (Lyons 2007, Winchester et al. 2009). Several persistent organic pollutants were measured in the blubber of subsistence harvested belugas from Cook Inlet, and both polybrominated diphenyl ethers (PBDEs) and semi-quantitatively hexabromocyclododecanes (HBCDs, in particular, α-HBCD) significantly increased from 1995 to 2005 for both sexes (Burek-Huntington et al. 2022). α-HBCD, a flame retardant used in upholstery fabric, building insulation, and many other products, has been found to be toxic to aquatic organisms, bioaccumulates, and has harmful effects on thyroid function and offspring development, though it is unclear if current levels would significantly affect beluga fetal development. The location of their primary foraging habitat, near Anchorage, the largest city in Alaska, as well as airports, military bases, oil and gas development, and a wastewater treatment facility, which uses only primary treatment (McGuire et al. 2020), suggests further testing for pollutants is needed. Congenital defects often have a genetic component, and lack of genetic diversity has been suggested as a possible cause of increased congenital defects in other species (Trupkiewicz et al. 1997). Considering the Critically Endangered status of CIBs and the occurrence of these congenital defects, investigation into possible contaminant exposure, genetic diversity, and nutritional factors is warranted.

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