NATURALLY OCCURRING ETIOLOGIC FACTORS AFFECTING THE HEALTH OF BREEDING SEABIRDS IN THE BERING SEA

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Abstract

Seabird populations across the globe have experienced both significant instability and consistent overall declines in recent history. Seabirds in the Bering Sea of Alaska, USA appear to be severely affected by environmental changes, exhibiting large-scale shifts in behavior and distribution and increases in unusual mortality events (UMEs) in recent years. I analyze a selection of the naturally occurring pathogenic and toxicological factors affecting breeding seabirds in the Bering Sea region using an approach focusing on zoonoses and bioaccumulating toxins. Specimens were collected at three breeding colonies in the Bering Sea in 2018 and 2019, and were evaluated for the presence of several pathogens and toxins. First, I examined the frequency of Influenza A Virus (IAV) in several understudied clades of seabird host species (n =146 individuals) across the Bering Sea. Second, I used a novel set of genetic amplification and sequencing techniques for metagenomic analysis both to determine the respiratory microbiome and to detect the presence of potentially pathogenic microorganisms in northern fulmars (*Fulmarus glacialis*) on St. Matthew and Hall islands (n = 15). Finally, I sought to evaluate the levels of paralytic shellfish toxins (PSTs) in the digestive tracts of northern fulmars from St. Matthew and Hall islands (n = 14). Together, these studies detected several viral and bacterial pathogens, many with zoonotic potential. These included *Coxiella, Plasmodium, Toxoplasma,* and IAV. PSTs were also detected in birds sampled from 2019, indicating the presence of harmful algae in the Beringian food web. The detection of these etiologic factors along with the incidence of major morbidity and mortality events suggest these birds might serve as sentinel species, indicating variations in environmental change that can pose a significant risk to both ecological stability and human health in the region.

Table of Contents **Page**

List of Figures **Page**

List of Tables **Page**

Chapter 1: Introduction

1.1 The Bering Sea

The Bering Sea is located between Alaska, USA and Russia, and encompasses an estimated 2,304,000 km^2 (Alekseev et al., 2019). This region lies directly below the Arctic Circle and is separated from the much deeper Pacific Ocean by the Aleutian Islands to the south. The Bering Sea is also a comparatively shallow environment, with an average depth of 30 to 50 m (Alekseev et al., 2019). This region experiences dramatic temperature changes accompanied by sea ice formation and thaw cycles annually. The Bering Sea area is also characterized by high primary productivity. This is fueled by the highest levels of nitrate, phosphate, and silicate observed in any marine environment on the globe, and compounded by long summer daylight hours and a perpetual influx of warm, nutrient rich waters from the Pacific Ocean (Aydin & Mueter, 2007). The availability of consumable biomass in this area supports a plethora of higher-trophic-level animals, including many migratory species that travel to this region to breed and remain throughout the summer months (Aydin & Mueter, 2007).

The majority of the Bering Sea is sparsely inhabited by humans, many of which are Alaska Native Peoples residing in rural villages that pre-date modern settlements. Many of these communities are very isolated, with limited transportation opportunities to urban areas of the state. The people in these areas rely heavily on a traditional lifestyle for survival, including subsistence harvest of marine mammals, birds, fish, and invertebrates (Fall et al., 2013; Naves, 2018; Nelson et al., 2019). Although it is remote, the Bering Sea is also home to some of the nation's largest fisheries. In 2019, Dutch Harbor, Alaska topped the charts for the 23rd consecutive year with the highest seafood production in the US at more than 346,000,000 kg valued at $190,000,000. The remaining ports in the Aleutian Islands came in a close second at

more than 267,000,000 kg valued at $142,000,000 (National Oceanic and Atmospheric Adminstration, 2021).

The Bering Sea region has experienced drastic changes in recent years. Within the last decade, this area has seen some of its warmest temperatures on record (Duffy-Anderson et al., 2019; Stabeno et al., 2017; Stabeno & Bell, 2019; Walsh et al., 2017). Primary productivity in the southern portions of this region has decreased significantly, theoretically attributed, in part, to the loss of sea ice cover (Frey et al., 2020; Huntington et al., 2020). In 2017-2019, observations of significantly lower sea-ice cover in the winters and warmer water temperatures year round were recorded in both the northern Bering and Chukchi seas of 2017-2018 and 2018-2019 (Duffy-Anderson et al., 2019; Huntington et al., 2020; Stabeno & Bell, 2019). Current models predict that this warming will increase primary productivity in northern regions and decrease productivity in the southerly subarctic regions. Forecasted outcomes of this trend indicate that a slow and steady decline of biomass in the region is likely under current conditions for much of the Bering Sea (Huntington et al., 2020; Whitehouse et al., 2021).

Alaska hosts more than 20% of the world's seabirds across an estimated 1/3 of the nation's overall coastal habitat (Smith et al., 2014). The Alaska Maritime National Wildlife Refuge (AMNWR) encompasses much of coastal Bering Sea and is home to approximately 40 million breeding seabirds during summer, and an estimated 73% of Alaska's total seabird population. Many of these populations either migrate or disperse during the winter and aggregate in large breeding colonies during summer (Smith et al., 2014). The AMNWR also lies at an intersection of several migratory flyways used by birds from both North America and Eurasia. This includes the West Pacific, East Asian/Australasian, and Pacific Americas flyways (Lycett et

al., 2019). Numerous migratory bird species migrate through this region to breed on an annual basis, creating potential for pathogen transfer to regional seabird species (Lycett et al., 2019).

1.2 Zoonotic Potential

A zoonotic disease is one that can be transmitted between animal species, particularly between humans and other animals. These diseases may be viral, bacterial, or fungal, and can be transmitted through numerous vectors. An estimated 60% of the current diseases affecting humans are zoonotic, and 75% of the emerging infectious diseases observed in humans are animal in origin (Centers for Disease Control and Prevention, 2021b). As human populations expand in both size and geographic range, zoonotic infectious diseases are likely to emerge at an increasing rate (Jones et al., 2008). The concept of OneHealth addresses zoonotic disease and many other health related topics, with a focus on the overall wellbeing of people, animals, and the environment (Centers for Disease Control and Prevention, 2021a). Surveillance of wildlife is critically important to maintain a clear picture of zoonoses from a OneHealth perspective (Bird & Mazet, 2018).

Seabirds also share habitats with numerous other terrestrial and marine species. Contact with other avian species is common in coastal regions at high-density breeding sites (Byrd et al., 2005). Some species of seabirds also have a high degree of interaction with both humans and other mammals. Numerous species of marine mammals haul out in environments with immediate proximity to breeding seabird colonies, including northern fur seals (*Callorhinus ursinus*), walrus (*Odobenus rosmarus*), Steller sea lions (*Eumetopias jubatus*), and bearded seals (*Erignathus barbatus*) (Cameron et al., 2018; Gelatt & Gentry, 2018; Speckman et al., 2011; Sweeney et al., 2018). Arctic foxes (*Vulpes lagopus*), red foxes (*Vulpes vulpes*), and arctic ground squirrels (*Urocitellus parryii*) are also known to share these habitats, and frequently eat

breeding seabirds and their eggs (Bolton et al., 2017; Klein & Sowls, 2015; US Fish and Wildlife Service, 2006).

In the Bering Sea, many human residents use an extensive range of wild animal species as subsistence food sources. Seabirds in the family *Alcidae* are among the most represented avian species in the subsistence harvest of Alaska Native peoples. In the groups of murres and auklets, harvests comprise an estimated 15,000 birds and more than 65,000 eggs annually in the Bering Sea region (Naves, 2018). These seabirds, along with select groups of marine and terrestrial mammals, often comprise a significant portion of the diet of rural Alaskans in the region (Fall et al., 2013; Naves, 2018). Consequently, transmission risk of many zoonotic pathogens is elevated. Several seroprevalence analyses of archived samples from Alaska Natives demonstrated elevated exposure to a number of different pathogens, particularly among people engaging in subsistence harvest (Elmore & Jenkins, 2012; Kersh et al., 2020). Similarly, many etiologic agents have been detected in both humans and animals in the region, indicating that additional interspecific transmission is a possibility (Duncan et al., 2012, 2014; Elmore & Jenkins, 2012; Groom et al., 2014; Kersh et al., 2020; Lefebvre et al., 2016; Reed et al., 2014). Thus, continued subsistence use of birds and marine mammals in this region provides opportunity for zoonotic pathogen exchange (Fall et al., 2013; Naves, 2018; Nelson et al., 2019).

Interactions with fisheries also provide potential for disease transmission to human hosts (Eich et al., 2016). While current traditional subsistence use of northern fulmars (*Fulmarus glacialis*) is minimal, these seabirds are regularly observed interacting with marine industry, and they are among the most commonly recorded avian species on both fishing and at-sea processing vessels in the north Pacific Ocean (Eich et al., 2016; Naves, 2018). These birds consistently

comprise at least half of the avian groundfish bycatch, at a range of 811-7,758 individuals annually (Eich et al., 2016).

1.3 Seabird Population Stability, Morbidity, and Mortality

Seabird populations across the globe have experienced both significant instability and consistent overall decline in recent history (Paleczny et al., 2015). Recent dramatic fluctuations in the climate of the Bering Sea indicate significant changes may be occurring for this ecosystem (Huntington et al., 2020). Several species of seabirds in this region have been affected by these changes, exhibiting large-scale shifts in behavior, distribution, and an increase in unusual mortality events (UMEs; Jones et al., 2019; Kuletz et al., 2020; Robinson et al., 2018; Romano et al., 2020; Van Hemert et al., 2020; Will et al., 2020). From 2015-2020, five seabird UMEs were observed in the Bering Sea, affecting species using a broad range of habitats, trophic levels, and foraging behaviors (Dorresteijn et al., 2012; Hunt et al., 2002; Kokubun et al., 2010; Piatt et al., 2018; Sydeman et al., 2017). The causes of these events appear to be multi-factorial and might include responses to starvation, disease, or toxin exposure (Jones et al., 2019; Robinson et al., 2018; Romano et al., 2020; Van Hemert et al., 2020, 2021; Will et al., 2020).

Major morbidity and mortality events suggest these birds may serve as sentinel species, indicating variations in environmental change that can pose a significant risk to both ecological stability and human health in the Bering Sea region. On a global scale, birds are common carriers of a breadth of zoonoses affecting a large variety of species, and are a unique vector for pathogens affecting humans in particular, due to both their mobility and level of interaction with people across the planet (Contreras et al., 2016). The transmission of these pathogens has caused some of the major epidemics and epizootics in history (Hill et al., 2005; Morens et al., 2019; Petersen et al., 2013; Sutton, 2018). Birds are also a common indicator species of environmental

disturbances, exhibiting a heightened sensitivity to contaminants, disease, or major ecosystem shifts (Smits & Fernie, 2013). Thus birds are both the literal and figurative embodiment of the proverbial "canary in the coal mine" (Pollock, 2016). While Beringian seabirds are unlikely indicators of global conditions, they might serve as indicators of regional ecosystem health. It is for this reason that surveillance of the factors contributing to the health and habitat stability of these species is a crucial component to understanding the health of seabirds, the ecosystem, and the people within it.

1.4 Study Aims

I analyze a selection of the naturally occurring pathogenic and toxicological factors affecting breeding seabirds in the Bering Sea region using a OneHealth approach focusing on zoonoses and bioaccumulating toxins. I did this using 146 seabirds from 17 species. These birds were collected at three breeding colonies throughout the Bering Sea (Causey, pers. comm), and I examined them for the presence of various pathogens and toxins.

Following this general introduction, in chapter two I seek to fill a critical gap in current knowledge of Influenza A Virus (IAV) reservoirs by examining both the frequency and phylogeny of respiratory IAV in several understudied groups of seabird host species from breeding colonies of Beringian seabirds. This region occupies a critical migratory region of reservoir species overlap at the Eurasian to North American boundary. First, I evaluate the frequency and location of IAV infections in avian respiratory tissues from my samples using quantitative polymerase chain reaction (qPCR). Second, I generate sequence data for these positive samples when possible and assess the closest sequence identity to reconstruct the lineages of the isolated viruses. My study provides insight into the transmission dynamics of respiratory IAVs across this important intercontinental region among breeding seabirds, and

provides important baseline information on the IAV risks these birds face during the breeding season.

In chapter three, I use a novel set of genetic amplification and sequencing techniques for metagenomic analysis to determine the respiratory microbiome of the northern fulmar and to detect the presence of potentially pathogenic microorganisms. First, I profile the respiratory microbiota of Beringian northern fulmars. Second, I identify potential respiratory or systemic pathogens carried by these birds. Third, I establish proof of concept for a rapid and accurate portable method of assessing the total genetic composition of an avian microbiome sample. This method has the potential to be used in the field to improve mobile metagenomics.

In chapter four, I evaluate the levels of paralytic shellfish toxins (PSTs) in the digestive tracts of northern fulmars from St. Matthew and Hall islands in the Bering Sea. This study determines the endemic levels of PSTs in breeding northern fulmars of the Bering Sea in 2018 and 2019. I assess the presence of key toxins and create toxicological profiles in these wild, apparently healthy seabirds in one of the largest breeding colonies in the US to address two major objectives. First, I evaluate seabirds as an indicator of ecosystem stability and algal bloom prevalence in the broader ecological food web of Bering Sea. Second, I determine the prevalence of PSTs in one of the most abundant breeding seabirds of Beringia. This information provides valuable baseline data that can be used as an indicator of marine and coastal ecosystem dynamics in the Bering Sea, particularly those affecting both human and animal health and safety.

The goals of this study were created for their potential to contribute to the understanding of both the Bering Sea ecosystem and the population dynamics of the focal seabird species examined. A secondary major goal was to examine factors that might serve as indicators of a both human and overall ecosystem health. This OneHealth approach considers seabirds as

sentinel species in broad-range surveillance to examine emerging threats to the health and stability of the Beringian ecosystem and its occupants in a rapidly changing Arctic.

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Chapter 2: Identification of respiratory Influenza A Viruses in breeding seabirds of the Alaska Maritime National Wildlife Refuge (2018-2019) *

2.1 Abstract

Influenza A Virus (IAV) is among the most lethal global pathogens in modern history and remains a constant public health concern. Waterfowl (Anatidae) are the major reservoir for avian IAVs (also referred to as AIV), and avian migration patterns play a key role in global IAV distribution. Evidence indicates exchange between antigenically distinct Eurasian and North American IAV clades across the Bering Sea, between Alaska and Russia. This region hosts a plethora of seabird species, many of which aggregate in large breeding colonies during summer. These colonies are typically high-density and present a unique ecological niche in which the potential for pathogenic exchange is high. While much is known about waterfowl reservoirs in Alaska, little data exist about seabird species in this region, particularly during the breeding season. Here, we examine these respiratory IAVs in a selection of high-density breeding colonies of Beringian seabirds, at a migratory region of reservoir species overlap. We collected a total of 146 seabirds in 2018 (n = 65) and 2019 (n = 81), representing 17 species. qPCR results indicated an IAV prevalence of 17.8% (n = 26/146). The frequency of these positives varied between years, with most occurring in 2018 (54.8%, n = 23/65) versus 2019 (3.70%, n = 3/81). Best-match alignments of consensus sequences indicated closest identity to IAVs of both North

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American and Eurasian origin, suggesting intercontinental exchange in this region. Monitoring of IAV in seabirds of the Alaskan Arctic provides insight into the dynamics of this virus in an understudied and rapidly changing region.

2.2 Introduction

Influenza A Virus (IAV) is among the most lethal global pathogens in modern history, and remains an ongoing public health concern (Centers for Disease Control and Prevention, 2021). IAVs possess an eight-segmented genomic structure, and reassortment of segments occurs during replication. Reassortment is enhanced by co-infection of different strains and is thought to be the primary driver of the notably fast evolutionary rate of this virus (Shao et al., 2017). While all eight segments consistently recombine to form new viruses during replication, the segments encoding hemagglutinin (HA) and neuraminidase (NA) are the most noteworthy. These facilitate the infection process via both the initial entry into the host cell (HA) and the release of progeny virions (NA) (Dou et al., 2018). Strain pathogenicity is also largely determined by these two surface proteins. This is why IAVs are classified by subtype (HA-NA).

IAVs in birds show 16 HA and 9 NA polymorphisms, named H1-16 and N1-9. Waterfowl (Anatidae) and shorebirds (Scolopacidae) are considered the primary reservoir for IAVs, and these groups possess the highest variation of observed subtypes. Other avian groups such as gulls, noddies, and terns (Laridae) often harbor more specific subtypes (Lang et al., 2016; Wille & Holmes, 2020). Even with ongoing reassortment, some HA-NA combinations occur more frequently than others. Physiological determinants of the host species are also a key factor in IAV replication, and are mediated by factors such as receptor specificity in host cells (Dou et al., 2018). Many possible scenarios for transmission exist, however, and inter- and intra-specific viral coinfections can cause the generation of novel forms of IAV.

Pathogenicity varies by strain and host, and surveillance in both wild and domestic birds is a key component in monitoring and mitigating outbreaks of highly pathogenic avian influenzas (HPAIs), as these epizootics have the potential for tremendous ecological and economic damage. Avian migration plays a key role in the global distribution of IAVs (Causey & Edwards, 2008). Current theory views the distribution of IAVs among wild bird hosts as a somewhat fluid global metapopulation driven by seasonal movements of host species, although phylogeographic analysis of IAV lineages has also found distinct variations between IAVs in Eurasia and those in North America (Lycett et al., 2019). These two lineages appear to be somewhat discrete and encompass all segments and subtypes (Bahl et al., 2013; Webster et al., 1992). Current evidence indicates that they may also produce different antigenic recognition within hosts, even with identical subtypes (Bahl et al., 2013; Wille & Holmes, 2020). Some avian migratory flyways have intercontinental overlap, particularly in the Arctic (Lycett et al., 2019). Eurasian and North American birds mix extensively in Beringia, where intercontinental reassortment of IAVs occur, making Alaska a crucial region for IAV study (Lee et al., 2015; Ramey et al., 2015, 2016; Wille & Holmes, 2020). Furthermore, the current body of evidence increasingly suggests that a significant degree of the exchange between Eurasian and North American IAVs occurs across the Bering Sea, between Alaska and Russia (Jeong et al., 2019; Lee et al., 2015; Ramey et al., 2015, 2016).

The Bering Sea region of Alaska hosts a plethora of seabird species. The Alaska Maritime National Wildlife Refuge (AMNWR) encompasses much of this region and is home to an estimated 40 million breeding seabirds during summer. Annual movements include migration, dispersal and aggregation in large breeding colonies in summer (Smith et al., 2014). These colonies are typically high-density, and are an ecological niche with high potential for

pathogenic exchange (Chen & Holmes, 2009; Lang et al., 2016). These aggregations also have interactions with migratory birds of other species from both continents. While much is known about waterfowl IAV reservoirs in Alaska, little data exist for seabird species in this region.

We seek to expand current knowledge of IAV reservoirs by examining both the frequency and phylogeny of respiratory IAV in several understudied clades of seabird host species. We examine IAVs from high-density breeding colonies of Beringian seabirds, at a migratory region of host species overlap at the Eurasian to North American boundary. First, we evaluate the frequency and location of respiratory IAV infections in seabirds from the Bering Sea. Second, we sequence positive samples and determine the closest matches for the appropriate gene segments. Our study examines the prevalence of respiratory IAVs at a region of intercontinental migration and in the unique habitats of breeding seabirds, providing insight into IAV exposure and infection during breeding.

2.3 Materials and Methods

2.3.1 Field Sampling

Adult breeding seabirds (n = 146) were lethally collected during targeted sampling efforts under USFWS permit MB795841 aboard the USFWS *R/V Tiglax* in the summers of 2018 (n = 65) and 2019 (n = 81) as part of a larger seabird study in the AMNWR. All birds were collected at the major breeding colonies around St. Matthew Island, Hall Island, and Attu Island in the Bering Sea (Figure 2.1). After collection, birds were immediately frozen as whole specimens at -20C aboard the *R/V Tiglax* and transported to the University of Alaska Anchorage (UAA) for necropsy. Lung tissues were aseptically collected in 3-5cm^3 subsamples and stored with 1mL RNALater [Thermo Fisher] at -80C for subsequent genetic analysis.

2.3.2 Detection of IAV

Preserved lung tissues were homogenized by adding sterile glass beads [ThermoFisher], and vortexing for 15 seconds. The resulting homogenate was spun through QIAshredder cell lysate homogenizing columns [QIAGEN] at 12,000 rcf for 2 minutes to increase nucleic acid yield. Total viral RNA (vRNA) was isolated using the QIAamp Viral RNA Mini Kit [QIAGEN] and standard protocols. To detect IAV, a probe-based qRT-PCR was used to target and amplify highly conserved regions of the matrix (M) segment of the viral genome (Spackman et al., 2002). Isolated vRNA from A/WSN/1/1933 (H1N1) was used as a positive control, while nuclease-free water served as the negative control. All samples were analyzed in a randomized fashion, and resulting Ct values of 35 or less were considered "probe-positive" for influenza and were treated as positives.

2.3.3 Full-Genome Sequencing of IAV

Multi-segment RT-PCR (msRT-PCR) was conducted for all IAV probe-positive samples to generate full-length viral complementary DNA (cDNA) amplicons for all eight segments of the IAV genome using universal IAV optimized (opti) primers (Hoffmann et al., 2001). Amplicons were visualized using a Tapestation 4200 Series [Agilent]. Samples exhibiting successful full genome amplification were considered "sequence quality". These underwent library preparation using the LSK-109 and EXP-NBD103 PCR ligation sequencing kit [Oxford Nanopore Technology] and were sequenced on a MinION portable sequencing device [Oxford Nanopore Technology].

2.3.4 Bioinformatic Analysis

Raw sequence data were basecalled using Guppy Basecaller v4.0.11 [Oxford Nanopore Technology], and demultiplexed, trimmed, and filtered to reads longer than 50 nucleotides and

Q scores greater than 90 using Guppy Barcoder v4.0.11 [Oxford Nanopore Technology]. Metagenomic analysis using Kraken2 v2.1.1 taxonomic software (Wood et al., 2019) with a US National Center for Biotechnology Information (NCBI) reference database built by the UAA bioinformatics core was conducted to verify presence and read counts of IAV (Wood et al., 2019). These .fastq files were assessed for mean read lengths of greater than 500 nucleotides for evaluation as candidates for sequence assembly to determine best match using Assembly-stats (Challis, 2017). Alignments of these data were then conducted for each genomic segment for each sequence file with adequate data in *Minimap2* (Li, 2018). These alignments compared each viral segment sequence to a multi-.fasta reference bank of all avian IAV genome segments in the National Institute of Allergy and Infectious Diseases Influenza Research Database. Alignment files were individually assessed for closest sequence match using Samtools idxstats (Li et al., 2009) and Tablet (Milne et al., 2009) to determine the best match sequence for subtype estimates. All samples with sufficient data were analyzed through the Center for Disease Control's Iterative Refinement Meta-Assembler for generation of consensus sequences (Shepard et al., 2016). Consensus sequences generated were run through NCBI's Basic Local Alignment Search Tool (BLAST) to determine isolate lineage.

2.4 Results

We collected a total of 146 seabirds in 2018 (n = 65) and 2019 (n = 81), representing 17 species (Table 2.1). Positive qPCR results indicated an overall IAV prevalence of 17.8% (n = 26/146). The majority of detections occurred in 2018 (54.8%, n = 23/65) versus 2019 (3.70%, n = 3/81) (χ^2 = 22, df = 1, p = 2.7e-06). Of the three locations sampled, the highest prevalence of infection was among seabirds from St. Matthew Island (n = 16/34, 88.9%), with that of birds from Hall Island 10.3% (n = 4/43) and Attu Island 40.0% (n = 6/21) being significantly lower

($\chi^2 = 4.233$, df = 2, $p = 0.1204$). Distribution of positive detections also varied by species, with northern fulmars (*Fulmarus glacialis*), common murres (*Uria aalge*), thick-billed murres (*Uria lomvia*), and crested auklets (*Aethia cristatella*) exhibiting the highest incidence rates (Figure 2.2).

Analysis of sequenced samples ($n = 24$) indicated limited quality and quantity of genomic data. This resulted in a total of four samples for assembly analyses (Table 2.1, 2.2). These samples represented three northern fulmars and one crested auklet, all collected on St. Matthew Island on June 6-7, 2018. Alignments of all four isolates returned the same best matches. Best-match alignments of these samples indicated closest identity to IAVs of both North American and Eurasian origin (Table 2.2). Total HA alignments found best-match identity to A/ostrich/South Africa/IMP/2013(H7N7) at 3.30% coverage, and total NA alignments indicated best match to A/broiler chicken/Iran/MMV9-4/2013(H9N2) at 48% coverage. One of these samples (A/Northern Fulmar/Bering Sea/32/2018) returned two partial assemblies for PA and M segments. Best-match alignments of these samples using NCBI BLAST indicated closest identity to IAVs of both North American and Eurasian origin (Table 2.3). Due to incomplete coverage across all segments for all samples, phylogenetic analysis was not possible.

2.5 Discussion

The prevalence of IAV in the seabird colonies of the Bering Sea during 2018-2019 was relatively high compared to other Alaska IAV surveillance work (Table 2.1). This confirms that breeding colonies for Arctic and Subarctic seabirds can serve as high-transmission microcosms for IAV. IAV frequency varied dramatically by location, and a higher infection rate was observed in 2018 when compared against 2019. St. Matthew Island also had the highest frequency of positive IAV detections. The IAV isolates detected on St. Matthew indicate that this

IAV might have been part of a larger epizootic occurring on a global scale. Although limited in coverage, the best matches for each consensus segment indicated possible Eurasian and North American origins (Table 2.2, 2.3). Ancestors of these segments have likely been circulating for a decade or more and might have been transported across the globe by migrating birds across intersecting flyways, contributing to the global circulation and reassortment of IAVs. While these data are not sufficient to make a clear determination of subtype, this information provides baseline data for our understanding of IAVs and their hosts in Beringia.

Northern fulmars presented with the highest prevalence of IAVs (Figure 2.2). While northern fulmars are not a regular component of any subsistence harvest, they have interactions with fisheries and are the most encountered avian bycatch in commercial fishing. These birds are often recorded at high levels of interspecific interaction with humans at both offshore and shore-based commercial fishing activities (Eich et al., 2016). Current IAV surveillance in northern fulmars is limited, however, prior surveillance in this species also yielded detection rates similar to those we observed (Lang et al., 2016). Interactions with both subsistence harvesters and fisheries in the Bering Sea also provide potential for disease transmission to human hosts, further reinforcing the need for IAV surveillance in rural communities in this region.

Several species of Alcidae also presented with high prevalence (Figure 2.2). Investigations into IAV prevalence in both common and thick-billed murres indicates these species may be susceptible to a broad range of influenzas, and IAVs are often detected at higher rates in these birds than other seabird species (Huang et al., 2014; Ip et al., 2008; Lang et al., 2016). Alcids are also among the most represented species in the subsistence harvest of Alaska Native Peoples. Murre and auklet harvests comprise an estimated 15,000 birds and more than 65,000 eggs on an annual basis in the regions we sampled (Naves, 2018).

Birds used in this study were sampled opportunistically from an existing study, and were not collected exclusively for viral analyses. Therefore, storage methods were not ideal for preservation of the IAV RNA genome. As a result of higher than ideal storage temperatures and multiple freeze/thaw events, RNA was likely degraded during collection, storage, and transport. Overall, the storage methods used for these samples provided limited quality and quantity of genomic data for analysis. Several methods were attempted for re-amplification and further analyses, however, RNA appeared to be severely degraded and recovery of quality isolates was limited. Improved sequence quality of isolates would have increased the quality of analytical results, and allowed for full genome assembly and phylogenic analyses. Future collections efforts of this nature should include onsite sampling methods of euthanized birds, as well as -80C freezing capabilities and virus-compatible preservatives for immediate sample storage.

Wild birds play a key role in the circulation of IAVs, and monitoring these hosts is a crucial component to understanding the dynamics of this virus. Investigation of these viruses in unique habitats with high-risk potential provides insight into IAV transmission with global implications. The potential for zoonotic spillover and overall high evolutionary rate of IAVs make these viruses one of the most concerning zoonotic pathogens on the planet. Continued surveillance in Beringia is necessary to further expand our understanding of these viruses.

2.6 Acknowledgments

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2.8 Figures

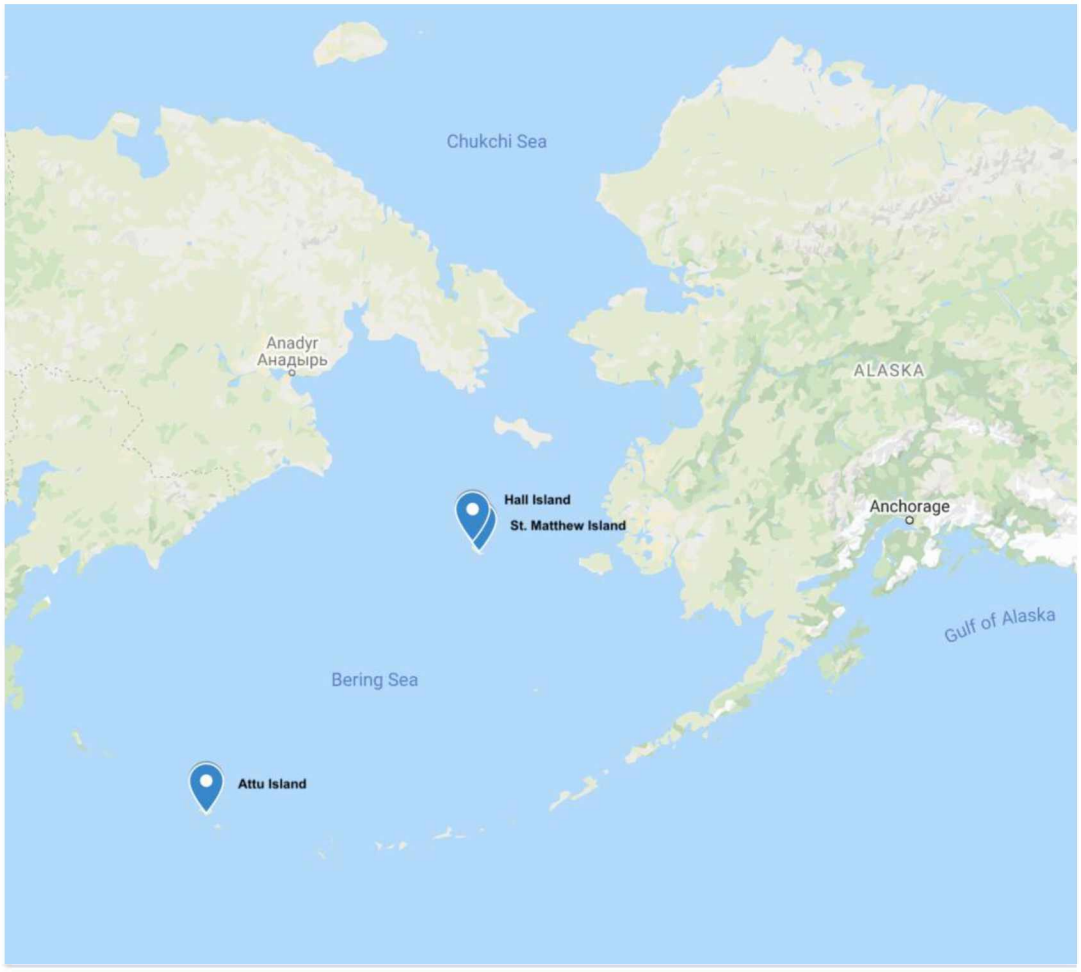

Figure 2.1. Seabirds analyzed in this study (n = 146) were collected at St. Matthew Island, Hall Island, and Attu Island in the Alaska Maritime National Wildlife Refuge aboard the USFWS R/V Tiglax.

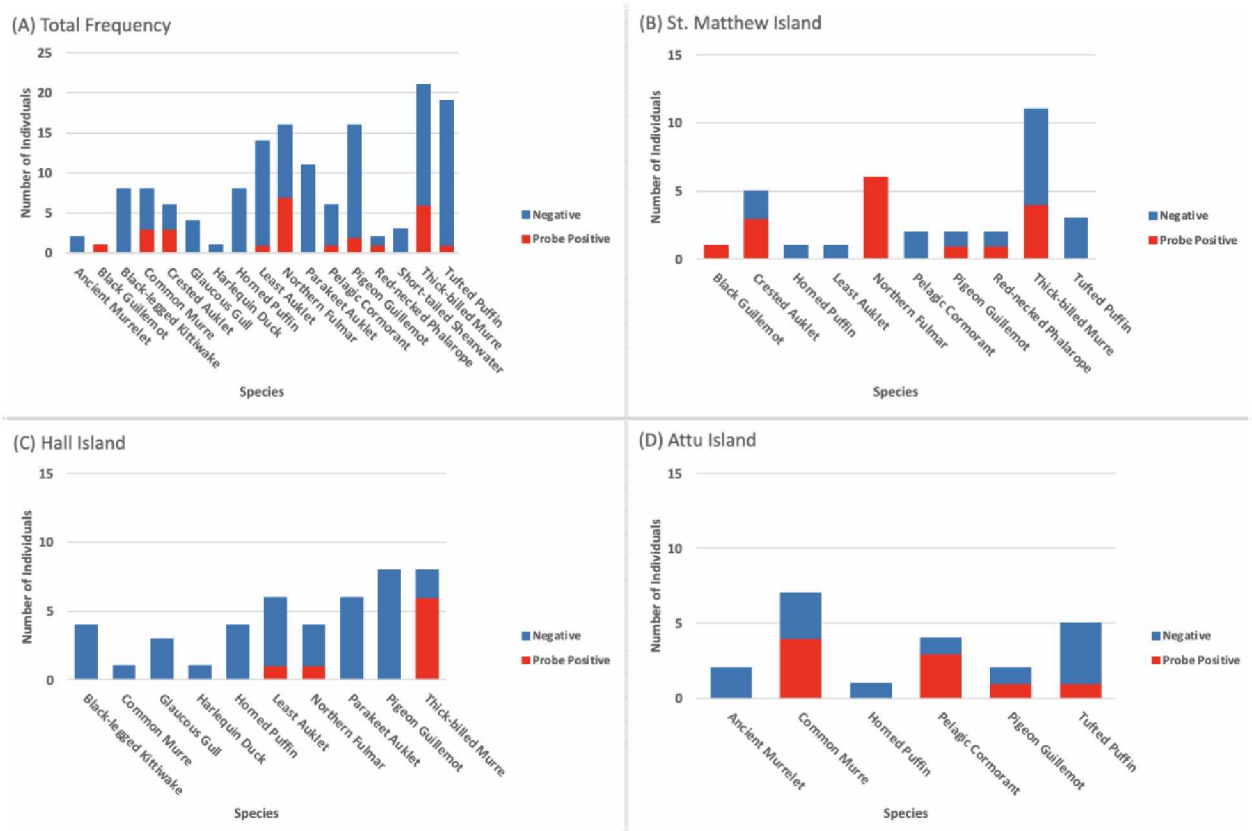

Figure 2.2. Frequency of Influenza A Virus (IAV) positives across sampled species for each sampling location. (A) indicates the total frequency of positives for each species, (B) is the IAV frequency in birds collected on St. Matthew Island, (C) is the IAV frequency in birds collected on Hall Island, and (D) is the IAV frequency in birds collected on Attu Island.

2.9 Tables

Table 2.1. Breeding seabirds (n = 146) collected in the Bering Sea in 2018 and 2019 and evaluated for Influenza A Virus. Data are species evaluated, number of individuals, number of positives, number of sequenced positives, and number of sequences suitable for subtyping.

Family	Species	Individuals Evaluated	Probe Positives	Sequence Positives	Quality Sequences for Subtyping
Alcidae	Ancient Murrelet (*Synthliboramphus antiquus*)	2	0	0	0
	Black Guillemot (*Cepphus grylle*)	1	1	1	0
	Common Murre (*Uria aalge*)	8	3	3	0
	Crested Auklet (*Aethia cristatella*)	6	3	1	1
	Horned Puffin (*Fratercula corniculata*)	8	0	0	0
	Least Auklet (*Aethia pusilla*)	14	1	0	0
	Parakeet Auklet (*Aethia psittacula*)	11	0	1	0
	Pigeon Guillemot (*Cepphus columba*)	16	2	3	0
	Thick-billed Murre (*Uria lomvia*)	21	6	6	0
	Tufted Puffin (*Fratercula cirrhata*)	19	1	0	0
Anatidae	Harlequin Duck (*Histrionicus histrionicus*)	1	0	0	0
Fregatidae	Pelagic Cormorant (*Phalacrocorax pelagicus*)	6	1	1	0
Laridae	Black-legged Kittiwake (*Rissa tridactyla*)	8	0	0	0
	Glaucous Gull (*Larus hyperboreus*)	4	0	0	0
Procelariidae	Northern Fulmar (*Fulmarus glacialis*)	16	7	7	3
	Short-tailed Shearwater (*Ardenna tenuirostris*)	3	0	0	0
Scolopacidae	Red-necked Phalarope (*Phalaropus lobatus*)	2	1	1	0
Totals	17	146	26	24	4

Table 2.2. Avian Influenza A Virus isolates with the highest alignment match for each genomic segment to all samples this study (n = 4). All samples examined for sequence identity were from St. Matthew Island in 2018.

Gene Segment	Subtype	Isolate with the highest sequence identity	Coverage (%)	Average Coverage Depth
PB2		A/barnacle goose/Netherlands/2/2014(H3N6)	28.1	8.7
PB1		A/white-rumped sandpiper/Lagoa do Peixe/RS1167/2012(H6N1)	21.0	10.4
PA		A/wild bird/Chile/1805/2008(H5N9)	9.2	39.1
HA	H7	A/ostrich/South Africa/IMP/2013(H7N7)	3.3	3.9
NP		No Data	No Data	No Data
NA	N2	A/broiler chicken/Iran/MMV9-4/2013(H9N2)	48.0	50.8
M		A/white-rumped sandpiper/Lagoa do Peixe/RS1167/2012(H6N1)	28.4	5.1
NS		No Data	No Data	No Data

Table 2.3. Avian Influenza A Virus partial segment isolates from (A/Northern Fulmar/Bering Sea/32/2018) collected from St. Matthew Island in 2018. Best matches for each genomic segment were determined using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST).

Gene Segment	NCBI Accession #	Isolate with the highest sequence identity	% Identity
PA	N/A	A/mallard duck/Netherlands/26/2010(H4N6)	98.8%
M	OK284600	A/duck/Bangladesh/38827/2019(H11N3)	99.5%

Chapter 3: Profiling the respiratory microbiome of northern fulmars (*Fulmarus glacialis*) in the Bering Sea using metagenomic analysis*

3.1 Abstract

Northern fulmars (*Fulmarus glacialis*) are among the most prevalent seabirds in the subarctic marine regions of Alaska. This species engages in seasonal dispersal within the northern hemisphere, and largely remains in the north Pacific Ocean. Throughout their range, northern fulmars have interactions with both humans and other mammalian species. They are well documented at sites of high anthropogenic activity, in addition to mammalian rookery habitats. Few investigations have explored the microbiome and pathological profiles of these birds. This study examines the respiratory microbiome using lung tissues from lethally collected northern fulmars on St. Matthew and Hall Islands, Alaska (n = 15). Total RNA extracted from these tissues was isothermally amplified with random hexamer universal primers and sequenced using next-generation sequencing. Full metagenomic profiles were generated for each individual and for the study population as a whole. Our study indicates the presence of several potential pathogens that might play a role in disease dynamics in both avian and mammalian species in the Bering Sea. Noteworthy detected genera include: *Coxiella, Escherichia, Plasmodium, Toxoplasma,* and influenza A virus (IAV). IAV (100%) and *Plasmodium* (100%) were the most prevalent pathogens, followed by *Toxoplasma* (64%), and *Coxiella* (in one bird). The presence

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and prevalence of zoonotic respiratory pathogens indicates that this species might serve as a vector for pathogenic organisms. As the levels of movement and interspecific interactions are high in this species, further investigation of its role as a host and in pathogenic transmission is warranted.

3.2 Introduction

Northern fulmars (*Fulmarus glacialis*) are among the most prevalent seabirds in the subarctic marine regions of Alaska. This species has limited seasonal dispersal within the northern hemisphere, and largely remains in the north Pacific Ocean. Birds typically move offshore during winter months and return to breed in high-density terrestrial colonies during the summer (Mallory et al., 2020; US Fish and Wildlife Service, 2006). In Alaska, the majority of northern fulmar breeding occurs at four major colonies in the Bering Sea region. In the summer breeding season, St. Matthew and Hall islands host the second largest of these colonies, at an estimated 450,000 individuals (Mallory et al., 2020).

Throughout their range, northern fulmars have substantial interactions with other species. Contact with other avian species is common in coastal regions at high-density breeding sites (Byrd et al., 2005). Northern fulmars also have a high degree of interaction with both humans and other mammalian species. Numerous species of marine mammals haul out in environments with immediate proximity to northern fulmar colonies, including northern fur seals (*Callorhinus ursinus*), walrus (*Odobenus rosmarus*), Steller sea lions (*Eumetopias jubatus*), and bearded seals (*Erignathus barbatus*) (Cameron et al., 2018; Gelatt & Gentry, 2018; Speckman et al., 2011; Sweeney et al., 2018). Arctic foxes (*Vulpes lagopus*), red foxes (*Vulpes vulpes*), and arctic ground squirrels (*Urocitellus parryii*) are also known to share these habitats, and frequently eat breeding northern fulmars and their eggs (US Fish and Wildlife Service, 2006). While current

traditional subsistence use of northern fulmars is minimal (Naves, 2018), these birds are regularly observed interacting with marine industry, and they are among the most commonly recorded avian species on fishing and at-sea processing vessels in the northern Pacific Ocean (Eich et al., 2016). Northern fulmars consistently comprise at least half of the avian groundfish bycatch, at a range of 811-7,758 individuals annually (Eich et al., 2016).

While much is known about the basic ecology of northern fulmars, few investigations have explored the microbiome of these birds. Emerging research demonstrates northern fulmars may carry a significant disease burden, including from avian cholera (*Pastuerella multocida*) (Bodenstein et al., 2015), avian papillomavirus (Gaynor et al., 2015), lyme disease (*Borrelia burgdorferi*) (Duneau et al., 2008), and influenza A virus (IAV) (Lang et al., 2016). The presence of these pathogens, along with substantial interspecific interactions, establishes these birds as a potential transmission vector for diseases to humans and numerous animal species. Microbiome evaluation in these birds will provide insight into microbiota in subarctic wild birds, and pathogen detection could indicate that this species might serve as a sentinel for broader marine ecosystem health in the Bering Sea.

Here, we use genetic amplification and sequencing techniques for metagenomic analysis both to determine the respiratory microbiome of the northern fulmar and to detect the presence of potentially pathogenic microorganisms. This project has three goals. First, we profile the respiratory microbiota of the Beringian northern fulmar. Second, we identify potential respiratory or systemic pathogens carried by these birds. Finally, we establish a rapid, portable method of assessing the genetic composition of an avian microbiome sample. This method has the potential to be used in the field to improve mobile metagenomics.

3.3 Materials and Methods

3.3.1 Sample Collection and Necropsy

Adult northern fulmars (n = 15) were collected using a shotgun aboard the USFWS *R/V Tiglax* in the summers of 2018 (n = 7) and 2019 (n = 8) as part of a larger seabird study in the Arctic Maritime National Wildlife Refuge of Alaska (Supplementary Table 3.1). All birds analyzed in this study were collected at the major breeding colonies around St. Matthew and Hall islands in the northern Bering Sea (Supplementary Table 3.1). After collection, northern fulmars were immediately frozen as whole specimens at -20C aboard the *R/V Tiglax* and transported to the University of Alaska Anchorage (UAA) for necropsy. Lung tissues were aseptically collected in 3-5cm^3 subsamples and stored with 1 mL RNALater [Thermo Fisher] at -80C for subsequent genetic analysis.

3.3.2 Sample Preparation and Genetic Analysis

Total RNA was extracted from northern fulmar lung tissues using the Invitrogen PureLink RNA Mini Kit and standard protocols [Invitrogen]. This was done with the goal of targeting both RNA organisms (viruses) and ribosomal RNA from bacteria, plants, protozoa, fungi, and archaea. Using extracted sample RNA, an initial DNA digest was incorporated using the ezDNase Kit and protocols to ensure purity of total RNA [Invitrogen]. Following DNA digest, a reverse transcription step was applied. To achieve this, 1 ul of random hexamer primers (6-mers) and 1 ul dNTPs [Invitrogen] were added to the total product and incubated at 65C for 5 minutes to anneal primers. Subsequent to this, 1 ul SuperScript IV Reverse Transcriptase (SSIV RT) and 4ul 5X SSIV RT Buffer were added to the reaction, along with 1 ul 100 uM DTT and 1 ul Ribonuclease Inhibitor [Invitrogen]. A total of 7 ul of this product was transferred to a new reaction tube, and incubated for 10 minutes at 23C (annealing), 50 minutes at 42C (first strand

synthesis), and 10 minutes at 70C (inactivation) to generate complementary DNA (cDNA) via reverse transcription. The final cDNA product was treated with 1ul RNase H [Invitrogen] at 37C for 20 minutes to ensure purity of cDNA product. Following reverse transcription, 5ul purified cDNA was transferred to a new reaction tube, and isothermally amplified with 6-mer primers using the Repli-G Kit and protocols [Invitrogen]. All thermal cycles were conducted on a CFX96 Real Time PCR system [Bio-Rad]. Total amplified product was visualized using a Tapestation 4400 [Agilent], and samples with successful amplification were sequenced using Oxford Nanopore Technology's portable LSK109 MinION next generation sequencer [Oxford Nanopore Technology].

3.3.3 Bioinformatic Analysis

Raw sequence data were basecalled using Guppy Basecaller v4.0.11 [Oxford Nanopore Technology], and demultiplexed, trimmed, and filtered to reads longer than 50 nucleotides and Q scores greater than 90 using Guppy Barcoder v4.0.11 [Oxford Nanopore Technology]. Bioinformatic analysis was conducted using Kraken2 v2.1.1 taxonomic software (Wood et al., 2019) with a US National Center for Biotechnology Information (NCBI) reference database built by the UAA bioinformatics core. Final visualization was completed with the Pavian v1.0.0 metagenomics package in R v3.6.2 (Breitwieser & Salzberg, 2019; R Core Team, 2020), using a concatenated file of total RNA from all birds sampled to produce a full microbial profile. This sequencing and classification method has greater than 99% accuracy at the genus level and 92% accuracy at the species level (Leidenfrost et al., 2020). Alignments of these data were then conducted for suspected pathogen with full species genomes using Minimap2 (Li, 2018) to confirm rRNA amplification. Data are deposited in the NCBI Sequence Read Archive (SRA) data repository (Supplementary Table 3.1).

3.3.4 Pathogen Validation

Prior to constructing a full RNA microbiome for all birds evaluated, a metagenomic profile was generated from each individual sample to identify unique pathogens on an individual level. IAV qPCR (Spackman et al., 2002) was conducted in the remaining lung tissues of all animals for a concurrent avian influenza study (Branson et al., Chapter 2). Due to limited tissue availability, this was used as a metric to validate the potential pathogens detected in this metagenomic analysis.

3.4 Results

Successful amplification occurred in 11 of the 15 northern fulmar lung tissue samples. A total of 305,102 non-host reads were recorded for all samples analyzed, and 221,168 of these reads were classified using the reference database (Table 3.1). These reads encompassed several major clades, including bacteria, plants, protozoa, viruses, fungi, and archaea. We focus on bacteria, protozoa, viruses, and fungi as indicators of the microbiome. Visualization utilized a single file of total RNA from all birds sampled to produce a full microbial profile (Figure 3.2). At the genus level, this profile included seven bacterial identifications, two protozoal identifications, and one viral identification. A single fungal identification was also included at the phylum level (Figure 3.2).

Genera identified at a 99% accuracy level with zoonotic potential included: *Coxiella, Plasmodium, Toxoplasma,* and IAV (Table 3.2). Further exploration of these genera identified species at the 92% accuracy level including *Coxiella burnetti*, *Toxoplasma gondii,* and nine species of *Plasmodium* (Supplementary Table 3.2). Alignments of these samples with full genomes from NCBI confirmed rRNA amplification for all suspected pathogens. Of these pathogens, qPCR validations were conducted for IAV (Table 3.2). As the only viral pathogen

detected in these samples, IAV was detected with both qPCR and the Repli-G method in more than half of the individuals sampled for this study (Table 3.2). A total of 11 samples demonstrated successful IAV amplification using Repli-G, while 8 of the same samples exhibited successful IAV amplification using qPCR (Table 3.2). Those samples that did not amplify were the same across both detection methods. Lack of amplification in 4 of the 15 northern fulmars for both Repli-G and qPCR was likely due to RNA degradation during storage or processing. This indicates a strong correlation between detection using qPCR and Repli-G amplification methods for detection. Sequencing and phylogenetic analyses of IAV was conducted in a concurrent IAV study in these individuals. These analyses determined that the IAVs sequenced were of both Eurasian and North American origins; these results are detailed in Chapter 2 of this dissertation.

3.5 Discussion

Lung microbiomes in northern fulmars from St. Matthew and Hall islands indicate a diverse number of organisms, many of which are unknown in both source and behavior. The breadth and classification of these genera suggest a relatively normal host microbiome. We focus on the prevalence of pathogens in this analysis. Detection of potentially pathogenic genera indicate a health concern for avian, mammalian, and human populations alike and suggests the need for further surveillance. Species-level identification indicated *Coxiella burnetti*, a common zoonotic pathogen known to cause widespread disease in numerous human and animal populations (Supplementary Table 3.2). *C. burnetti* has been detected in numerous species of seabirds and seabird parasites, as well as in environmental samples within the Bering Sea region (Duncan et al., 2012, 2013, 2014). Recent indications of a possible *C. burnetti* epizootic in Beringian northern fur seals (Duncan et al., 2012, 2014) have led to concerns for population

stability in this species and to broader public health concerns for zoonotic transmission of this pathogen to humans. In the Bering Sea, many human residents use an extensive range of wildlife species as subsistence food sources (Fall et al., 2013). Seroprevalence analyses of archived samples from Alaska Natives in this region demonstrated significantly elevated evidence of exposure to *C. burnetti*, particularly in those people engaging in subsistence harvest practices (Kersh et al., 2020).

IAV can also pose a threat to the stability of avian health, particularly within the high-transmission environments of breeding colony sites or in those regions with extensive migratory routes (Wille & Holmes, 2020). Due to the segmented genomic structure of IAV, both reassortment and mutation are a regular component of viral evolution, and recombination to form novel IAVs is a distinct possibility (Shao et al., 2017; Wille & Holmes, 2020). This viral evolution is of elevated concern due to the location of the Bering Sea at the Eurasian and North American interface. This region represents a crossroads between two antigenically distinct IAV lineages (Lee et al., 2015; Ramey et al., 2015, 2016). Therefore, both exchange and mutation of viruses carried across this space by migratory birds further exposes the region's seabirds to IAV risk. Pathogenic spillover of these novel viruses to humans is rare (Wille & Holmes, 2020) but remains a possibility, particularly among those interacting with northern fulmars in either subsistence or commercial settings.

Both *Toxoplasma* and *Plasmodium* are notable protozoal pathogens in avian and mammalian hosts. *T. gondii*, present with 64% prevalence in Beringian northern fulmars (Supplementary Table 3.2), has been detected across numerous avian species encompassing most orders (Dubey et al., 2021; Khademvatan et al., 2013; Wilson et al., 2020) and is a well-known zoonotic pathogen in mammals (Dubey, 2008; Elmore & Jenkins, 2012). In Alaska, numerous

species of terrestrial mammals have demonstrated either seropositivity or active infection (Elmore & Jenkins, 2012). Alaska Natives in rural and subsistence-based communities have also been recorded with significant levels of *Toxoplasma* seropositivity (Elmore & Jenkins, 2012). While not a likely zoonosis from avian to mammalian hosts, species belonging to the genus *Plasmodium* cause avian cholera. This is a prevalent avian pathogen in Alaska, and regular bird-to-bird transmission is likely (Loiseau et al., 2012; Meixell et al., 2016; Wilkinson et al., 2016). Recorded incidences of *Plasmodium* in subarctic seabirds are limited, and our study provides evidence for *Plasmodium* in this group at a high prevalence (100%).

Many of the other detected genera might play an important role in respiratory health. Some well-documented aquatic genera found in northern fulmars, such as *Halomicronema*, *Flavobacterium*, and *Agrobacterium* may be incidental to the consumption of marine organisms and ingestion of water (Liu et al., 2020; Mishra et al., 2018). We postulate that this was also likely the case with the numerous plant-based reads excluded from this analysis, which may have represented phytoplankton in the surrounding seawater. Organisms belonging to phyla *Actinobacteria*, *Bacteroides*, *Proteobacteria*, and *Firmicutes* (Table 3.2) have all been detected in other avian species (Simon et al., 2016; Škaraban et al., 2017). Fungal data did not yield specific enough classifications to make any inferences. Our pathogenic validations establish correlation between this metagenomic method and targeted qPCR detection, confirming the utility of this method for conducting comprehensive profiling of total microbiota.

Our description of the respiratory microbiome among northern fulmars, including the presence and prevalence of potential zoonotic pathogens, provides insight into the occurrence of these pathogens in arctic and subarctic coastal ecosystems. Detections of similar pathogens in a variety of mammalian and avian hosts suggests a large-scale transmission of these organisms

among wildlife. Seasonal movements of these animals further complicate transmission dynamics within-year and long-term, particularly in the rapidly changing environments of the coastal arctic and subarctic. Additionally, human-wildlife interaction in this region provides opportunity for zoonotic pathogen exchange (Fall et al., 2013; Eich et al., 2016; Naves, 2018; Nelson et al., 2019).

This project is a unique application that has potential to be used in the field (e.g., aboard research vessels, farms, mobile clinical settings, remote research stations, etc.) as an initial pathogen screening method (Gigante et al., 2020; Quick et al., 2016; Acharya et al., 2020; Rambo-Martin et al., 2020). Situations from epidemiological investigations, wildlife unusual mortality events, and environmental monitoring to both human and animal clinical scenarios can benefit from the application of this technique as a microbiome assessment method.

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3.8 Figures

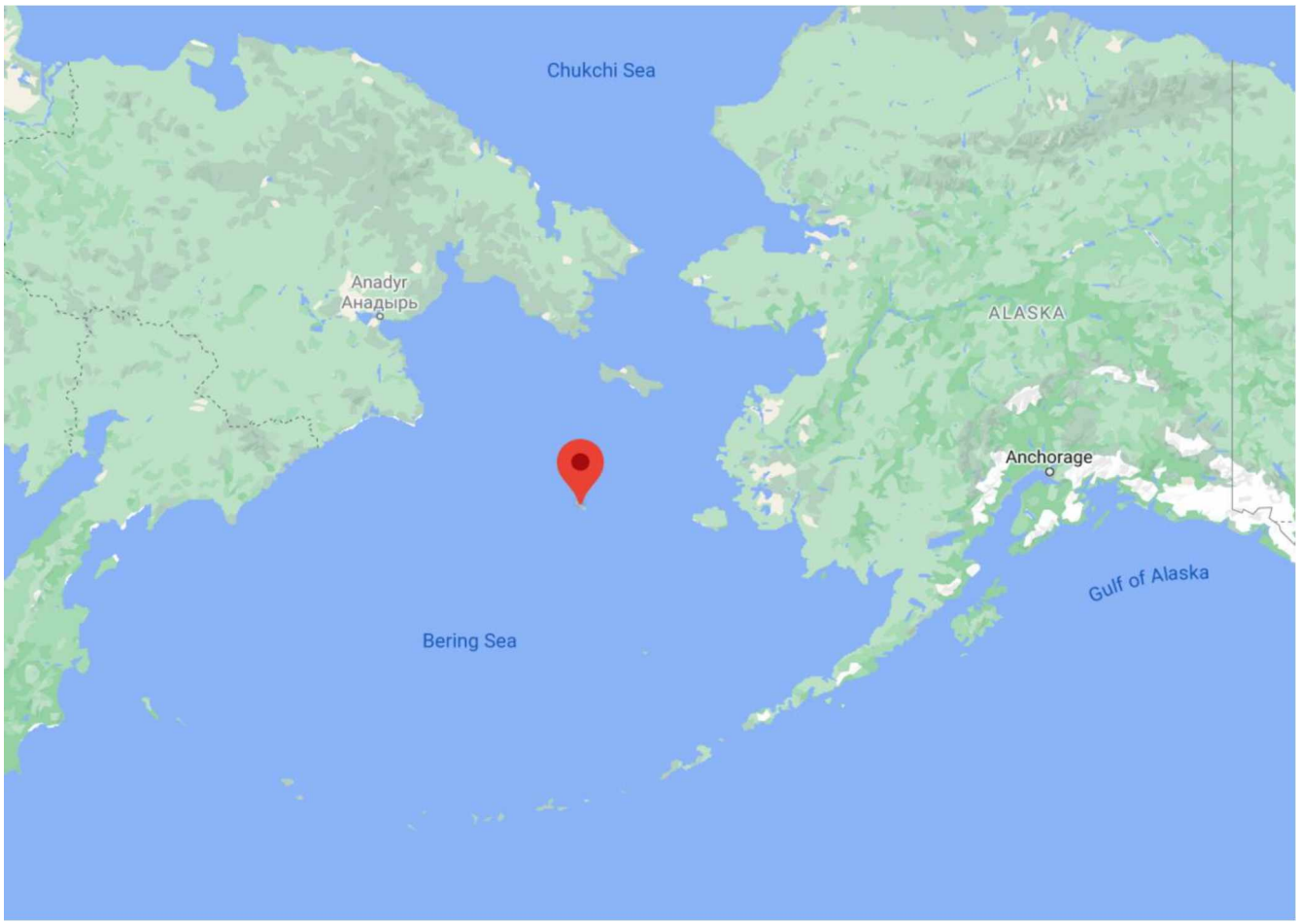

Figure 3.1. Northern fulmars (*Fulmarus glacialis*) analyzed in this study (n = 15) were from St. Matthew and Hall islands, Alaska in June and July of 2018 and 2019.

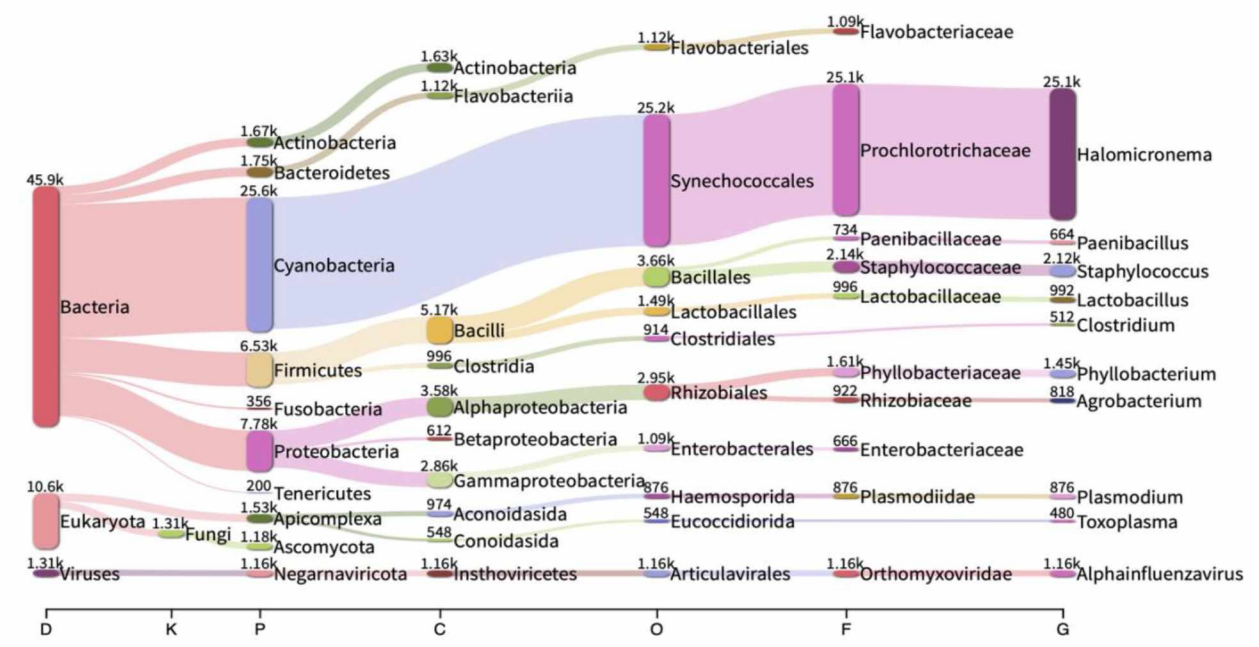

Figure 3.2. Respiratory microbiome of the Beringian northern fulmar (n = 11) using total RNA collected from lung tissue. Data are read counts versus taxonomic hierarchy (D = Domain, K = Kingdom, P = Phylum, C = Class, F = Family, O = Order, and G = Genus).

3.9 Tables

Table 3.1. Read counts for total RNA sequenced from northern fulmar (*Fulmarus glacialis*) lung tissues (n =11) sampled in the Bering Sea, Alaska (2018-2019). Shaded regions (Total, Classified, and Unclassified reads) represent overall sequencing data, while unshaded regions (Bacterial, Protozoal, Viral, and Fungal reads) indicate major clades selected for further analyses.

Total	Classified	Unclassified	Bacterial	Protozoal	Viral	Fungal
305,102	221,168	83,934	45,880	1,568	1,310	1,306

Table 3.2. Detected genera with pathogenic potential in total RNA sequenced from northern fulmar (*Fulmarus glacialis*) lung tissues (n = 15) sampled in the Bering Sea, Alaska (2018-2019). A positive metagenomic analysis detection (MA = +) indicates read counts of 5 or greater. qPCR results are reported as Ct (Cycle threshold) values. A positive qPCR result indicates Ct < 35 using probe-based detection. No qPCR amplification is reported as (NA = No Amplification). Samples that did not yield adequate sequence data (ND = No Data) were excluded from further analysis.

Sample	*Coxiella*	*Toxoplasma*	*Plasmodium*	Influenza A	
	MA	MA	MA	MA	qPCR
Total RNA Read Count	88	480	812	1158	
2018-31	-	-	+	+	33
2018-32	-	-	+	+	31
2018-33	-	-	+	+	34
2018-34	-	+	+	+	34
2018-35	-	+	+	+	32
2018-48	-	+	+	+	35
2019-17	-	-	+	+	NA
2019-29	-	+	+	+	36
2019-30	ND	ND	ND	ND	NA
2019-31	ND	ND	ND	ND	NA
2019-39	ND	ND	ND	ND	NA
2019-40	+	+	+	+	NA
2019-41	ND	ND	ND	ND	NA
2019-50	-	+	+	+	35
2019-51	-	+	+	+	NA

Supplementary Table 3.1. Metadata for successfully sequenced Beringian northern fulmar (*Fulmarus glacialis*) lung tissue samples (*n* =11). Sequences have been archived in the US National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) under database accession number PRJNA765567. SMI = St. Matthew Island, AK, USA, HI = Hall Island, AK, USA

Sample ID	Sample Accession	Organism	Host	Isolation Source	Collection Date	Location	Lat/Long
201831	SAMN21566271	Metagenome	northern fulmar	Lung Tissue	2018-06-06	SMI	60.442463319856906 N, -172.73885482325672 W
201832	SAMN21566272	Metagenome	northern fulmar	Lung Tissue	2018-06-06	SMI	60.442463319856906 N, -172.73885482325674 W
201833	SAMN21566273	Metagenome	northern fulmar	Lung Tissue	2018-06-06	SMI	60.442463319856905 N, -172.73885482325673 W
201834	SAMN21566274	Metagenome	northern fulmar	Lung Tissue	2018-06-06	SMI	60.442463319856905 N, -172.73885482325674 W
201835	SAMN21566275	Metagenome	northern fulmar	Lung Tissue	2018-06-06	SMI	60.442463319856906 N, -172.73885482325673 W
201848	SAMN21566276	Metagenome	northern fulmar	Lung Tissue	2018-06-07	SMI	60.442463319856905 N, -172.73885482325672 W
201917	SAMN21566277	Metagenome	northern fulmar	Lung Tissue	2019-07-24	HI	60.68717727881287 N, -173.07260808397132 W
201929	SAMN21566278	Metagenome	northern fulmar	Lung Tissue	2019-07-25	SMI	60.442463319856907 N, -172.73885482325673 W
201940	SAMN21566279	Metagenome	northern fulmar	Lung Tissue	2019-07-26	SMI	60.442463319856903 N, -172.73885482325673 W
201950	SAMN21566280	Metagenome	northern fulmar	Lung Tissue	2019-07-27	HI	60.68717727881287 N, -173.07260808397133 W
201951	SAMN21566281	Metagenome	northern fulmar	Lung Tissue	2019-07-27	HI	60.68717727881287 N, -173.07260808397134 W

Supplementary Table 3.2. Genus and species level classifications for non-viral pathogens detected in Beringian northern fulmar (*Fulmarus glacialis*) lung tissues (*n* =11).

Genus	Species	
Coxiella	*burnetti*	Duncan et al., 2013; Wilkinson et al., 2014, Tokarevich et al., 2019
Toxoplasma	*gondii*	Dubey et al., 2021; Khademvatan et al., 2013; Wilson et al., 2020
Plasmodium	*vivax* *malariae* *coatneyi* *cynomolgi* *relictum* *berghei* *chabaudi* *yoelii* *knowlesi*	Loiseau et al., 2012; Meixell et al., 2016; Wilkinson et al., 2016

Chapter 4: Paralytic shellfish toxins in the digestive tracts of northern fulmars (*Fulmarus glacialis*) in the Bering Sea 2018-2019*

4.1 Abstract

Harmful algal blooms (HABs) are of increasing concern in coastal Alaskan ecosystems. In the Bering Sea of Alaska, commonly recorded algal genera include a suite of potentially harmful phytoplankton. Of these, *Alexandrium* spp. are among the most frequently observed. *Alexandrium* spp. are dinoflagellate algae known to produce harmful paralytic shellfish toxins (PSTs), which are defined as saxitoxin (STX) and its congeners. Recent dramatic fluctuations in the climate of the Bering Sea suggest substantial changes might be on the horizon for this ecosystem, including a potential increase in HABs. Seabirds in this region appear to be affected by these climatic changes, exhibiting large-scale shifts in behavior and distribution, and an increase in unusual mortality events. Northern fulmars are commonly used as a sentinel species to monitor the health of the Arctic marine ecosystems, and we focus on this species in the Bering Sea region. We examined the levels of PSTs in the digestive tracts of northern fulmars (n = 14) from St. Matthew and Hall islands in the Bering Sea of Alaska, USA in June-July of 2018 and 2019. None of the northern fulmars collected in 2018 (0%, n = 0/6), and half of those from 2019 (50%, n = 4/8) had detectable quantities of PSTs. Three had measurable levels of STX (4.08 $\pm$ 0.335 μg/100g), and one had measurable GTX4 (2.96 μg/100g). These detections reflect the

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presence of PSTs in the Bering Sea during 2019, suggesting the occurrence of a harmful algal bloom during 2019.

4.2 Introduction

Harmful algal blooms (HABs) are becoming of increasing concern in coastal Alaskan ecosystems, ranking as the number one zoonotic health concern by the Alaskan Centers for Disease Control One-Health working group (Centers for Disease Control and Prevention, 2019). These blooms can comprise a variety of algal species, which synthesize biotoxins that cause health risks to both humans and wildlife. In the Bering Sea, commonly recorded HABs include a suite of phytoplankton genera. Of these, *Alexandrium* spp. are among the most frequently observed (Anderson et al., 2021). *Alexandrium* spp. are a dinoflagellate algae known to produce harmful paralytic shellfish toxins (PSTs), which are defined as saxitoxin (STX) and a suite of structurally related neurotoxins. These neurotoxins function by blocking sodium channels and inhibiting the generation of neuronal action potential, resulting in the acute paralytic condition known as paralytic shellfish poisoning (PSP) when ingested (Anderson et al., 2021; Wiese et al., 2010). PSTs are most commonly observed in lower aquatic trophic levels, and are regularly found in shellfish, mollusks, and fish (Deeds, 2008). However, limited bioaccumulation of these toxins has been recorded in higher trophic levels of both aquatic and terrestrial organisms, indicating the potential for substantial risk to coastal and marine fauna (Lefebvre et al., 2016; Turner et al., 2018; Van Hemert et al., 2020). This includes humans utilizing coastal marine resources for subsistence or recreational harvests (Anderson et al., 2021).

On a global scale, PSTs are responsible for health concerns across many coastal and marine species, in addition to widespread human health, ecological, and economic damage (Anderson et al., 2021; Centers for Disease Control and Prevention, 2019). Across Alaska,

elevated levels of PSTs have caused several cases of human illness and even death in shellfish harvesters (Anderson et al., 2021). *Alexandrium* spp. blooms have been documented in the Bering Sea and are regularly observed in coastal habitats (Deeds, 2008; Harley et al., 2020; Natsuike et al., 2013; Trainer et al., 2014). In Alaska, HAB monitoring programs frequently record biotoxin levels well above the regulatory limits in shellfish tissues (Harley et al., 2020; Trainer et al., 2014). While several of these monitoring programs exist for human health and shellfish consumption in Alaska, investigation of harmful algae in wildlife remains a relatively novel field.

HABs have been documented in coastal Alaska; however, recent data demonstrate both an increase in frequency and an expansion of range for many algal species (Gobler, 2020; Natsuike et al., 2013). While current data suggest several components as primary drivers for HABs, the causes of these blooms are still unclear. Several environmental conditions have been identified prior to HAB events, and evidence is emerging to suggest that climate change has contributed to the increase in the duration, frequency, and geographical distribution of HABs (Gobler, 2020). Warmer temperatures are a dominant driver of many of the recorded ecological perturbations contributing to HABs. Thus, the increase in HABs is hypothesized to worsen as climate change continues to rapidly alter ecosystem balance (Gobler, 2020). Alaskan waters have recently seen some of the warmest temperatures on record (Stabeno et al., 2017; Walsh et al., 2017).

Dramatic fluctuations in the climate of the Bering Sea indicate long-term changes may be on the horizon for this ecosystem, including a potential increase in HABs. Seabirds are affected by these changes, exhibiting large-scale shifts in behavior and distribution and an increase in unusual mortality events (UMEs) (Jones et al., 2019; Kuletz et al., 2020; Robinson et al., 2018;

Romano et al., 2020; Van Hemert et al., 2020; Will et al., 2020). From 2015-2020, five seabird UMEs occurred in the Bering Sea (Jones et al., 2019; Robinson et al., 2018; Romano et al., 2020; Van Hemert et al., 2020; Will et al., 2020). While the causes of these events are still unknown, they appear to be multi-factorial and may include starvation, disease, or toxins (Jones et al., 2019; Robinson et al., 2018; Romano et al., 2020; Van Hemert et al., 2020, 2021; Will et al., 2020). Harmful algal toxins, including PSTs, have been documented in several species of Beringian seabirds (Van Hemert et al., 2020, 2021) and marine mammals (Lefebvre et al., 2016), indicating that toxin loads in the food web are causing bioaccumulation.

We evaluated the levels of PSTs in the digestive tracts of northern fulmars from St. Matthew and Hall islands in the Bering Sea of Alaska, USA during 2018 and 2019. We assessed the presence and toxicological profiles in these sub-clinical birds at one of the largest breeding colonies in the U.S. to address two objectives. First, we evaluate northern fulmars as indicators of algal bloom presence in the ecological food web of the Bering Sea at this time. Second, we determine the prevalence of PST in these northern fulmars. Our results provide valuable data useful as an indicator of the health of the marine and coastal ecosystem of the Bering Sea, a region of considerable importance to wildlife and U.S. seafood production (Smith et al., 2014).

4.3 Materials and Methods

4.3.1 Sample Collection and Necropsy

Adult northern fulmars (n = 14) were collected using a shotgun during sampling aboard the USFWS *R/V Tiglax* in June and July of 2018 (n = 6) and 2019 (n = 8) in the Arctic Maritime National Wildlife Refuge of Alaska, USA. These birds were collected at the major breeding colonies around St. Matthew and Hall islands in the northern Bering Sea (Figure 4.1). All birds were taken while active and in visibly healthy condition. After collection, the birds were

immediately frozen as whole specimens at -20C aboard the *R/V Tiglax* and transported to the University of Alaska Anchorage (UAA) for laboratory necropsy. Gastrointestinal (GI) contents were aseptically collected in 1-5 mL subsamples and stored at -80C for subsequent analyses.

4.3.2 High Pressure Liquid Chromatography Post Column Oxidation Analysis (HPLC PCOX)

GI subsamples were analyzed for PSTs at the Alaska Department of Environmental Conservation Environmental Health Laboratory (DEC EHL) in Anchorage, AK, USA. Extraction and analysis with High Pressure Liquid Chromatography Post Column Oxidation (HPLC PCOX) were conducted according to Association for Official Analytical Chemists (AOAC) methods for PSTs (Van De Riet et al., 2011). PST congeners targeted for this assay included 12 chemical structures most commonly observed in association with the harmful algae species recorded in Alaska (Deeds, 2008). These include the following toxins, in order of least to greatest toxicity (Deeds, 2008): sulfocarbomyl toxins 1-2 (C1-2), decarbamoylated gonyautoxins 2-3 (dcGTX2-3), decarbamoylated saxitoxins (dcSTX), gonyautoxins 1-5 (GTX1-5), neosaxitoxin (NEO), and saxitoxin (STX).

4.3.3 QA/QC

Quality assurance and quality control were conducted using AOAC protocols detailed in (Van De Riet et al., 2011). Technique-specific sample concentration reporting limits of detection (RL) for HPLC PCOX were unique to the DEC EHL, and were calculated using eight repeated measurements of each sample to generate a mean sample value. Assay buffer was used to create a zero standard, and sample values greater than two standard deviations from the mean of the zero standard were considered above the RL. RLs for each congener were as follows (all in units of μg/100g): C1 < 0.0990, C2 < 0.476, dcGTX2 < 1.18, dcGTX3 < 0.845, dcSTX < 2.58, GTX1

< 8.62, GTX2 < 2.17, GTX3 < 1.64, GTX4 < 1.99, GTX5 < 0.558, NEO < 9.25, and STX < 5.05. Results were recorded on a wet weight basis as μg/100 g.

4.3.4 Statistical Analysis

Prevalence of PST congeners was assessed for northern fulmars collected in 2018 and 2019. Morphometric data were recorded for northern fulmars collected in 2018 and 2019 and included sex, body mass, and spleen mass when available to measure. These were used to calculate the splenic-somatic index [spleen mass/(body mass-spleen mass)*100]. These data were evaluated to compare splenic-somatic index across birds with and without a positive PST detection in 2019 using a two-tailed t-test with 95% confidence interval in R v3.6.2 (R Core Team, 2020).

4.4 Results

None of the northern fulmars collected in 2018 (0%, n = 0/6) had measurable concentrations of any of the PST congeners evaluated (Table 4.1). The presence of at least one congener was detected in half of the individuals examined in 2019 (50%, n = 4/8). Of these, three had measurable concentrations of STX, and one had a measurable concentration of GTX4 (Table 4.1). All of these had measurable and quantifiable concentrations, however all three STX measurements fell below the RL for this assay. Mean ($\pm$SD) STX concentration from positive samples was (4.08 ± 0.335 μg/100g). The single bird with GTX4 showed 2.96 μg/100g. None of the other PST congeners tested were detected for any birds examined (Table 4.1).

Analysis of the morphometrics found that PST-positive birds had mean ($\pm$SD) splenic-somatic index values of 0.079 ($\pm$ 0.035) and individuals with no detectable levels of PSTs had somewhat lower splenic-somatic indices (0.040 $\pm$ 0.012), but these differences were not significant (Table 4.2).

4.5 Discussion

Our study indicates the absence of PSTs at detectable levels in Beringian northern fulmars during 2018, and the presence at levels nearing the lower reportable limit of detection during 2019. Although these data are largely indicative of an ecosystem with substantially low or absent harmful algal toxins in higher trophic levels, our findings from 2019 suggest the occurrence of at least one HAB event within the St. Matthew and Hall islands region during the sample period. Coincident with this evidence, there were observations of lower sea ice, warmer waters, and seabird die-offs in this same year (Duffy-Anderson et al., 2019; R. Kaler, personal communication, 2020).

Although detected quantities of STX were below RL for the accuracy of the analytical technique, they were within ranges observed in several Alaskan seabird studies, e.g., 1.3-4.6 μg/100 g in sub-clinical common murres (*Uria aalge*) and black-legged kittiwakes (*Rissa tridactyla*) from the Gulf of Alaska during 2015-2017 (Van Hemert et al., 2020). Our values were also within the 2.1-11.1 μg/100 g range found in dead northern fulmars from a Bering Sea UME in 2017 (Van Hemert et al., 2021). Similar quantities of STX were also found in the stomach, livers, and muscle tissue of these birds, indicating that a relatively acute systemic toxicity is possible at these exposure levels. Limited data exist on PST concentrations in wild birds, but our data, coupled with those of Van Hemert et al. (2021), suggest the potential for PST toxicity in lethal concentrations in Bering Sea fulmars.

Splenic sizes in birds are strongly correlated with immune function and are often used as an indicator of general health (Fairbrother et al., 2004; Powers, 2000; Smith & Hunt, 2004). Changes in splenic-somatic indices have been recorded with exposure to numerous antigenic agents, including viruses, parasites, and toxins (Dänicke et al., 2011; Fairbrother et al., 2004;

Fernie et al., 2005; Marteinson et al., 2017; Wang et al., 2009). Although our sample sizes were limited, the observed trend was concordant with this prior work. Future study efforts should include surveillance of PSTs in multiple trophic levels in the Bering Sea.

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4.8 Tables

Table 4.1. Paralytic shellfish toxin (PST) congener profiles for northern fulmars (*Fulmarus glacialis*) sampled in June and July of 2018 and 2019 at St. Matthew and Hall islands, Alaska (n = 14). Sulfocarbomyl toxins 1-2 (C1-2), decarbamoylated gonyautoxins 2-3 (dcGTX2-3), decarbamoylated saxitoxins (dcSTX), gonyautoxins 1-5 (GTX1-5), neosaxitoxin (NEO), and saxitoxin (STX). All concentration values are expressed in μg/100 g.

Year	Sample ID	C1	C2	dcGTX 2	dcGTX 3	dcSTX	GTX 1	GTX 2	GTX3	GTX 4	GTX5	NEO	STX
2018	31	-	-	-	-	-	-	-	-	-	-	-	-
2018	32	-	-	-	-	-	-	-	-	-	-	-	-
2018	33	-	-	-	-	-	-	-	-	-	-	-	-
2018	34	-	-	-	-	-	-	-	-	-	-	-	-
2018	35	-	-	-	-	-	-	-	-	-	-	-	-
2018	48	-	-	-	-	-	-	-	-	-	-	-	-
2019	17	-	-	-	-	-	-	-	-	-	-	-	3.70
2019	29	-	-	-	-	-	-	-	-	2.96	-	-	-
2019	30	-	-	-	-	-	-	-	-	-	-	-	-
2019	31	-	-	-	-	-	-	-	-	-	-	-	-
2019	40	-	-	-	-	-	-	-	-	-	-	-	-
2019	41	-	-	-	-	-	-	-	-	-	-	-	-
2019	50	-	-	-	-	-	-	-	-	-	-	-	4.19
2019	51	-	-	-	-	-	-	-	-	-	-	-	4.34
Mean (±SD) Congener Concentration										2.96			4.08 ±0.335

Table 4.2. Metrics associated with northern fulmars (*Fulmarus glacialis*) sampled in June and July of 2019 in St. Matthew and Hall Islands, Alaska (n = 8). These include sex, spleen mass, body mass, splenic-somatic index, and Paralytic Shellfish Toxin (PST) detection (P = positive for PSTs, N = Negative for PSTs).

Sample ID	Sex	Spleen Weight (g)	Body Weight (g)	Splenic-Somatic Index (%)	PST
17	Male	0.300	950	0.032	P
29	Female	0.800	698	0.115	P
30	Male	0.300	761	0.039	N
31	Male	0.500	905	0.055	N
40	Female	0.300	747	0.040	N
41	Female	0.200	756	0.026	N
50	Female	0.600	712	0.084	P
51	Female	0.600	704	0.085	P

Chapter 5: Conclusion

5.1 General Conclusion

My results provide important insight into the naturally occurring pathogens and toxins in breeding seabirds in the Bering Sea. In chapter two, I examined incidences of respiratory IAV at several seabird colonies in the region, including St. Matthew Island, Hall Island, and Attu Island. I found the presence of respiratory IAVs circulating in numerous species of birds. Incidences of IAV positive birds in this study were much higher than those in the current seabird IAV surveillance literature (Lang et al., 2016). Furthermore, the incidence of IAV positives was higher in 2018 than 2019, indicating a possible epizootic event in 2018. These influenzas were of both Eurasian and North American lineages, indicating some mixing of antigenically distinct phylogenetic descent is taking place in the region. Sequence data suggest that the isolates represent H7 and N2 subtypes, however coverage was insufficient to accurately determine phylogeny.

In chapter three, I investigated the respiratory microbiome of northern fulmars from St. Mathew Island using next-generation sequencing and found a diverse microorganism community. Both non-pathogenic genera and those with pathogenic potential were detected. These microbiomes indicate organisms that might play an important role in respiratory health, including phyla *Actinobacteria*, *Bacteroides*, *Proteobacteria*, and *Firmicutes* (Simon et al., 2016; Škaraban et al., 2017). Several well-documented aquatic genera such as *Halomicronema*, *Flavobacterium*, and *Agrobacterium* were also detected, likely incidental to the consumption of marine organisms and ingestion of water (Liu et al., 2020; Mishra et al., 2018). The most noteworthy finding was pathogens with zoonotic potential. These included *Coxiella, Plasmodium, Toxoplasma,* and IAV.

The source and nature of many of the remaining genera were unknown.

In chapter four, I evaluated the levels of paralytic shellfish toxins (PSTs) in the gastrointestinal (GI) contents of northern fulmars from St. Mathew and Hall Islands. I found PSTs in 50% of the birds from 2019, suggesting the occurrence of at least one harmful algal bloom (HAB) event that year. These toxins occurred at levels similar to those observed in the GI contents of sub-clinical seabirds from the Gulf of Alaska and in deceased birds from the Bering Sea (Van Hemert et al., 2020, 2021). While data on PST concentrations in wild birds are limited, information from my study suggests the potential for PST toxicity in lethal concentrations in these birds. A seabird unusual mortality event (UME) was observed from May to October 2019 in the Bering Sea, from the southern Chukchi Sea to the eastern Aleutian Islands. This UME affected shearwaters (*Puffinus* spp.), murres (*Uria* spp.), puffins *(Fratercula* spp.), and auklets (*Aethia* spp.; R. Kaler, personal communication, 2020). While my data suggest a possible co-occurrence, it is not known whether these events were related.

My results provide baseline information about key pathogen and toxin threats to several seabird species in the Bering Sea. Seabirds here are exposed to numerous etiologic agents during the breeding season, when most congregate in large numbers. Breeding colonies are typically high-density groupings and present a high potential for pathogenic exchange (Chen & Holmes, 2009). Furthermore, concentrated populations of these birds also overlap in foraging areas (Kuletz et al., 2020). While not a true zoonotic pathogen, PSTs are also potentially transferrable agents. The distributions of these biotoxins in the food web indicate that a single bloom near a colony or rookery habitat might affect a wide range of organisms. The occurrence of these or similar etiological agents in these ecologically important habitats could precipitate a large-scale morbidity or mortality event, as has been seen elsewhere (Van Hemert et al., 2020; 2021).

My research examines key pathogen and toxin threats to Bering Sea seabirds, and my data serve as an indicator of ecological and, by extension, human health in the region insofar as it is connected to that ecosystem. Although many of these diseases are naturally circulating in wild animal populations, birds are relatively mobile and can transmit many of these pathogens regionally and even globally. Baseline data on the pathogens carried by avian hosts can provide important information on the movement of pathogens in a rapidly changing environment. Many of the etiologic agents I found have been detected in both humans and non-avian animals in the region, indicating that interspecific transmissions are likely (Duncan et al., 2012, 2014; Elmore & Jenkins, 2012; Groom et al., 2014; Kersh et al., 2020; Lefebvre et al., 2016; Reed et al., 2014). Thus, subsistence use of birds and marine mammals in this region provides opportunity for zoonotic pathogen exchange (Fall et al., 2013; Naves, 2018; Nelson et al., 2019). Substantial interactions of these birds with fisheries also provide potential for disease transmission to humans (Eich et al., 2016).

My results suggest the etiologic agents detected might pose great risk to seabirds, other animals, humans, and the ecosystem as whole. As the climate continues to change, feeding behaviors, migration patterns, spatial distribution, and anthropogenic interactions with the species I studied will undoubtedly change as well. Furthermore, increasing temperatures might broaden the geographic ranges of vector species and pathogens, exposing this ecosystem to new host-pathogen dynamics. As human populations expand, zoonotic infectious diseases are likely to emerge at an increasing rate (Jones et al., 2008). Thus, baseline data and ongoing surveillance of wildlife is critically important to maintain a clear picture of zoonoses from a OneHealth perspective (Bird & Mazet, 2018).

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