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Epizootiology of a *Cryptococcus gattii* outbreak in porpoises and dolphins from the Salish Sea

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ABSTRACT: *Cryptococcus gattii* is a fungal pathogen that primarily affects the respiratory and nervous systems of humans and other animals. *C. gattii* emerged in temperate North America in 1999 as a multispecies outbreak of cryptococcosis in British Columbia (Canada) and Washington State and Oregon (USA), affecting humans, domestic animals, and wildlife. Here we describe the *C. gattii* epizootic in odontocetes. Cases of *C. gattii* were identified in 42 odontocetes in Washington and British Columbia between 1997 and 2016. Species affected included harbor porpoises *Phocoena phocoena* (n = 26), Dall's porpoises *Phocoenoides dalli* (n = 14), and Pacific white-sided dolphins *Lagenorhynchus obliquidens* (n = 2). The probable index case was identified in an adult male Dall's porpoise in 1997, 2 yr prior to the initial terrestrial outbreak. The spatiotemporal extent of the *C. gattii* epizootic was defined, and cases in odontocetes were found to be clustered around terrestrial *C. gattii* hotspots. Case-control analyses with stranded, uninfected odontocetes revealed that risk factors for infection were species (Dall's porpoises), age class (adult animals), and season (winter). This study suggests that mycoses are an emerging source of mortality for odontocetes, and that outbreaks may be associated with anthropogenic environmental disturbance.

KEY WORDS: *Cryptococcus gattii* · Cryptococcosis · Salish Sea · Odontocete · Epizootic · Zoonosis

1. INTRODUCTION

Cryptococcosis is a fungal disease caused by pathogenic organisms in the genus *Cryptococcus* that are divided into 2 main species complexes: *C. neoformans* and *C. gattii* (formerly called *C. neoformans gattii* or var. *gattii*). The monogeneric complex *C. neoformans* was identified to species as *C. neoformans* and *C. gattii* in 2002 (Kwon-Chung et al. 2002, 2017, D'Souza et al. 2011). Unlike *C. neoformans*,

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which is globally distributed and infects immunosuppressed individuals, *C. gattii* has historically been found primarily in tropical and subtropical areas where it infects immunocompetent individuals (Stephen et al. 2002, Byrnes et al. 2009, Harris et al. 2012, Brito-Santos et al. 2015). Infection targets the respiratory and central nervous systems in all affected species (Galanis et al. 2009, Harris et al. 2012, Andreou et al. 2020) and can also cause localized dermatitis, cellulitis, cutaneous ulcers, lymphangitis, and multisystemic fungemia (Duncan et al. 2006a, Galanis et al. 2009, Lester et al. 2011, Rosenberg et al. 2016). Cryptococcosis is acquired through environmental exposure via the inhalation of airborne basidiospores or yeasts. In British Columbia (Canada) and the US Pacific Northwest, these cells sporulate from cryptococci that reside in decaying material in soil or trees such as Douglas fir *Pseudotsuga menziesii*, red alder *Alnus rubra*, Pacific madrone *Arbutus menziesii*, Western red cedar *Thuja plicata*, grand fir *Abies grandis*, and Garry oak *Quercus garryana* (Sorrell 2001, MacDougall & Fyfe 2006, Kidd et al. 2007a, Datta et al. 2009, Harris et al. 2012, May et al. 2016). In British Columbia and Washington, the predominant genotype of *C. gattii* is VGII, with 90–95% of infections resulting from the more virulent molecular type VGIIa and 5–10% of infections resulting from molecular type VGIIb (Kidd et al. 2004, Datta et al. 2009, Byrnes et al. 2009, 2010, Ngamskulrungroj et al. 2011, Engelthaler et al. 2014, Roe et al. 2018). While not contagious, *C. gattii* is of particular concern in North America due to its increased prevalence in multiple species and in the environment since the late 1990s (Stephen et al. 2002, Datta et al. 2009).

In 1999, a multispecies terrestrial *C. gattii* epidemic began in British Columbia and the US Pacific Northwest (Stephen et al. 2002). At least 59 human cases of *C. gattii* were recorded in mostly immunocompetent people living on Vancouver Island from 1999 to 2002 (Hoang et al. 2004). By the end of March 2002, there were 45 laboratory-confirmed cases of cryptococcosis in domestic animals and wildlife on Vancouver Island (Stephen et al. 2002). By 2004, *C. gattii* had infected at least 100 people that lived on Vancouver Island or had traveled there within a year prior to onset of symptoms (MacDougall & Fyfe 2006). The first recorded cases of cryptococcosis in people who had not recently traveled to Vancouver Island occurred on the lower mainland of British Columbia between September and December of 2004 (MacDougall et al. 2007). This coincided with *C. gattii*-positive air samples collected on the mainland in 2002 and 2004 (Kidd et al. 2007a,b, MacDougall et al. 2007). In 2004 and 2005, the first human cases of *C. gattii* were recorded in the USA (Oregon and Washington) that were not associated with travel to Vancouver Island or mainland British Columbia (MacDougall et al. 2007, Upton et al. 2007, DeBess et al. 2010). In 2005, the first positive *C. gattii* environmental samples (tree and soil) were recorded in the USA (Washington State) (MacDougall et al. 2007). By 2006, there were 313 cases recorded in animals in British Columbia. These primarily occurred in domestic dogs and cats but also included horses, pet ferrets *Mustela putorius furo*, llamas *Lama glama*, and eastern gray squirrels *Sciurus carolinensis* (Stephen et al. 2002, Kidd et al. 2004, Lester et al. 2004, 2011, Duncan et al. 2005a, 2006b). In the USA, reported animal cases of *C. gattii* included 2 dogs, 1 parrot (undisclosed species), and at least 5 cats in Washington from 2005 to 2008; and 1 cat, 1 dog, and 2 alpacas *Vicugna pacos* in Oregon in 2007 (MacDougall et al. 2007, Datta et al. 2009). By 2007, at least 218 human cases of cryptococcosis were recorded in British Columbia (Galanis et al. 2010). By July 2010, at least 60 human cases of *C. gattii* were recorded in the USA from Oregon, Washington, Idaho, and California, of which 88% were not associated with travel to Vancouver Island or mainland British Columbia (DeBess et al. 2010). Reported cases of *C. gattii* in any species are infrequent in the literature after 2013, and it has been suggested that confirmed cases may have decreased in both the USA and Canada (Espinel-Ingroff & Kidd 2015). Nevertheless, continued monitoring for the disease is important (Acheson et al. 2018, Cohen et al. 2020).

While cryptococcosis has been well-studied in humans and terrestrial animals, the disease is less understood in marine mammals (Danesi et al. 2021). Previous studies have reported isolated instances of cryptococcosis in free-ranging marine mammals in Western Australia (Gales et al. 1985), Hawaii (Rotstein et al. 2010), South Africa (Mouton et al. 2009), and California (Huckabone et al. 2015). Beginning in 2000, there were reports of various numbers of infected odontocetes that died from *C. gattii* in British Columbia and Washington, including harbor porpoises *Phocoena phocoena* (Stephen et al. 2002, Huggins et al. 2015, Fenton et al. 2017, Danesi et al. 2021), Dall's porpoises *Phocoenoides dalli* (Stephen et al. 2002, Kidd et al. 2004, Duncan et al. 2006b, Huggins et al. 2015, Danesi et al. 2021), and Pacific white-sided dolphins *Lagenorhynchus obliquidens* (Norman et al. 2011) In 2007, a case of maternal–fetal transmission of *C. gattii* was documented in a preg-

nant adult female harbor porpoise in Washington (Norman et al. 2011). In Oregon, cryptococcosis was documented in 3 porpoises (species undisclosed) from 2007 to 2008 (Engelhard et al. 2012). Cases of *C. gattii* were also documented in harbor seals *Phoca vitulina*, including a subadult in Washington in 2007 (Ashley et al. 2020), and a female pup and adult male in British Columbia in 2014 and 2015, respectively (Rosenberg et al. 2016).

While previous studies examined the *C. gattii* outbreak in North America in terrestrial ecosystems and wildlife, the epizootiology of the disease in marine mammals had not been characterized. To better understand this outbreak in marine mammals, we retrospectively evaluated stranding and necropsy reports from small odontocetes infected with *Cryptococcus* spp. in Washington and British Columbia between 1997 and 2020. This included an evaluation of the spatiotemporal extent of the outbreak and a case-control study to identify factors associated with increased risk of infection.

2. MATERIALS AND METHODS

We reviewed cases for *Cryptococcus* spp. infection from necropsies performed on stranded marine mammals between 1997 and 2020 as authorized by the Department of Fisheries and Oceans (Canada) and the National Oceanic and Atmospheric Administration's Marine Mammal Health and Stranding Response Program (USA). As part of ongoing disease surveillance efforts, complete postmortem examinations were performed on dead marine mammals in fresh (Code 2) to moderate (Code 3) postmortem condition (Geraci & Lounsbury 2005) from in and near the Salish Sea, the 16 925 km^2 inland sea shared by Washington State and British Columbia. Complete necropsies were performed according to established protocols with the goal of determining cause of death and identifying ancillary lesions, e.g. as described by Raverty et al. (2018). Representative samples from available tissues, including lesions, were collected and preserved in 10% neutral buffered formalin. Fresh samples were also placed in sterile packs and frozen.

For histological examination, tissue samples were embedded in paraffin, sectioned at 3–5 µm, mounted on glass slides, stained with hematoxylin and eosin, and examined by a veterinary pathologist (M.M.G. or S.R.). When tissue samples showed microscopic evidence of intralesional yeast morphologically consistent with *Cryptococcus* spp., additional diagnostic tests such as fungal culture and/or molecular studies on isolates using PCR were performed when possible. Multiple methods for fungal culture were employed depending on the laboratory. Swabs from fresh frozen tissue were either (1) inoculated onto Sabaraud's media, incubated at room temperature, and identified as *Cryptococcus* spp. using Auxacolor 2 (Sanofi Diagnostics Pasteur) or Uni-Yeast-Tek (Corning Medical) yeast identification kits (Bowman & Ahearn 1975, Davey et al. 1995, Chen et al. 2014), or (2) plated onto Columbia agar with 5% sheep blood (Oxoid), incubated at 35–37°C with 5–10% CO_2 for up to 7 d, and identified as *Cryptococcus* spp. using API Aux (BioMerieux) from 2007 to 2018 or MALDI-TOF (Bruker) from 2018 to 2020 (Willemsen et al. 1997, Sivasangeetha et al. 2007, Firacative et al. 2012). Identification to species, e.g. distinguishing *C. neoformans* and *C. gattii*, was accomplished at a reference laboratory (British Columbia Centre for Disease Control or Washington Animal Disease-Diagnostic Laboratory) using canavine-glycine-bromthymol (CGB) agar plates (Klein et al. 2009) or PCR. PCR was used to identify *C. gattii* genotypes and molecular types as previously described (Kidd et al. 2004, 2005, Lee et al. 2010, Norman et al. 2011). Restriction fragment length polymorphism targeted the *ura5* gene and samples were tested by PCR with primers amplifying the *ura5* gene (Meyer et al. 2003). Subsequent restriction enzyme analysis of PCR products using 2 panels of restriction enzymes or multilocus sequence typing based on partial sequences of 7 housekeeping genes (*cap59*, *gpd1*, *lac1*, *plb1*, *sod1*, *ura5*, and *igs1*) allowed for the identification of molecular types (Meyer et al. 2009, Cogliati 2013). Sequencing was performed using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and ABI Prism 310 Genetic Analyzer (Applied Biosystems), and sequence analysis was performed using Geneious software (https://www.geneious.com).

For this study, we defined a case as any odontocete species within, or near (i.e. Washington's outer coast or the southwest coast of Vancouver Island), the Salish Sea diagnosed with confirmed or probable *C. gattii* infection between 1997 and 2020. Confirmed cases included those in which (1) histologic lesions compatible with cryptococcosis were diagnosed and (2) *C. gattii* was cultured using CGB agar plates and/or *C. gattii* was identified using PCR. Probable cases included those in which cryptococcosis was diagnosed by histologic examination, but differentiation between *C. gattii* and *C. neoformans* species complexes was not performed by culture or molecular tests. We listed these cases as probable for *C. gattii*

because they occurred within the spatiotemporal extent of the terrestrial *C. gattii* outbreak in British Columbia and Washington, during which 100% of odontocete cases identified to species were *C. gattii*.

We calculated the proportional mortality ratio ([number of deaths from *C. gattii* / total number of deaths] × 100) for odontocetes in British Columbia and Washington from 1997 to 2020 using the total number of probable and confirmed cases. We grouped odontocetes into categories by species, sex, estimated age class (juvenile, subadult, and adult) (Gearin et al. 1994, Ferrero & Walker 1996, Ferrero & Walker 1999), and season of stranding. We did not include fetuses in total counts because their infection was contingent upon maternal exposure. We classified season as winter (January–March), spring (April–May), summer (June–August), or autumn (September–December) (Norman et al. 2008). We used SaTScan software (SaTScan Information Management Services, version 9.6.1) to identify spatiotemporal clusters of *C. gattii* cases and adjusted for temporal and demographic covariates. Retrospective space–time permutation models performed 999 replications of Monte Carlo simulations to scan for both high and low rates of clusters within one-year aggregations across the entire sampling period. We identified clusters using Euclidean and non-Euclidean proximity measures. For Euclidean measures, SaTScan constructed a centroid around each point and identified its closest neighbors sequentially until it reached the maximum window size (Kulldorff 2021). For adjusted, non-Euclidean measures, SaTScan detected clusters by identifying cases in relation to their 8 closest neighbors without being constrained to Euclidean distances. We used non-Euclidean measures to account for the shoreline geography of cases as opposed to Euclidean measures, which find the shortest linear distance between cases (Kvit et al. 2019). We adjusted spatiotemporal models based on univariate and multivariate parameters to account for the relationship between species, sex, age, and/or season. We considered clusters to be statistically significant at $p < 0.10$ and examined demographic and temporal similarities within significant clusters. We visually displayed cases with the use of a geographic information system (ArcGIS, ESRI).

We performed Fisher's exact tests (R Core Team 2018, version 3.5.0) to determine associations between covariates (species, sex, age, and season) for cases and considered tests to be statistically significant at a 2-sided p-value of ≤0.05. Next, we performed Pearson's chi-squared tests for independence with Yates' continuity correction to determine differences in covariate distribution among cases and controls and considered differences to be statistically significant at $p \leq 0.05$. We defined controls as harbor porpoises, Dall's porpoises, or Pacific white-sided dolphins that stranded in the inland waters of the Salish Sea between 2000 and 2019, had a complete gross and histologic examination, and had a cause of death that was attributed to trauma (predation, entanglement, vessel strike) or was undetermined, but underlying infectious disease, including *C. gattii*, was excluded to remove any confounding effects from similar infectious agents. For analyses that considered only adult females, we included pregnancy as a covariate and defined it as positive for individuals that were pregnant or displayed signs of recent pregnancy (including lactation or dystocia) and negative for individuals that were not pregnant or for which pregnancy was not identified. To compare potential risk factors for infection by *C. gattii* in cases and controls, we used univariate, bivariate, and multivariate logistic regression approaches. Variables included species, sex, estimated age class, season, and pregnancy (for regression on adult females only). To evaluate the potential effect of small sample size, we performed a separate sub-analysis excluding Pacific white-sided dolphins. We assessed overall fit of each univariate model with $p < 0.05$ with the Hosmer-Lemeshow goodness-of-fit test (Hosmer & Lemeshow 2000), and we cross validated goodness of fit for nested models using likelihood ratio tests. We evaluated final model fit using Akaike's information criterion (AIC) and calculated the odds ratio (OR) and 95% confidence interval (CI) for the final logistic regression model.

3. RESULTS

Between 1997 and 2020, 717 necropsies were conducted on stranded harbor porpoises, Dall's porpoises, and Pacific white-sided dolphins in Washington and British Columbia, and all cases were screened for *Cryptococcus* spp. (Table 1, Fig. 1). We identified 42 cases of *C. gattii* (22 confirmed, 20 probable) in odontocetes in the marine waters of British Columbia and Washington for a proportional mortality ratio of 5.9%. The first case occurred in 1997 and the last in 2016 (Table 2). For 3 of the 20 probable cases, CGB culture was performed but neither *C. gattii* nor *C. neoformans* was isolated, despite histologic detection of large numbers of yeasts consistent with *Cryptococcus* spp. in various organs.

Table 1. Total number of necropsies and *Cryptococcus gattii* cases (in parentheses) in British Columbia (Canada) and Washington (USA) between 1997 and 2020

Species	British Columbia	Washington	Total
Harbor porpoise	108 (15)	532 (11)	640 (26)
Dall's porpoise	14 (8)	33 (6)	47 (14)
Pacific white-sided dolphin	25 (2)	5 (0)	30 (2)
Total	147 (25)	570 (17)	717 (42)

Forty cases occurred within the Salish Sea and 2 cases occurred proximal to, but outside of, the Salish Sea, including one on the southwest coast of Vancouver Island (2003) and another on Washington's outer coast (2015). Outbreak hotspots where cases were clustered included Metro Vancouver Regional District with 19.0% of cases (n = 8), Nanaimo Regional District (14.3%, n = 6), and Capital Regional District (11.9%, n = 5; Table 2). Cases included 26 harbor porpoises (61.9%), 14 Dall's porpoises (33.3%), and 2 Pacific white-sided dolphins (4.8%) (Table 2). The majority of cases in harbor porpoises (69.2%, 18/26) occurred between 2006 and 2012 and in Dall's porpoises (64.3%, 9/14), between 2000 and 2005 (Fig. 2). The highest proportion of cases occurred in the winter (35.7%, n = 15) followed by autumn (28.6%, n = 12), spring (23.8%, n = 10), and summer (11.9%, n = 5). Genotypes of *C. gattii* were identified for 40.5% of cases (n = 17/42) and included VGIIa (n = 12), VGIIb (n = 3), and VGII, molecular type undetermined (n = 2).

Cumulatively, 47.6% of cases were female (n = 20), 50.0% were male (n = 21), and 2.4% (n = 1) were of unknown sex. For cases of *C. gattii* in harbor and Dall's porpoises, Fisher's exact tests revealed a significant association between species and sex (p = 0.048). Female harbor porpoises had the greatest occurrence of infection (38.1%, n = 16) compared to other demographics. Male harbor porpoises (21.4%, n = 9) had similar occurrence of infection to male Dall's porpoises (23.8%, n = 10), while female harbor

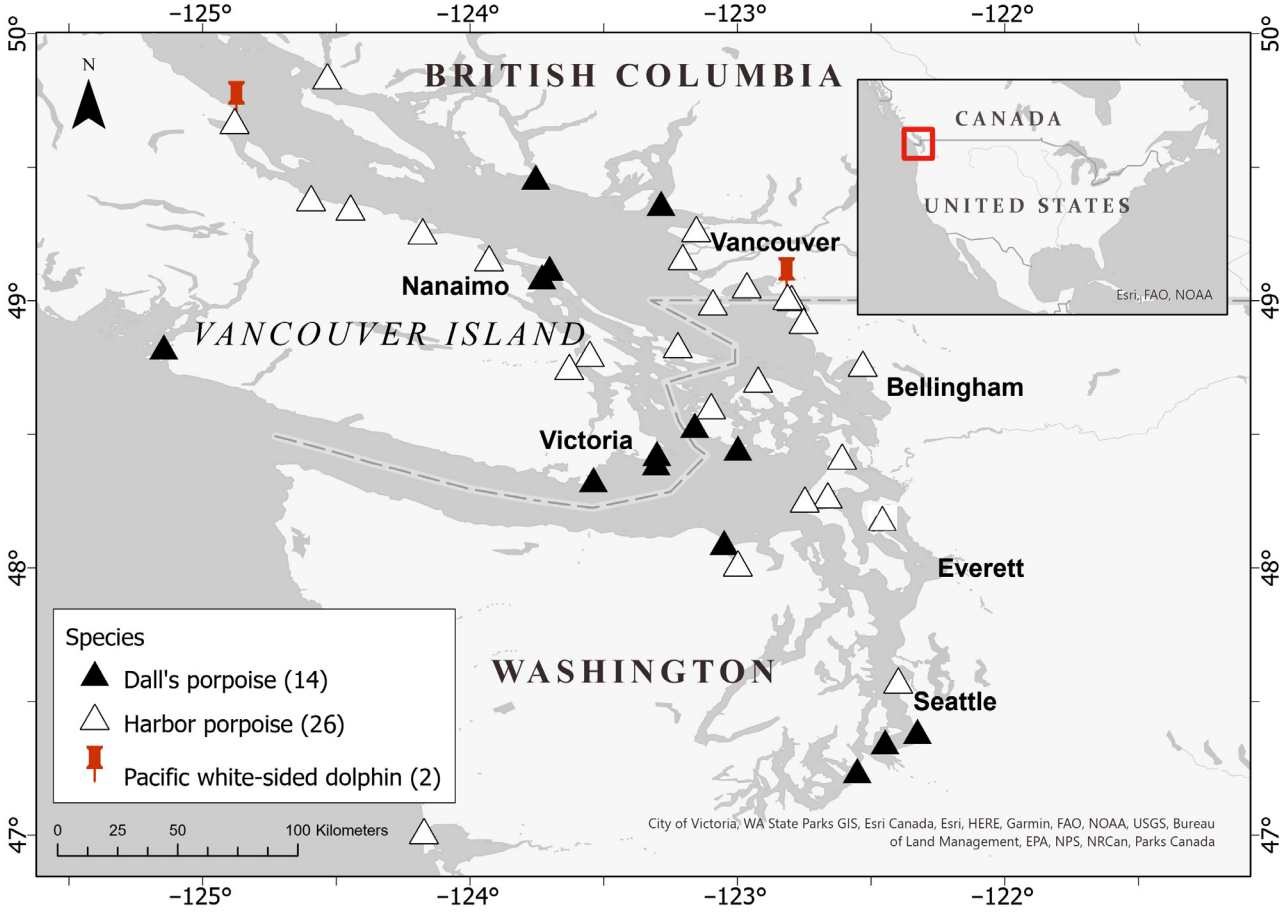

Fig. 1. Cases of *Cryptococcus gattii* in odontocetes in the Salish Sea, 1997–2016

Table 2. Details of 42 cases of *Cryptococcus gattii* in odontocetes in Washington (WA; USA) and British Columbia (BC; Canada), 1997–2016, in order of occurrence. NA: not applicable; C: confirmed case; P: probable case; CGB: canavine-glycine-bromthymol agar culture. * indicates maternal–fetal transmission of *C. gattii*

Case no.	Accession number(s)	Common name	Season	Year	Subarea	County or regional district	Sex	Estimated age class	Pregnant	Level of confirmation (method[s])	Molecular type
Pd-1	MMP97-36, G97-1997	Dall's porpoise	Autumn	1997	WA	Pierce	Male	Adult	NA	P	
Pp-1	DFO 3607, AHC 00-01915, BMS PP199	Harbor porpoise	Winter	1999	BC	Nanaimo	Female	Adult	No	P	
Pd-2	DFO 3520, AHC 00-0645 and AHC 00-0654 [sic]	Dall's porpoise	Winter	2000	BC	Nanaimo	Male	Adult	NA	P	
Pp-2	DFO 4376, AHC 00-1915	Harbor porpoise	Spring	2000	BC	Nanaimo	Female	Adult	No	P	
Pd-3	DFO 4631, AHC 02-0609	Dall's porpoise	Winter	2002	BC	Nanaimo	Male	Adult	NA	C (CGB culture)	
Pd-4	DFO 3859, AHC 02-0369	Dall's porpoise	Winter	2002	BC	Metro Vancouver	Male	Adult	NA	P	
Pp-3	DFO 4644, AHC 03-1246, LBL03-21	Harbor porpoise	Winter	2003	BC	Nanaimo	Male	Juvenile	NA	C (CGB culture)	
Pd-5	DFO 4545, AHC 03-1465	Dall's porpoise	Spring	2003	BC	Sunshine Coast	Male	Adult	NA	P	
Pd-6	DFO 1075, AHC 03-2768, LBL03-14, 030BDM06	Dall's porpoise	Spring	2003	BC	Capital	Male	Subadult	NA	P	
Pp-4	DFO 1081, AHC 03-2186, LBL03-19	Harbor porpoise	Spring	2003	BC	Metro Vancouver	Male	Juvenile	NA	C (CGB culture)	
Pd-7	DFO 6444, AHC 03-2770, 030WCPPF10	Dall's porpoise	Summer	2003	BC	Alberni-Clayoquot	Male	Adult	NA	P	
Pd-8	DFO 1812, AHC 04-0362, LBL04-18	Dall's porpoise	Winter	2004	BC	Capital	Female	Adult	No	P	
Pd-9	2004-SJ004, AHC 02-1780	Dall's porpoise	Winter	2004	WA	San Juan	Female	Adult	Yes	C (CGB culture)	
Pp-5	DFO 2250, 05PpSSF23, AHC 05-2076	Harbor porpoise	Summer	2005	BC	Capital	Female	Adult	Yes*	P	
Pd-10	MBHPd112105, AHC02-1775	Dall's porpoise	Autumn	2005	WA	Clallam	Female	Adult	No	C (CGB culture)	
Lo-1	DFO 2306, AHC 05-4357	Pacific white-sided dolphin	Autumn	2005	BC	Comox Valley	Male	Adult	NA	P	
Pp-6	06Pp01MarFI-01	Harbor porpoise	Winter	2006	WA	Skagit	Female	Adult	No	P	
Pp-7	060412-JST-PHPH, AHC 06-02388	Harbor porpoise	Spring	2006	WA	Clallam	Female	Adult	No	P	
Pp-8	DFO 2412, AHC 06-3635	Harbor porpoise	Spring	2006	BC	Nanaimo	Male	Adult	NA	C (CGB culture, PCR)	VGIIa
Pp-9	06Pp11AugWH-03, AHC 06-3059	Harbor porpoise	Summer	2006	WA	Whatcom	Female	Adult	Postpartum, lactating	P	
Lo-2	DFO 2568, AHC 06-3700	Pacific white-sided dolphin	Autumn	2006	BC	Metro Vancouver	Male	Adult	NA	P	
Pp-10	DFO 2650, AHC 07-2065	Harbor porpoise	Autumn	2006	BC	Comox Valley	Female	Adult	Yes	C (CGB culture, PCR)	VGIIa
Pp-11	07-WC-001, AHC 07-01119	Harbor porpoise	Winter	2007	WA	Whatcom	Male	Adult	NA	C (CGB culture, PCR)	VGIIa
Pp-12	07Pp22FebWI-01, AHC 07-1555	Harbor porpoise	Winter	2007	WA	Island	Female	Adult	Yes*	C (CGB culture, PCR)	VGIIa
Pp-13	DFO 3217, AHC 08-1378, ORR: 08-0033	Harbor porpoise	Spring	2008	BC	Metro Vancouver	Female	Adult	Yes	C (CGB culture, PCR)	VGIIa
Pp-14	DFO 3241, AHC 08-2855, LBL08-25, ORR: 08-0055 and ORR: 08-0056 [sic]	Harbor porpoise	Spring	2008	BC	Cowichan Valley	Female	Adult	No	P (CGB culture performed but fungi not isolated)	
Pp-15	2009-SJ001, AHC 09-1252	Harbor porpoise	Winter	2009	WA	San Juan	Female	Adult	Yes	C (CGB culture, PCR)	VGII, molecular type undetermined
Pp-16	DFO 4841, AHC 09-1907	Harbor porpoise	Spring	2009	BC	Metro Vancouver	Male	Juvenile	NA	P	
Pp-17	DFO 5026, AHC 09-2921, ORR: 09-0103	Harbor porpoise	Summer	2009	BC	qathet	Unknown	Adult	Unknown	C (CGB culture, PCR)	VGIIa
Pd-11	DFO 5289, AHC 09-4544	Dall's porpoise	Autumn	2009	BC	Capital	Male	Adult	NA	C (CGB culture, PCR)	VGIIb
Pd-12	CRC-1020, AHC 10-00109	Dall's porpoise	Autumn	2009	WA	King	Male	Subadult	NA	P (CGB culture performed but fungi not isolated)	
Pp-18	DFO 5657, AHC 10-3819	Harbor porpoise	Summer	2010	BC	Metro Vancouver	Female	Subadult	No	C (CGB culture, PCR)	VGIIa
Pp-19	DFO 5733, AHC 10-4591	Harbor porpoise	Autumn	2010	BC	Capital	Male	Adult	NA	C (PCR)	VGIIa
Pp-20	10Pp31DecWI-07	Harbor porpoise	Autumn	2010	WA	Island	Female	Adult	Yes*	C (CGB culture, PCR)	VGIIb
Pp-21	DFO 6285, AHC 11-0930	Harbor porpoise	Winter	2011	BC	Metro Vancouver	Female	Adult	No	C (CGB culture, PCR)	VGIIa
Pd-13	2011-MAST-003, AHC 11-02408	Dall's porpoise	Spring	2011	WA	King	Male	Adult	NA	C (CGB culture, PCR)	VGIIa
Pp-22	SSW031712, AHC 12-01424	Harbor porpoise	Winter	2012	WA	King	Female	Adult	No, lactating	C (CGB culture, PCR)	VGIIa
Pp-23	12Pp28MarCI-01	Harbor porpoise	Winter	2012	WA	Island	Male	Subadult	NA	C (PCR)	VGIIb
Pd-14	2014-SJ089	Dall's porpoise	Autumn	2014	WA	San Juan	Female	Adult	Yes	P (CGB culture performed but fungi not isolated)	
Pp-24	CRC-1467, G17-0612	Harbor porpoise	Winter	2015	WA	Grays Harbor	Male	Adult	NA	P	
Pp-25	DFO 15-505, AHC 15-6261	Harbor porpoise	Autumn	2015	BC	Metro Vancouver	Male	Juvenile	NA	C (CGB culture, PCR)	VGIIa
Pp-26	2016-SJ084	Harbor porpoise	Autumn	2016	WA	San Juan	Female	Adult	No	C (CGB culture, PCR)	VGII, molecular type undetermined

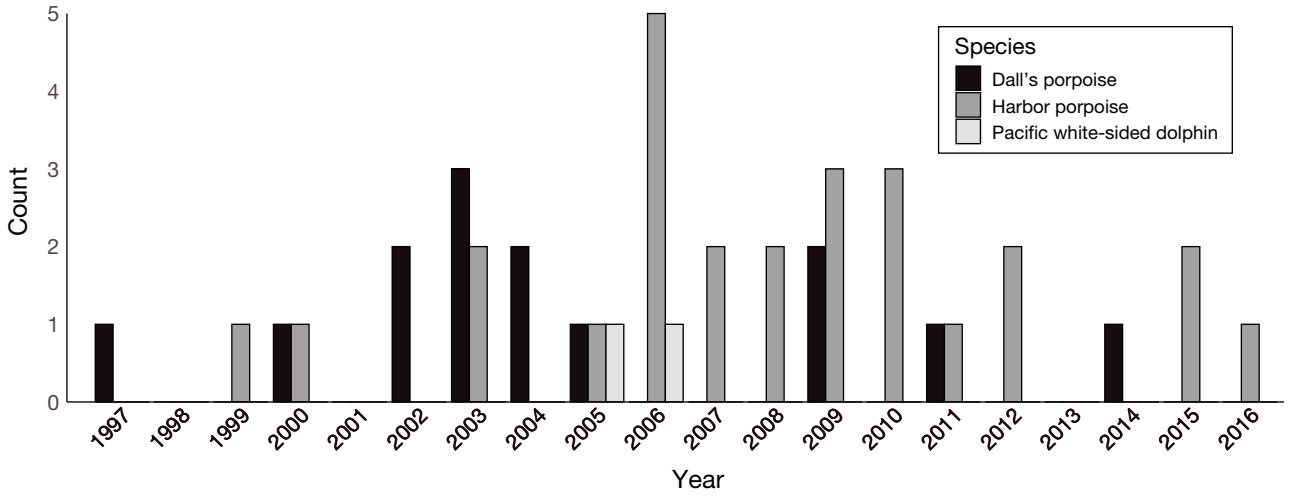

Fig. 2. Cases of *Cryptococcus gattii* in odontocetes by year and species in Washington (USA) and British Columbia (Canada), 1997–2016

porpoises had a much greater occurrence (38.1%, n = 16) than female Dall's porpoises (9.5%, n = 4).

Across all species, 81.0% of cases were adults (n = 34), 9.5% were subadults (n = 4), and 9.5% were juveniles (n = 4). For cases of *C. gattii* in harbor and Dall's porpoises, Fisher's exact tests revealed a significant association ($p \leq 0.05$) between sex and age ($p = 0.045$). Adult female porpoises had the greatest occurrence of infection (45.2%, n = 19) compared to other demographics. For harbor porpoises and Dall's porpoises, there was a sex-based bias ($p = 0.045$) for juveniles and subadults (grouped as 'non-adults'), with a greater proportion of non-adults being male (7/8, 87.5%) compared to adults (14/33, 42.4%). Across all species (including Pacific white-sided dolphins), infection by *C. gattii* was more common in adult females (45.2%, n = 19) than in adult males (33.3%, n = 14). Juvenile and subadult cases were more common in males (16.7%, n = 7) than females (2.4%, n = 1).

Females that were pregnant (19.0%, n = 8) or were lactating with signs of a recent pregnancy (4.8%, n = 2) comprised 23.8% of cases. This included 3 cases of maternal–fetal transmission of *Cryptococcus* spp. (e.g. Norman et al. 2011) and 1 pregnant animal in which histologic evaluation of the fetal tissues did not indicate vertical transmission (Table 2).

We identified 138 control cases in the inland waters of Washington between 2000 and 2019. These included 131 harbor porpoises, 6 Dall's porpoises, and 1 Pacific white-sided dolphin. Comparing controls to cases of *C. gattii*, Pearson's chi-squared test for independence showed significant differences by species ($\chi^2 = 25.989$, $p < 0.0001$). For harbor porpoises, season ($\chi^2 = 11.94$, $p = 0.0005$) using 'winter' as the reference, and age class ($\chi^2 = 10.176$, $p = 0.001$), using 'adult' as the reference, were significant. Chi-squared tests between harbor porpoise cases and controls showed no significant differences by sex or pregnancy status. Fisher's exact tests between Dall's porpoise cases and controls showed no significant differences among the sexes, age classes, or seasons.

The logistic regression model that best fit the data ($\Delta AIC = 0.00$; Hosmer and Lemeshow $\chi^2 = 0.18558$, $p = 1.00$) was the model that included species, age, and season (Table 3). Odontocetes that had a higher probability of infection by *C. gattii* were adult (OR = 4.31, 95% CI 1.79–11.32) Dall's porpoises (OR = 10.41, 95% CI 3.36–37.40) that stranded in the winter (OR = 5.24, 95% CI 1.94–14.47). Harbor porpoises had a lower probability of infection (OR = 0.10, 95%

Table 3. Multivariate logistic regression analysis of significant risk factors for *Cryptococcus gattii* in stranded odontocetes in the Salish Sea, where Pacific white-sided dolphin cases (n = 2) and controls (n = 1) were included

Risk factor	p	Odds ratio	95% CI
Species	<0.0001		
Harbor porpoise		0.10	0.03–0.29
Dall's porpoise		10.41	3.36–37.40
Season	0.00113		
Winter		5.24	1.94–14.47
Summer		0.18	0.05–0.52
Age	0.00171		
Adult		4.31	1.79–11.32

CI 0.03–0.29) compared to Dall's porpoises and Pacific white-sided dolphins, when adjusted for season and age class. Odontocetes had a lower probability of infection in the summer (OR = 0.18, 95% CI 0.05–0.52), when adjusted for species and age class.

Similar results were obtained when Pacific white-sided dolphins were removed from the analysis. Species ($p < 0.0001$), age ($p < 0.0001$), and season ($p < 0.0001$) were still significant predictors of probability of infection. Adult (OR = 5.71, 95% CI 2.55–14.18) Dall's porpoises (OR = 12.13, 95% CI 4.43–37.15) in the winter (OR = 6.46, 95% CI 2.71–15.82) had a higher probability of infection and harbor porpoises had a lower probability of infection (OR = 0.08, 95% CI 0.03–0.23). For analyses that included and excluded Pacific white-sided dolphins, sex ($p = 0.750$) and pregnancy ($p = 0.552$, adjusted for sex and age class) were not significant predictors of infection.

Ten significant ($p < 0.10$) spatiotemporal Euclidean models were identified across the entire sampling period (Table 4). These included the univariate models adjusted for species, sex, season, and age; the bivariate models adjusted for sex and season, age and season, and species and sex (cluster 1: $p = 0.048$; cluster 2: $p = 0.097$); the multivariate model adjusted for species, age, and sex; and the unadjusted model.

Seven out of 10 significant Euclidean models identified 11 cases (Cluster A) that were centered in Qualicum Bay, Nanaimo Regional District, between 1999 and 2006 (Fig. 3, Table 5). Cluster A consisted of female harbor porpoises (n = 4), male harbor porpoises (n = 2), male Dall's porpoises (n = 4), and male Pacific white-sided dolphin (n = 1). Two out of 10 significant Euclidean models identified 6 cases (Cluster B) that were centered in the waters of Nanaimo Regional District between 1999 and 2003 (Fig. 3, Table 5). Cluster B consisted of female harbor porpoises (n = 2) and male Dall's porpoises (n = 4). All 6 cases in Cluster B were included within Cluster A.

A total of 5 significant ($p < 0.10$) spatiotemporal non-Euclidean models, detected using case proximity to its 8 nearest neighbors, were identified across the entire sampling period (Table 4). These included the univariate models adjusted for sex, age, and species; the bivariate model adjusted for age and season; and the unadjusted model. Four out of 5 significant non-Euclidean models identified Cluster B as a significant cluster in the waters of Nanaimo Regional District between 1999 and 2003 (Fig. 3, Table 5).

4. DISCUSSION

Retrospectively, odontocetes were a sentinel group for the multi-species *Cryptococcus gattii* epizootic in British Columbia and Washington State which began on Vancouver Island during the late 1990s. Despite limited resources for diagnostic tests in some stranding networks, 22 of the 42 cases of *Cryptococcus gattii* were confirmed histologically, and culture or

Table 4. Significant ($p < 0.10$) spatiotemporal models from 1 January 1997 to 31 December 2016, in order of significance. Non-Euclidean models were based on nearest neighbor and thus do not provide radius or center coordinates. Note that there were 2 significant clusters for the Euclidean model adjusted for species and sex (labeled '1' and '2'). Dates are given as yr/mo/d

Model	p	Test statistic	Date Start	Date End	Radius (km)	Center coordinates (°N, °W)	Cases n	Cluster
Euclidean								
Species × Age × Sex	0.038	4.62	2007/1/1	2016/12/31	68.46	49.2669, 124.1776	11	A
Species × Sex (1)	0.048	5.08	2007/1/1	2016/12/31	68.46	49.2669, 124.1776	11	A
Sex × Season	0.048	5.19	2007/1/1	2016/12/31	68.46	49.2669, 124.1776	11	A
Species	0.064	5.14	2007/1/1	2016/12/31	68.46	49.2669, 124.1776	11	A
Sex	0.064	5.34	1999/1/1	2002/12/31	41.53	49.4704, 123.7545	6	B
Age × Season	0.064	4.63	2007/1/1	2016/12/31	68.46	49.2669, 124.1776	11	A
No parameters	0.065	5.67	1999/1/1	2002/12/31	41.53	49.4704, 123.7545	6	B
Season	0.076	5.44	2007/1/1	2016/12/31	68.46	49.2669, 124.1776	11	A
Age	0.091	5.22	2007/1/1	2016/12/31	68.46	49.2669, 124.1776	11	A
Species × Sex (2)	0.097	4.73	1999/1/1	2004/12/31	135.44	47.3561, 122.4478	13	Neither A nor B
Non-Euclidean								
No parameters	0.011	5.67	1999/1/1	2002/12/31	–	–	6	B
Sex	0.015	5.34	1999/1/1	2002/12/31	–	–	6	B
Age	0.032	5.01	1999/1/1	2003/12/31	–	–	6	B
Age × Season	0.069	4.08	2004/1/1	2012/12/31	–	–	6	B
Species	0.083	4.43	1999/1/1	2002/12/31	–	–	6	B

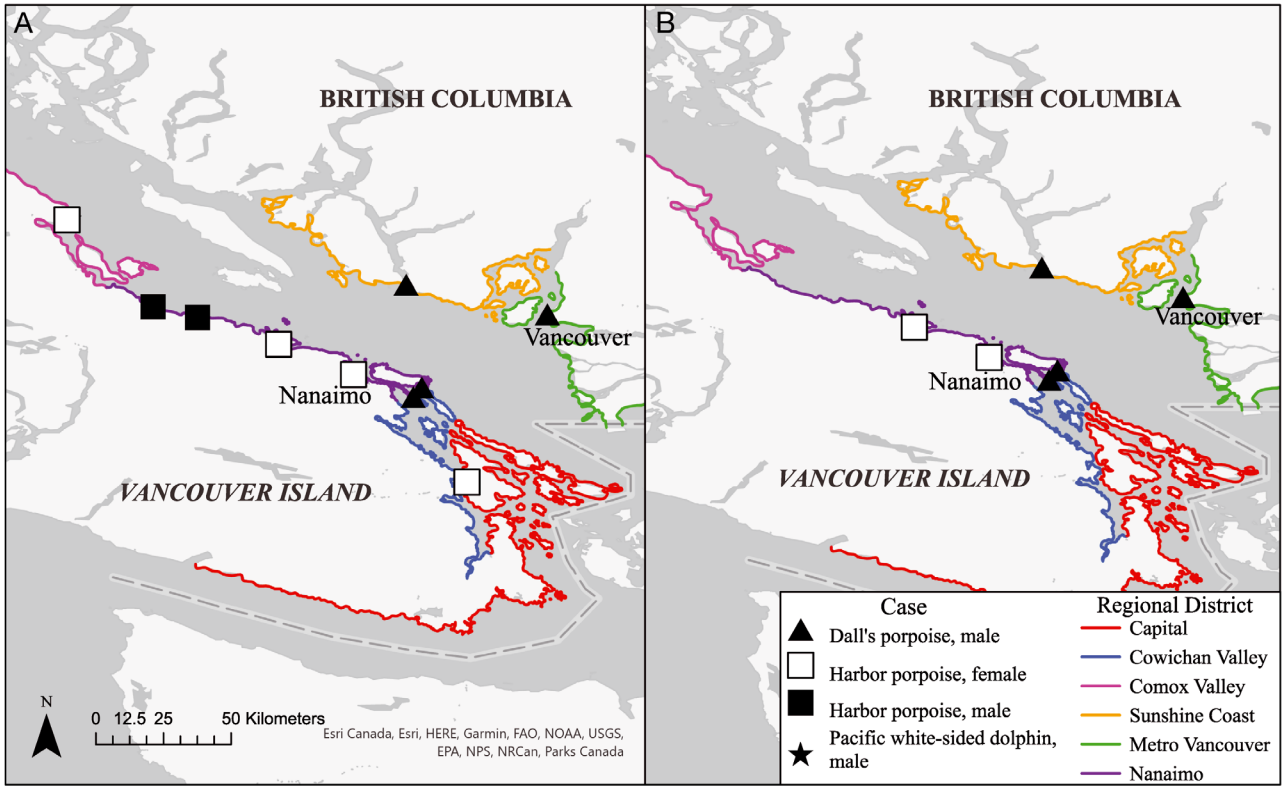

Fig. 3. Cases of *Cryptococcus gattii* in odontocetes that were identified as significant clusters by SaTScan software. (A) Cases in Cluster A (n = 11) that were detected by 7 Euclidean models. (B) Cases in Cluster B (n = 6) that were detected by 2 Euclidean and 4 non-Euclidean models. All cases in Cluster B were also included in Cluster A

Table 5. Number of cases by British Columbia (Canada) regional district that were identified in significant clusters by SaTScan software ($p < 0.010$): Cluster A and Cluster B). Note that all cases in Cluster B were also identified in Cluster A

	Nanaimo	Sunshine Coast	Metro Vancouver	Comox Valley	Capital	Total
Cluster A	6	1	1	2	1	11
Cluster B	4	1	1	0	0	6

molecular techniques did not identify any cases of *C. neoformans.* Although not identified to species, the first probable case of cryptococcosis in the epizootic occurred in an adult male Dall's porpoise in Tacoma, Washington, in 1997, 2 yr prior to the recognition of the *C. gattii* outbreak in humans in 1999 (Galanis et al. 2009). Histologically, the cause of death in this animal was attributed to pulmonary cryptococcosis caused by *C. neoformans*; however, culture was not performed to differentiate *C. neoformans* from *C. gattii*, as this was 5 yr before *C. gattii* and *C. neoformans* were differentiated into 2 species complexes (Kwon-Chung et al. 2002). Two decades later, attempts were made to amplify *Cryptococcus* spp. nucleic acid from paraffin-embedded tissue from this case at the Washington Animal Disease Diagnostic Laboratory; however, no nucleic acid could be amplified, possibly due to degradation of DNA over time. Because *C. neoformans* was not isolated from odontocetes in the Salish Sea during this time period, and due to the onset of widespread *C. gattii* cases identified just after this case, it is possible that this individual represents the earliest recorded case of *C. gattii* in the epizootic.

Beach-cast or floating carcasses of *C. gattii*-infected odontocetes were recovered near the terrestrial *C. gattii* hotspots identified by Kidd et al. (2004, 2007a) in British Columbia and Washington. From 1997 to 2005, 16 cases of *C. gattii* in odontocetes appeared in Washington and British Columbia, largely on eastern Vancouver Island where the outbreak was originally identified (Stephen et al.

2002). Particularly, between 1999 and 2003, it appears that significant risk factors for infection by *C. gattii* in odontocetes included proximity to the coastlines of Nanaimo Regional District on eastern Vancouver Island, Metro Vancouver Regional District on mainland British Columbia, or the Sunshine Coast Regional District on mainland British Columbia (Cluster B, Fig. 3). Odontocetes that were positive for *C. gattii* in these regions were recovered in close proximity to areas identified by Kidd et al. (2007a) as having high rates of human and nonhuman cases, and environmental isolates of *C. gattii* in coastal Douglas fir and coastal western hemlock *Tsuga heterophylla* biogeoclimatic zones (Kidd et al. 2007a). This included 2 Dall's porpoises and 2 harbor porpoises that stranded in the Canadian Gulf Islands, which was identified as a hotspot for *C. gattii* environmental isolates (MacDougall et al. 2007).

Beginning in 2006, odontocete cases of cryptococcosis markedly increased in Washington, including in the lower Puget Sound, which coincided with the increase in cases of *C. gattii* in humans and domestic animals and the identification of *C. gattii*-positive tree and soil samples in Washington in 2005 (MacDougall et al. 2007). While it is possible that *C. gattii*-infected odontocetes that stranded in Washington may have acquired the disease in British Columbia, it is likely that infected harbor porpoises and Dall's porpoises stranded near the locations where they acquired the disease due to their relatively small home range and site fidelity (Hanson 2007). This supports the concept of multispatial and multitemporal disease acquisition of *C. gattii* in British Columbia and Washington over the duration of the epizootic.

From 2006 to 2016, 26 new cases of *C. gattii* in odontocetes were recorded in Washington, Vancouver Island, and the southern mainland of British Columbia. These were largely harbor porpoises (n = 21). Initially, between 1997 and 2005, there were more infected Dall's porpoises than harbor porpoises (n = 10 and n = 5, respectively); however, between 2006 and 2016, infection increased for harbor porpoises (n = 21) compared to Dall's porpoises (n = 4). This may be explained by the increased abundance of harbor porpoises and the corresponding decrease in abundance of Dall's porpoises in some areas of the Salish Sea, especially Puget Sound, from 1994 to 2014 (Evenson et al. 2016, Jefferson et al. 2016, A. J. Warlick et al. unpubl.). Cases of *C. gattii* in odontocetes began to taper off in 2011, which coincided with the 2012–2013 decline of *C. gattii* in humans and terrestrial animals in Canada and the USA (Espinel-Ingroff & Kidd 2015). As of 1 January 2021, the last recorded case of *C. gattii* in odontocetes occurred in an adult female harbor porpoise that died and stranded on San Juan Island in October 2016. There is no apparent rationale for the relatively sudden decline in cases of *C. gattii* in small odontocetes. The decline in cases in humans and terrestrial animals since 2012–2013 is also poorly understood and could have been attributed to changes in low numbers and that only confirmed cases were reported (Espinel-Ingroff & Kidd 2015). As *C. gattii* seemed to decline in small odontocetes, another novel fungal disease, mucormycosis, emerged in the Salish Sea in 2012, affecting harbor seals, harbor porpoises, and an endangered southern resident killer whale *Orcinus orca* (Huggins et al. 2020).

All *C. gattii* genotypes identified in odontocetes (n = 17) were VGII, the predominant genotype of the *C. gattii* epizootic in British Columbia and Washington (Kidd et al. 2004, Ngamskulrungroj et al. 2011, Engelthaler et al. 2014, Roe et al. 2018). This included 12 cases of the more virulent, major molecular type VGIIa (90–95% of infections, Byrnes et al. 2009); 3 cases of the less virulent, minor molecular type VGIIb (5–10% of infections); and 2 cases with genotype VGII (molecular type undetermined). To our knowledge, *C. gattii* molecular type VGIIb had not been reported in marine mammals prior to this study, expanding the number of possible molecular types of *C. gattii* that can infect marine mammals.

Our spatial and temporal analyses of odontocete cases suggest that multiple sporulation events likely occurred over time and space during this epizootic, with individuals closest to the point source for airborne basidiospores or yeast cell distribution most likely to be exposed. Certain biogeoclimatic conditions are strongly associated with the distribution of *C. gattii* in British Columbia, including daily average January temperatures >0°C, low elevation (<770 m and average 100 m), coastal Douglas fir forests, and very dry regions of coastal western hemlock forests (Mak et al. 2010). Anthropogenic factors also might have played a role in the epizootic of *C. gattii* in British Columbia and the US Pacific Northwest. For example, soil disturbances associated with construction and deforestation have been hypothesized as actions that could incite basidiospores or yeast cell aerosolization (Duncan et al. 2006c, Fyfe et al. 2008). Also, it has been hypothesized that temperate range expansion of *C. gattii* from tropical and subtropical areas to the site of this epizootic may have been associated

with warmer average global temperatures that increase the susceptibility of a tree to fungal colonization (Cohen et al. 2002, Benedict & Park 2014). Dispersal mechanisms of *C. gattii* in temperate areas include aerosolization during forestry and municipal activities such as wood chipping, as well as human-mediated dispersal from footwear (Kidd et al. 2007b). It has been shown that domestic animals that were active outdoors or lived near a commercial environmental disturbance such as soil disruption or logging during the *C. gattii* epizootic had a significantly increased risk of infection (Duncan et al. 2006a). Kidd et al. (2007a) found that seawater samples were positive for *C. gattii* around Vancouver Island, near areas with high concentrations of *C. gattii* in trees and soil. Odontocetes likely acquired the disease by inhaling basidiospores or yeasts at the air–water surface interface during respiration. The large tidal volume of air exchange at each surface respiration may play a role in increasing the possibility of initial exposure to *C. gattii* (Danesi et al. 2021).

While there were more cases in harbor porpoises compared to Dall's porpoises (n = 26 and 14, respectively), case control comparison revealed that Dall's porpoises were at greater risk of infection (OR 10.41) compared to harbor porpoises (OR = 0.10; Table 3). This is particularly interesting considering that harbor porpoises stranded more than Dall's porpoises in Washington from 2000 to 2019 (n = 814 and 86, respectively; A. J. Warlick et al. unpubl.). Of particular note are 3 cases of Dall's porpoises in Puget Sound, an area where harbor porpoises have increased in recent years while Dall's porpoises have decreased (Evenson et al. 2016). It is uncertain why Dall's porpoises had greater risk of infection, and it may be due to behavioral, physiological, cellular, and/or molecular processes. It is possible that surface activity behaviors increased the risk of infection for Dall's porpoises as they are known to display such behaviors, e.g. bow-riding, that entail surfacing in short intervals which may increase the possibility of exposure and susceptibility to infection (Law & Blake 1994, Hall 2011).

The case control study shows that odontocetes with a higher risk of infection were adults (OR = 4.31) during winter (OR = 5.24; Table 3). Adults may have acquired the fungal infection more than other age classes because they had a greater time period over which to be exposed, as porpoises undergo rapid development and mature at an earlier age than other odontocetes (Read & Hohn 1995, Noren et al. 2014). The greater number of cases in the winter, particularly adult females, aligns with the seasonal calving trends of harbor porpoises in the Salish Sea in which adult female harbor porpoises are either pregnant or raising calves in the winter (Norman et al. 2018). This could be associated with energy costs related to maternal investment, including gestation and lactation, which are physiological stressors that negatively impact maternal odontocete energy budgets (Read 2001). Finally, it is worth noting that the habitats and locations used by pregnant and postpartum porpoises may pose a greater risk for infection than pregnancy itself, particularly if these are near sites of construction or deforestation.

Reporting bias of marine mammal strandings is heavily influenced by human populations, geographic elements, prevailing currents, and temporal animal movements, but likely did not affect our analyses. While odontocete strandings are reported year-round in Washington and British Columbia, the majority are reported in the summer, likely in part due to increased human presence at coasts, seasonal animal movement, and oceanographic features (Norman et al. 2004). An analysis by Norman et al. (2004) of marine mammal strandings in Washington and Oregon from 1930 to 2002 found that harbor porpoises primarily stranded in the summer (50%) and Dall's porpoises stranded in the spring (44%) and summer (32%). Conversely, in this case series, *C. gattii*-infected odontocetes were recovered more in the winter (35.7%, 15/42) and less in the summer (11.9%, 5/42). The case control study also supported these findings and showed that winter was a risk factor for infection (OR = 5.24). The pathogenesis of *C. gattii* in odontocetes is not well known, including the interval between infection and death, so it is possible that odontocetes acquired the infection in summer or autumn and had a slow disease progression that resulted in their death during winter. We do not know the incubation period, the period between initial infection and development of clinical signs, for *C. gattii* in odontocetes. Humans exposed to *C. gattii* in British Columbia had variable incubation periods that ranged from 6 wk (Lindberg et al. 2007) to 13 mo (Georgi et al. 2009), with a median of 6 to 7 mo (MacDougall & Fyfe 2006, Galanis et al. 2009). Incubation periods in domestic animals are variable (Maccolini et al. 2017); for instance, 2 cats progressed to clinical disease between 4 and 6 mo after exposure to *C. gattii* (Duncan et al. 2005b) and another cat developed disease >8 yr post-exposure (Castrodale et al. 2013).

Other fungal diseases reported in odontocetes include blastomycosis, lacaziosis, and, more recently, mucormycosis (Higgins 2000, Waltzek et al. 2012, Huggins et al. 2020). Previously, mycoses were usu-

ally secondarily associated with immunosuppressive morbillivirus infections and rarely primary epizootics in marine mammals, perhaps because environmental exposure and potential for contagious spread are low; however, epizootic and other data suggest that fungal pathogens are emerging as primary pathogens in odontocetes, particularly in nearshore environments associated with human disturbances such as agriculture, construction, and forestry (Reidarson et al. 2018). Continued monitoring for *Cryptococcus gattii* and other fungal pathogens is important for understanding disease risks for marine mammal populations in the Salish Sea, including endangered southern resident killer whales. Further research is needed to fully characterize the pathogenesis of *C. gattii*-associated cryptococcosis in cetaceans and to examine the seroprevalence of *C. gattii* in cetaceans in order to better understand the risk factors for mortality. Identification of a presumed *C. gattii*-infected Dall's porpoise 2 yr prior to the first case in humans demonstrates how marine mammals can be sentinels for diseases of humans and domestic animals and supports the benefits of taking a 'one-health' approach (Fenton et al. 2017, Mackenzie & Jeggo 2019).

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