DYNAMICS OF DISEASE: ORIGINS AND ECOLOGY OF AVIAN CHOLERA IN THE EASTERN CANADIAN ARCTIC

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By

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ABSTRACT

Avian cholera, caused by infection with *Pasteurella multocida,* is an important infectious disease of wild birds in North America. Since it was first confirmed in 2005, annual outbreaks of avian cholera have had a dramatic effect on common eiders on East Bay Island, Nunavut, one of the largest breeding colonies of northern common eiders (*Somateria mollissima borealis*) in the eastern Arctic. I investigated potential avian and environmental reservoirs of *P. multocida* on East Bay Island and other locations in the eastern Canadian Arctic by collecting cloacal and oral swabs from live or harvested, apparently healthy, common eiders, lesser snow geese, Ross's geese, king eiders, herring gulls, and snow buntings. Water and sediment from ponds on East Bay Island were sampled before and during outbreaks. Avian and environmental samples were tested using a real-time polymerase chain reaction (PCR) assay to detect *P. multocida*. PCR positive birds were found in every species except for snow buntings, and PCR positive common eiders were found in most locations, supporting the hypothesis that apparently healthy wild birds can act as a reservoir for avian cholera. In all years, *P. multocida* DNA was detected in ponds both before and after the avian cholera outbreak began each year, suggesting that the environment also plays a role in outbreak dynamics. Contrary to our expectations, model results revealed that ponds were generally more likely to be positive earlier in the season, before the outbreaks began. Whereas average air temperature at the beginning of the breeding season was not an important predictor for detecting *P. multocida* in ponds, eiders were more likely to be PCR positive under cooler conditions, pointing to an important link between disease and weather. Potential origins of *P. multocida* causing avian cholera in Arctic eider colonies were investigated by comparing

eastern Arctic isolates of *P. multocida* to isolates from wild birds across Canada, and the central flyway in the United States. Using repetitive extragenic palindromic-PCR (REP-PCR) and multi-locus sequence typing (MLST), we detected a low degree of genetic diversity among isolates, and *P. multocida* genotypes were correlated with somatic serotype. Isolates from East Bay Island were distinct from *P. multocida* from eider colonies in the St. Lawrence Estuary, Quebec, however, East Bay Island isolates were indistinguishable from isolates collected from a 2007 pelagic avian cholera outbreak on the east coast of Canada. Isolates from East Bay Island and Nunavik shared sequence types, indicating possible transmission of isolates among eider colonies in the eastern Arctic. Previously, feather corticosterone in eiders was found to be significantly associated with environmental temperature during the moulting period. In my study, path analysis revealed that environmental conditions experienced during the moulting period had direct impacts on arrival date and pre-breeding body condition of common eiders during the subsequent breeding period on East Bay Island, with indirect impacts on both reproductive success and survival. Higher temperatures experienced during the fall moulting period appear to impose significant costs to eiders, with subsequent carry-over effects on both survival and reproduction many months later during avian cholera outbreaks. This thesis describes several important features of the host, agent and environmental dynamics of avian cholera in North America with a particular focus on the disease in the eastern Canadian Arctic. Continued exploration of infectious wildlife disease dynamics is needed to better predict, detect, manage, and mitigate disease emergence that can threaten human and animal health and species conservation.

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DEDICATION

To my parents

"Make your choice, adventurous Stranger,
Strike the bell and bide the danger,
Or wonder, till it drives you mad,
What would have followed if you had."

— C.S. Lewis, *The Magician's Nephew*

"When I came to consciousness my whole interest was in wild animals."

— Ted Hughes

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CHAPTER 1
GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Emerging avian diseases

Infectious diseases in wildlife are receiving increasing attention, perhaps because the emergence and re-emergence of infectious diseases that affect wildlife populations, or for which wildlife act as reservoirs, appear to be increasing in frequency (Rhyan and Spraker, 2001, Karesh et al., 2012). A high percentage of diseases in wildlife have the potential to cause disease in humans (Daszack et al., 2001; Karesh et al., 2012). In part, the transmission of pathogens from wildlife to humans or other species is a result of the evolution and ecology of pathogens as they adapt to new environments and hosts (Karesh et al., 2012). Wildlife pathogens may also be transmitted to domestic animals (Miller et al., 2013), or from domestic or feral animals to wildlife (Dobson and Foufopoulos 2001; Hughes et al., 2013; Mentaberre et al., 2013). Infectious diseases have been implicated as causes of marked declines or even local extinctions of wildlife species (Frick et al., 2010; Descamps et al., 2012; Rosa et al., 2013; Hollings et al., 2014). Indeed, disease in wildlife species may have important consequences at the ecosystem scale, particularly if the disease affects a keystone species (Tompkins et al., 2011). Human activities including changes in land use, expansion of agriculture, resource extraction, climate change, and global trade, appear to be important underlying mediators in the emergence of disease(Karesh et al., 2012).

Emerging infectious diseases can be defined as “diseases that have recently increased in incidence or geographic range, recently moved into new host populations, recently been discovered, or are caused by newly-evolved pathogens” (Daszak et al., 2001). In wildlife, emerging diseases include novel pathogens that have recently invaded a wildlife population;

pathogens that are spreading in their host population due to new external factors; or pathogens that emerge as a result of combinations of factors, such as movement of a novel pathogen into a population that is rendered more susceptible to disease due to external factors (Dobson and Foufopoulos, 2001). As in other species, emerging infectious diseases may affect the survival or reproductive success of individual birds, which may have significant implications for populations as well as subsequent impacts on ecosystem health and biodiversity (Friend et al., 2001; Daszak et al., 2001; Takekawa et al., 2010).

Infectious diseases result from a pathogen (or pathogens) invading a host and negatively affecting the host's ability to function (Burek et al., 2008). Frequently, very little is understood about the ecology of wildlife diseases, in part because of the challenges inherent in identifying parameters of wildlife populations, evaluating exposure to and infection with pathogens, and accurately assessing mortality in wildlife (Wobeser, 2006). Interaction among factors specific to the host, the pathogen, and the environment that increase the host's exposure or susceptibility to a pathogen, or increase the virulence or transmission rate of a pathogen, may influence the incidence, severity, and extent of a disease in a wildlife population (Wobeser 2006; Burek et al., 2008).

An important factor in the ecology of some diseases may be the response of individual hosts to stressors and their ability to manage energy expenditure. 'Stressors' are environmental stimuli that threaten an individual's survival and homeostasis, or are perceived to do so (Boonstra, 2012). One of the key adjustments animals make to disturbances or stressors is increasing the activity of the hypothalamic-pituitary-adrenal axis, which elevates circulating glucocorticoids and mediates the stress response (Taylor et al., 2014). While the stress-response is often a life-saving response that acts to return the animal to physiological homeostasis, chronic

high levels of glucocorticoids have been shown to negatively impact health through increasing susceptibility to disease, decreased fertility, and impaired growth and development (Romero, 2004). The stress response and the associated increase in glucocorticoids have been explored as a mechanism for how wildlife react to stressors in their immediate environment, and it has been shown to have effects on immune function in birds (Lindstrom et al., 2005; Borgeon et al., 2006a; Buttler et al., 2010).

Variation in access to resources and exposure to stressors may have immediate or long-term effects on wildlife, and have implications for individual fitness and population health. In most cases of wildlife disease, the factors that influence the disease and its outcomes are many, and may be better thought of as a web of causation, in which many factors interact to result in disease (Wobeser, 2007). Mechanisms that drive infection, transmission and maintenance of pathogens in wildlife populations must be identified and characterized if any management actions are to be taken (Cowled et al., 2012), and early methods of detection improved.

Many bird species are migratory, and make seasonal long-distance movements to access resources and habitats. Long-distance migration is very expensive energetically. Any changes to migratory routes, such as decreased or altered stop-over sites, and/or increased migration distance, can increase costs and result in a reduced ability of migrants to mount efficient immune responses (Moller and Erritzoe, 1998). This may increase susceptibility of migrants to infections, and/or may increase the cost of a disease or infection to animals that must migrate (Altizer et al., 2011). Mechanisms by which a migrant may become increasingly susceptible to disease include immunomodulation, when components of the immune response are adjusted to a lower level, or immunosuppression, when energetic demands, such as those of migration, decrease the effectiveness of immune responses (Altizer et al., 2011). Migratory species or groups are also

likely exposed to higher levels and diversity of pathogens than non-migratory animals. However, the net effect of migration on pathogen prevalence will depend in a large part on the mode of transmission and the degree of host specificity. While some pathogens may decline as a result of migration, others, particularly those that infect many species or have density-dependent transmission, may exert more pressure on migrant species which travel in dense aggregations, and through their interaction with other species, may be more susceptible to cross-species transmission (Altizer et al. 2011).

Long-distance migratory birds are prime candidates for the dispersal of infectious agents along migratory pathways and at breeding and wintering sites (Gaidet et al., 2010). Migration has the potential to connect geographically distant habitats, move pathogens, and facilitate cross-species disease transmission (Altizer et al., 2011). Gaidet et al., (2010) found that migratory waterfowl have the potential to disperse highly pathogenic avian influenza viruses over long distances, since some infected birds have a period of asymptomatic shedding. Migratory patterns may be influenced by changes in climate, anthropogenic alteration of landscapes, and changing food resources, leading to new variations in disease dynamics. For example, growing populations of lesser snow geese (*Chen caerulescens caerulescens*), which are potential carriers of virulent *Pasteurella multocida* (Samuel et al., 2005), in conjunction with increasingly available agriculture food resources and shrinking wetland stop-over sites, have resulted in very high densities of these birds on wetland habitats during migration (Blanchong et al., 2006). These staging sites are likely important areas for pathogen transmission and spread, and have been locations of large-scale mortality events due to avian cholera (Blanchong et al., 2006). In addition to moving pathogens, migratory species may themselves come into contact with a broad range of pathogens and their host species from spatially and geographically diverse locations.

Some pathogens associated with migratory birds also have serious implications for human and animal health (e.g. West Nile virus and avian influenza virus) (Gaidet et al., 2010).

In the Arctic, host and pathogen diversity is considered to be relatively low, which may increase the sensitivity of Arctic ecosystems to environmental change and invasions of new biota (Davidson et al., 2011). The climate in Arctic regions is warming at least twice as fast as the global average (ICPP, 2007). With ongoing climate change, range expansion to higher latitudes of some wildlife pathogens is expected, and in some cases has already been documented (Harvell et al., 2002; Kutz et al., 2013).

Although infectious diseases in wildlife occur around the globe, it is interesting to note that diseases affecting wild bird populations are now occurring in locations considered pristine or relatively untouched by anthopogenic influences, including the Galapagos Islands (Vargas, 2000), Antarctica (Gardner et al., 1997), Alaska (Bodenstein et al., 2015), and the Canadian Arctic (Buttler, 2009). Increasing anthropogenic influences such as resource exploration and extraction, climate change, introduction of domestic and invasive animal species, environmental contaminants, and tourism all have the potential to alter these ecosystems and affect the health of polar species (Kutz et al., 2009). Responses to environmental changes by seabird populations can vary from subtle and sublethal effects to massive die-offs (Mallory et al., 2010). Improving understanding about the ecology of diseases in avian populations, including pathogen origins, distribution, dispersal, potential impacts on populations, and transmission dynamics, is key to developing disease management strategies and predicting future occurrences and possible effects of these diseases.

1.2 *Pasteurella multocida* and avian cholera

Avian cholera, caused by *Pasteurella multocida*, is a significant cause of mortality in numerous waterfowl species (Samuel et al., 2007) and is considered to be one of the most important diseases of North American waterfowl (Samuel et al., 2005; Blanchong et al., 2006b). The disease in wild birds was first confirmed in North America in the 1940's in Texas (Windingstad et al., 1988), and is now found throughout the continent and along all major migratory flyways (Botzler, 1991). Avian cholera outbreaks are frequent in the Central Valley in California, along the central flyways at migratory stopover sites in Nebraska (Blanchong et al., 2006), and in Texas (Samuel et al., 2005), Minnesota, central Canada (Wobeser et al., 1982, Samuel et al., 2005), and the Canadian Arctic (Samuel et al., 1999, Descamps et al., 2012). Avian cholera occurs globally, and has been reported in locations including Europe (Pedersen et al., 2003), South Africa (Waller and Underhill, 2010), Korea (Kwon and Kang, 2003), New Zealand (de Lisle et al., 1990), Antarctica (Leotta et al., 2006), and Mongolia (Wang et al., 2009). Over 190 avian species have been reported with *P. multocida* infections (Samuel et al., 2007), although waterfowl species have proven to be the most susceptible to the disease. North American eider duck species have been affected by avian cholera in several locations, including Alaska (*Somateria* spp.) (Bodenstein et al., 2015), along the east coast of the United States (*S. m. dresseri*) (Korschgen et al., 1978), the St. Lawrence estuary of Quebec, Canada (*S. m. dresseri*) (The Joint Working Group on the Management of the Common Eider, 2004, S. Lair, G. Seguin, unpubl. data), and the eastern Canadian Arctic (*S. m. borealis*) (Gaston, 2004; Buttler, 2009; Descamps, 2009, Chapter 2). Avian cholera outbreaks may result in massive mortality in North America (i.e., >100,000 birds in a single outbreak) (Blanchong et al., 2006b), and epizootics have resulted in over 80% mortality on eider colonies in Denmark (Pedersen et al., 2003). In some locations, such as breeding colonies or migratory stop-over sites, avian cholera causes

annual mortality (Blanchong et al., 2006b; Descamps et al., 2012), and probably causes ongoing, low level mortality in some waterfowl populations that is difficult to detect (Wobeser, 1992; Samuel et al., 2005). Avian cholera outbreaks in waterfowl tend to occur, and are usually detected, when large aggregations of birds gather (Blanchong et al., 2006). Outbreaks can also result in rapid and large-scale mortality in multiple avian species in affected areas (Samuel et al., 2007).

Pasteurella multocida is a gram-negative, nonmotile, nonspore-forming, facultatively aerobic, bacterial pathogen that produces septicemic and respiratory diseases in a wide range of wild and domestic animals. Diseases caused by *P. multocida* include fowl cholera (in domestic poultry) and avian cholera (in wild birds), progressive atrophic rhinitis in pigs, pneumonia in cattle and sheep, and hemorrhagic septicemia in cattle and water buffaloes. *P. multocida* is also responsible for human infections subsequent to cat and dog bites (Davies, 2004). Strains of *P. multocida* vary in their host predilection, pathogenicity, morphology, and antigenic specificity (Townsend et al., 2001), and this variation has resulted in challenges in grouping or typing similar strains of the species. *P. multocida* has been traditionally characterized by assigning isolates to one of 5 capsular antigen serogroups and to one of 16 somatic serotypes. Depending on the capsular serogroup, capsules may contain hyaluronic acid, heparin, chondroitin sulfate, arabinose, mannose, or galactose (Townsend et al., 2001). Conventionally, serotyping of capsular serogroups has been done by the hemagglutination test (Carter, 1979; Rimler and Rhodes, 1989), however, more recent work showing the genetic basis for capsule biosynthesis also described a multiplex polymerase chain reaction (PCR) assay that can be used to determine the capsular serogroup of isolates (Townsend et al., 2001). Capsular serogroups have been shown

to be associated with specific animal species or disease; for example, most isolates from cases of fowl or avian cholera are capular serogroup A (Davies, 2004).

Somatic serotyping uses a gel diffusion precipitin test (Heddleston et al., 1972), with most of the pathogenic avian strains classified as somatic serotypes 1, 3, 4, and 3x4 (Samuel et al., 2007), and rarely serotype 2 (Eigaard et al., 2006). *P. multocida* strains have been further classified into three subspecies based on DNA-DNA hybridization and fermentation of ducitol and sorbitol, *P. multocida* subsp. *multocida, P. multocida* subsp. *gallicida,* and *P. multocida* subsp. *septica* (Mutters et al., 1985). However, these distinctions appear to be unimportant from a clinical standpoint, and furthermore, the separation of subspecies is not clearly reflected in the genetic relatedness among isolates (Blackall et al., 1998, Davies, 2004). The entire genomic sequence of a *P. multocida* isolate was first published in 2001, leading to important insights into genes associated with virulence factors, capsular biosynthesis, and iron metabolism (May et al., 2001). Virulence genes include production of toxins, capsules, hemogglutinins and hemophysins (Boyce et al., 2002). Further genomic analysis of additional *P. multocida* isolates from avian sources revealed genes that encoded for L-fucose transport and utilization, sugar transport systems, and hemagglutinins, which may be important for virulent strains to cause disease (Johnson et al., 2013).

The pathogenesis of *P. multocida* infection in wild birds is not well studied, but some information on pathogenesis in domestic birds is available (Wilkie et al., 2012). The pathogenesis is complex, and depends on a range of factors including host species, virulence and pathogenicity of the strain of bacterium, transmission route, and infectious dose (Samuel et al., 2007). Mildly virulent strains of *P. multocida* may be transformed into highly virulent strains via passage through birds over a short period of time (Matsumoto and Strain, 1993). The route of

infection for birds affected by avian cholera is most likely via the respiratory tract, with *P. multocida* colonizing the upper respiratory tract and then spreading to the lungs and air sacs (Wilkie et al., 2012). The bacteria are able to evade the phagocytic host defense, and enter the blood stream where infection results in septicemia (Samuel et al., 2007). To date, no exotoxin production associated with avian strains of *P. multocida* have been identified, and much of the tissue damage associated with infection may be due to the host immune response (Wilkie et al., 2012). However, the capsule appears to be an important determinant of virulence, with encapsulated strains able to multiply faster in blood and tissue, and to resist phagocytosis and complement (Christensen and Bisgaard, 2000). Other key virulence factors in avian strains of *P. multocida* include endotoxin (Christensen and Bisgaard, 2000) and various adhesion molecules that mediate colonization and invasion of host tissues (Boglarka et al., 2010).

Depending on host and agent properties, infection with *P. multocida* may kill birds acutely, as is seen with most waterfowl species, or may result in chronic disease, as seen in corvid, gull and raptor species. In acute cases, birds are usually in very good body condition, and lesions include petechial hemorrhages over serosal surfaces and the epicardium, or, in peracute cases, there may be no gross lesions (Samuel et al., 2007). When the infection has persisted for a slightly longer duration, lesions include multifocal hepatic and splenic necrosis, pulmonary edema, and mucoid intestinal contents (Samuel et al., 2007). More chronic disease results in fibrinous pericarditis, air sacculitis, and pneumonia (Samuel et al., 2007).

Wild birds which have survived infection with *P. multocida* produce detectable antibodies to the pathogen, as was seen in a population of greater white-fronted geese (*Anser albifrons frontalis*) in Alaska (Samuel et al., 2005) and in lesser snow geese (*Chen caerulescens caerulescens*) on Wrangle Island, Russia, and Banks Island, Canada (Samuel et al., 1999).

Experimental infection of mallard ducks indicated that most infected birds seroconvert, but that antibodies decrease over time to background levels within approximately 3-4 months after infection (Samuel et al., 2003a).

Exposure of susceptible birds to the bacteria during an outbreak likely occurs through direct bird-to-bird contact, inhalation of water droplets in aerosols from contaminated wetlands, or ingestion of the bacteria from contaminated water and vegetation (Botzler, 1991; Wobeser, 1992). Large numbers of bacteria are shed from infected and dead birds or scavenged carcasses through nasal discharge and from intestinal contents, which contaminate the surrounding environment (Botzler, 1991). Increasing concentration of waterfowl species at some sites, such as migration stop-over sites, is occurring due to loss of habitat, development of man-made wetland areas, and changes in agriculture that result in additional food resources (Wobeser, 1992), all of which may enhance the risk of transmission of pathogens such as *P. multocida* (Botzler, 1991; Blanchong et al., 2006b).

Two hypotheses have been proposed to explain the recurrence of avian cholera in specific geographical locations: 1) *P. multocida* persists within the soil, water, or other environmental elements in wetlands, which serve as a reservoir for susceptible birds; and 2) one or more species of waterfowl can act as carriers of the bacteria, and shed *P. multocida* in nasal secretions or feces, initiating disease outbreaks in groups of susceptible waterfowl (Samuel et al., 2005). In domestic poultry, the presence of carrier birds is relatively well described, usually associated with chronically infected birds from flocks previously affected by fowl cholera (Carpenter et al., 1989; Christensen and Bisgaard, 2000). Carrier birds have also been detected in poultry flocks with no history of fowl cholera (Muhairwa et al., 2000). *P. multocida* isolates have been recovered from live, apparently healthy lesser snow geese nesting in northern Canada (Samuel et

al., 1997), and lesser snow geese and Ross`s geese (*Chen rossii*) at wintering sites in Central US (Samuel et al., 2005). Blanchong et al., (2006b) found that mortality of other waterfowl species was correlated with lesser snow goose mortality during avian cholera outbreaks in Nebraska, suggesting a link between lesser snow geese and avian cholera outbreaks. *P. multocida* isolates were also recovered from apparently healthy American coots (*Fulica americana*) in Missouri (Vaught et al., 1967), nesting common eiders (*Somateria mollissima*) in Maine (Korschgen et al., 1978), and gulls (*Larus californicus*) in California (Titche, 1979). In most of these studies, virulence of the recovered isolates was not determined. However, Samuel et al., (1999; 2005) recovered *P. multocida* from apparently healthy geese and determined that the serotype 1 isolates were virulent to ducks in experimental challenges. Furthermore, an experimental study showed that mallards that were inoculated with *P. multocida,* and mallards in contact with inoculated birds, shed virulent *P.multocida* (Samuel et al., 2003a) and could serve as carrier birds.

Wetland environments (e.g., water, sediment, vegetation) become contaminated with *P. multocida* during an avian cholera outbreak, and environmental exposure to *P. multocida* is an important route of transmission and perpetuation of outbreaks in wild birds (Price et al., 1985; Botzler, 1991; Samuel et al., 2004; Lehr et al., 2005). Laboratory studies have also shown that under certain conditions, *P. multocida* can remain viable in water for over 1 year (Bredy and Botzler, 1989). Virulent strains of *P. multocida* have been found in wetlands associated with avian cholera outbreaks along the Central flyway (Samuel et al., 2003b). However, several studies have examined the potential for wetland environments at avian cholera outbreak sites to remain contaminated with viable *P. multocida* year-round, and concluded that it is unlikely that these wetlands serve as a year-round source of *P. multocida* (Samuel et al., 2004; Lehr et al., 2005; Blanchong et al., 2006a). Blanchong et al. (2006a) collected water and sediment samples

from wetlands during and shortly after avian cholera outbreaks, and found that they could recover *P. multocida* from environmental samples up to 7 weeks following an outbreak. This evidence suggests that wetlands at the sites of avian cholera outbreaks cannot maintain the pathogen between outbreaks, without re-introduction of *P. multocida* from avian hosts.

1.3 Emergence of avian cholera in the eastern Canadian Arctic

Avian cholera has caused annual large-scale mortality at the largest northern common eider breeding colony in the eastern Canadian Arctic since it was first detected there in 2005. The colony is located in Hudson Bay, Nunavut, on a small island named Mitivik Island (64°02'N, 81° 47' W), hereafter called East Bay Island, within the East Bay Migratory Bird Sanctuary (Figure 1-1). East Bay Island is a small (24 ha) island that supports a colony of over 4000 nesting pairs of common eiders (Buttler, 2009) which overwinter in southwest Greenland and along the east coast of Atlantic Canada (Mosbech et al., 2006). The rocky island is characterized by low tundra vegetation and several shallow freshwater ponds, which provide drinking water for nesting eiders (Buttler, 2009). Eiders are observed throughout the breeding season through the use of 8 observation blinds, strategically placed around the colony to maximize the observable area and minimize disturbance to the birds. The annual outbreaks begin in late June or early July of each year, several weeks after breeding eiders have arrived on the colony. The disease predominantly resulted in mortality of nesting female eiders, and a smaller number of male eiders, herring gulls (*Larus argentatus*), black guillemots (*Cepphus grylle*), brant geese (*Branta bernicla*), Canada geese (*Branta canadensis*), and snow buntings (*Plectrophenax nivalis*) (Buttler, 2009). Adult eider survival rates and reproductive success on East Bay Island have plummeted since the first confirmed avian cholera outbreak. The number of breeding pairs was lowest in 2011, since population surveys began in 2001 (Buttler, 2009;

Descamps et al., 2009, Iverson 2015). Researchers have been present on East Bay Island each summer since 1996, but no disease outbreaks were observed on the colony until 2005. Outbreaks of avian cholera had also been detected in common eider colonies in Nunavik (northern Quebec) near the communities of Ivujivik in 2004 (Gaston, 2004), Kangiqsujuak in 2006, and Aupaluk in 2006 and 2011 (Iverson, 2015). Interviews with Inuit residents from communities near the affected colonies in Nunavut indicated that large die offs of eiders had not been seen previously during the respondents' lifetimes (Henri et al., 2010).

In addition to the high rates of female common eider mortality at the East Bay Island colony (Buttler, 2009), avian cholera outbreaks also had a negative effect on reproduction and duckling survival on East Bay Island (Descamps et al., 2009; 2010). Prior to avian cholera emergence on the colony, the population of eiders had risen to an estimated high of 8600 breeding pairs (442±70 SE pairs/ha) in 2005 (Iverson, 2015). Mortality rates of eiders peaked in 2006, with a minimum percent mortality of 36.3% for female eider (Iverson, 2015). Mortality was also high in 2008 (minimum percent mortality of 26.9%). In subsequent years, eider minimum percent mortality has steadily decreased; 2009 (5.8%), 2010 (4.6%), 2011 (0.8%), 2012 (0.3%) (Iverson, 2015). Calculation of minimum percent mortality uses the end-of-season survey counts of female common eider carcasses on East Bay Island. Since an unknown fraction of birds may have died away from the colony, this estimate is considered a minimum measure of directly observable mortality. Minimum percent mortality is calculated by dividing the number of recovered carcasses by the annual breeding pair abundance on the island (Iverson, 2015). East Bay Island eider numbers have also decreased over time, and the estimated number of nesting pairs in 2012 was 4522 (233±22 SE pairs/ha) (Iverson, 2015). In 2013, avian cholera was not diagnosed in any birds on East Bay Island during the summer field season.

1.4 Northern Common Eiders (*Somateria mollissima borealis*)

Northern common eiders breed in the eastern Canadian Arctic and in Greenland and are a large, long-lived sea-duck with relatively low recruitment and reproduction rates (Merkel et al., 2004). Female common eiders are highly philopatric, with 70-100% of birds returning to the same nesting colony over successive breeding seasons (Goudie et al., 2000). Male eiders leave the colony to moult once egg incubation has commenced (Swennen, 1990). During the average 26 day incubation, female eiders fast, and only leave the nest for short periods of time to drink (Parker and Holm, 1990, Bottitta et al., 2003). Incubating female eiders may lose up to 45% of their body mass (Goudie et al., 2000), and incubation in eiders has been associated with a decrease in immune function (Hanssen et al., 2005; Borgeon et al., 2006b; 2006c). Northern common eiders breeding in northern Canada overwinter in eastern Atlantic Canada and the southwest coast of Greenland (Mosbech et al., 2006).

In northern Canada and western Greenland, common eiders are an important part of subsistence harvests in some communities, and are also hunted by sport hunters in their eastern wintering sites (Gilliland et al., 2009). Eiders are an integral component of northern ecosystems and one of the most heavily harvested birds in the Arctic (Gilliland et al., 2009). Eider populations in Greenland and in parts of the Canadian Arctic declined during the latter part of the 20th century, in part due to harvesting levels (Robertson and Gilchrist, 1998; Merkel et al., 2004; Gilliland et al., 2009; Merkel, 2010). Currently, eider populations appear to be recovering (Chaulk et al., 2005; Merkel, 2010), but continued monitoring of eider populations in both countries is ongoing, as other factors, such as climate change, increasing industrialization and shipping routes in the north, and disease will continue to affect eider population dynamics.

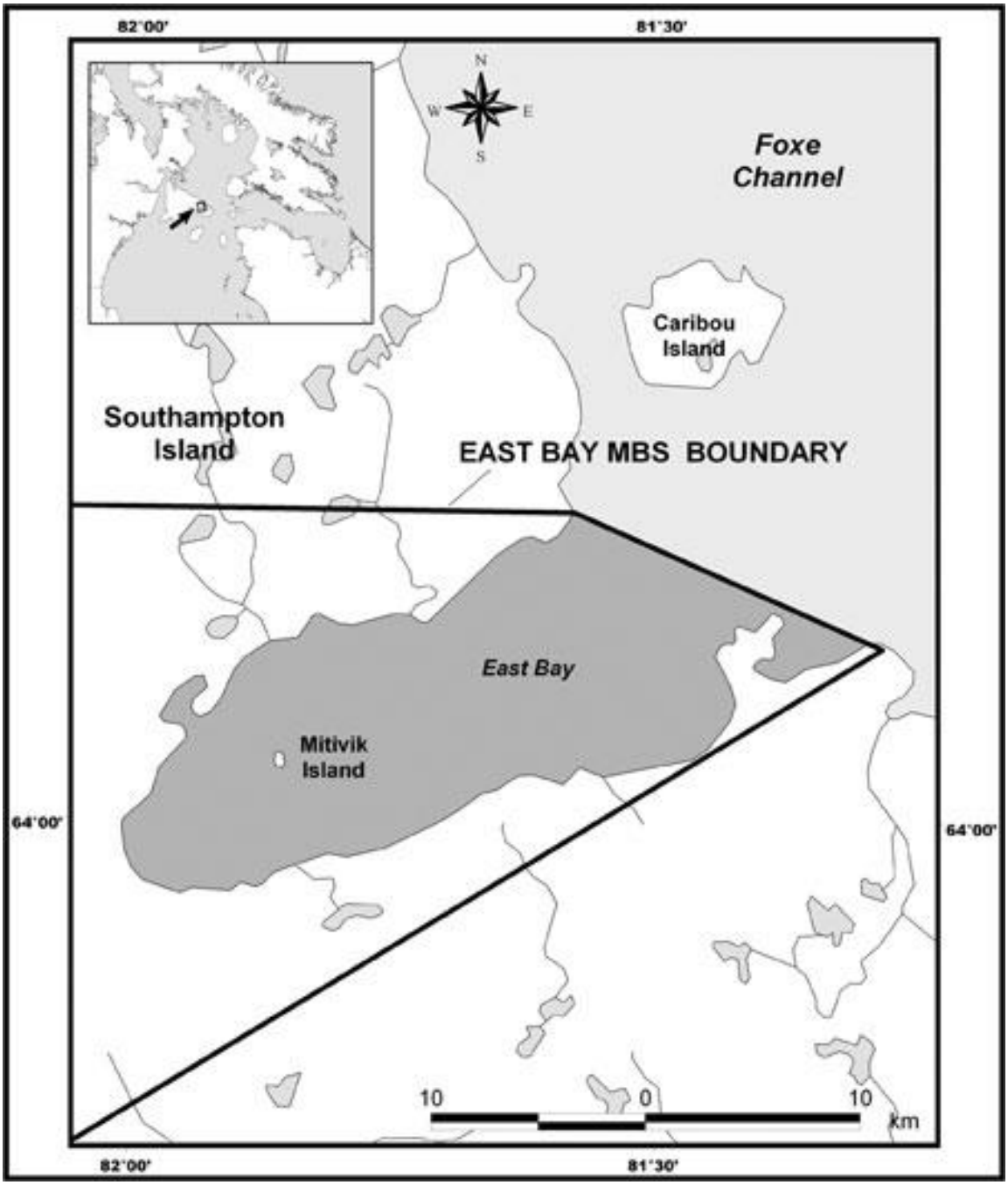

Figure 1-1. East Bay Migratory Bird Sanctuary, Nunavut, Canada, showing the location of Mitivik Island (East Bay Island). From: Mallory, M., and Fontaine, A.J., (2004).

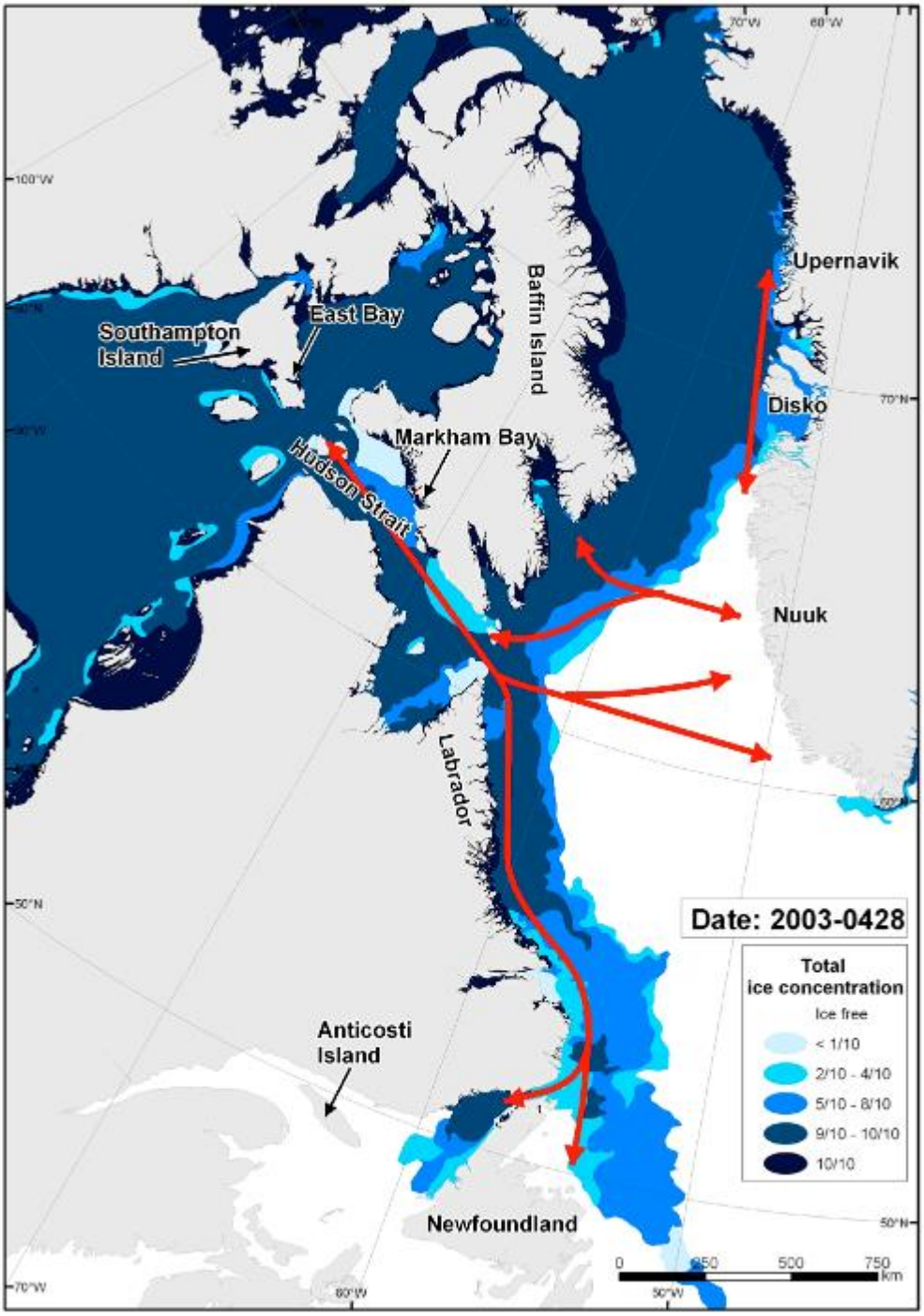

Figure 1-2. Map of the eastern Canadian Arctic and Greenland showing the main migration routes for northern common eiders. Map from Mosbech et al., (2006). Used with permission.

1.5 Objectives and thesis topics

The objective of this study was to investigate the disease dynamics of avian cholera in the eastern Canadian Arctic. To that end, I investigated three main aspects of the disease: distribution and ecological predictors of avian and environmental reservoirs of *P. multocida* in the eastern Canadian Arctic; potential origins of *P. multocida* causing avian cholera outbreaks in the eastern Canadian Arctic; and carry-over effects of past energy expenditure on survival and reproductive success of eiders experiencing annual avian cholera outbreaks.

In chapter 2, I explored the possible roles of both wild birds and the environment as reservoirs for *P. multocida* in the eastern Canadian Arctic. The main objectives for this chapter were to: 1. investigate whether common eiders or other sympatric avian species (e.g., lesser snow geese) are carriers of *P. multocida*, and therefore possible sources of the organism for initiating avian cholera outbreaks, 2. investigate whether the environment is a source of *P. multocida* for initiating and/or perpetuating outbreaks, 3. examine ecological and demographic factors in common eiders associated with being infected with (or "carrying") *P. multocida*, and 4. examine ecological factors associated with variation in prevalence of *P. multocida* in environmental samples. In this chapter, I used a recently developed real-time PCR assay (Corney et al., 2007) to test thousands of oral and cloacal swab samples for the presence of *P. multocida* DNA. Samples were collected from common eiders on East Bay Island and in other locations in the eastern Canadian Arctic including near Cape Dorset, Nunavut, and Ungava Bay, Nunavik. Samples from other species, such as lesser snow geese and Ross`s geese, herring gulls, king eiders, and snow buntings were also collected from East Bay Island, and some of these species were also sampled in other locations in the eastern Canadian Arctic. I also examined the effects of several factors, including sex, body condition, sample date, eider density, and air temperature

on the probability of common eiders being PCR positive for *P. multocida*. Although no mortality of lesser snow geese or Ross's geese due to avian cholera has been documented near East Bay or in Nunavut, healthy carrier birds may be shedding the bacteria during brief stop-overs on East Bay Island, or contaminating near-by environments that are accessed by eiders. To examine this possibility, I collected pond water and sediment samples from East Bay Island and other eider colonies and tested for *P. multocida*, to assess the potential for the environment to act as a reservoir for *P. multocida* and a source of the bacteria to initiate outbreaks in wild birds in the Arctic. Ecological factors such as environmental temperatures and sampling dates in relation to the onset of outbreaks were also examined as potential factors affecting the probability that environmental samples would contain *P. multocida*.

Following the investigation of *P. multocida* reservoirs of potential importance for initiating and maintaining avian cholera outbreaks in the eastern Canadian Arctic (i.e., the proximate cause of an avian cholera outbreak), I was interested in investigating the ultimate sources of the bacteria causing avian cholera in the eastern Canadian Arctic. Thus, in chapter 3, I explored the molecular epidemiology and potential origins of avian cholera in the eastern Canadian Arctic by examining the genetic relationships between *P. multocida* isolates from our study sites in the eastern Arctic, and isolates from across Canada and the Central flyway in the United States. In this chapter, I used two genotyping techniques to detect genetic variation in a suite of *P. multocida* isolates from wild birds from Canada and the US: repetitive extragenic palindromic PCR (REP-PCR) and multilocus sequence typing (MLST). The genetic relatedness of these *P. multocida* isolates was explored to better understand how *P. multocida* may have spread to the eastern Arctic, and also to improve our understanding of the genetic diversity of this pathogen in North America. Potential sources of *P. multocida* include wild carrier birds,

(Samuel et al., 2003) such as lesser snow geese or other bird species that migrate from the United States through southern Canada to nesting sites in the Canadian Arctic. Small numbers of snow geese make short stopovers on East Bay Island in the spring each year, prior to the arrival of eiders (Buttler, 2009), and large numbers nest within the East Bay Migratory Bird Sanctuary approximately 4 km away from East Bay Island. Other potential sources of bacteria include avian cholera outbreaks occurring in eiders and gull species in southern Canada along the east coast or within the St. Lawrence estuary in Quebec, since some eiders from East Bay Island are known to migrate and overwinter in eastern Canada (Mosbech et al., 2006) and may overlap spatially with other eiders as well as snow geese and gull species. Carrier birds or environmental sources from previous outbreaks in eider wintering areas could possibly spread the bacteria to eiders that eventually return to East Bay, therefore acting as a source of the disease.

In chapters 2 and 3, my investigations focused on the emergence, recurrence, and dynamics of avian cholera in the eastern Canadian Arctic and on features of the pathogen, *P. multocida*, itself. In chapter 4, I shifted the perspective away from the disease to focus on the host, and investigated factors that may affect the susceptibility of the common eider ducks, ultimately affecting their survival and reproductive success in the face of annual avian cholera outbreaks.

In chapter 4, I used corticosterone measured in feathers collected from eiders on East Bay Island to examine potential carry-over effects of moulting conditions experienced by the ducks in the autumn, on their subsequent reproductive output and survival while they experienced annual avian cholera outbreaks. Corticosterone is the main hormone produced in birds following activation of the hypothalamus-pituitary-adrenal (HPA) axis (Bortolotti et al., 2008). In birds, corticosterone can be measured in feathers and provides an index of an individual's HPA activity

during the growth of that feather and an integrated measure of circulating concentrations of corticosterone (Bortolotti et al., 2008; 2009). Corticosterone has a range of important physiological functions including response to stressors, effects on immune function (McEwan et al., 1997), and the modulation of whole body energy usage (Romero, 2004). Variation in access to resources and exposure to stressors, potentially reflected in feather corticosterone, could have immediate and/or long-term effects on individual fitness and population health.

In this chapter, I applied a relatively new area of study to eiders experiencing annual disease outbreaks on East Bay Island, and explored how energy expenditure in the fall while moulting may result in carry-over effects (COE). COEs are the result of events or processes occurring in one season that influence an individual's performance or condition in a subsequent season (Norris, 2005; Harrison et al., 2011). Using feather corticosterone, which provides an estimate of the energy expenditure and response to stressors experienced by an individual many months before the feather was collected, COEs on reproductive success, body condition, and survival of eiders were explored.

1.6 Ethics statement

This study adhered to the guidelines of the Canadian Council on Animal Care. Birds were captured, banded, and sampled under the authority of Environment Canada- Environment Canada Animal Care Committee permits (Gilchrist, EC-PN-07-008, EC-PN-08-026, EC-PN-09-026, EC-PN-10-26, EC-PN-11-026; Leafloor, EC-PN-10-01 and EC-PN-11-01; Alisauskas, NUN-SCI-11-02 and NUN-MBS-11-03), University of Saskatchewan Animal Research Ethics Board Certificates of Approval and the University Committee on Animal Care and Use (UCACU) Animal Use Protocol numbers (Soos, 20100063; Alisauskas, 19960014), Wildlife Research Permits from the Nunavut Department of Environment (Gilchrist, 000833, 2008-1028,

2009-029, 2010-004, 2011-029; Leafloor, 2010-018, 2010-019, 2011-024, 2011-025; Alisauskas, 2011-019). The mouse inoculation study was performed under the UCACU Animal Use Protocol number (Soos, 20110110).

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CHAPTER 2
IDENTIFYING RESERVOIRS FOR AVIAN CHOLERA IN AN ARCTIC ECOSYSTEM

Key words: avian cholera, Common eiders, lesser snow geese, disease reservoir, *Pasteurella multocida*, carriers

2.1 ABSTRACT

Avian cholera, a bacterial disease caused by *Pasteurella multocida*, is a significant cause of mortality in numerous species of waterfowl. Since it was first confirmed in 2005, avian cholera has had a dramatic effect on the largest common eider breeding colony in Arctic Canada. Annual outbreaks of the disease have resulted in substantial mortality of female eiders, raising questions about sources of *P. multocida* in the eastern Canadian Arctic. I investigated whether common eiders or other sympatric avian species were carriers of *P. multocida*, and/or whether the environment was a source of *P. multocida* for initiating and/or perpetuating outbreaks over time. In addition, I identified ecological and demographic factors associated with infection with *P. multocida* in eiders, and ecological correlates of *P. multocida* in ponds on the nesting island. To investigate the importance of avian reservoirs for initiating outbreaks, samples from apparently healthy common eiders, king eiders, lesser snow geese, Ross's geese, herring gulls and snow buntings from East Bay Island and other sites in the eastern Arctic, were collected from 2007-2011. Oral and cloacal swab samples were tested using a real-time PCR assay to detect *P. multocida*. Overall, using data from 2007-2011, 4.8% of common eiders arriving on the East Bay Island breeding colony tested positive for *P. multocida*. Prevalence ranged from 1.1-11.1% in lesser snow geese, 0-1.9% in Ross's geese, 0-14.3% in king eiders, 0-62.5% in herring gulls, and 0-13.8% in common eiders, depending on the location and year of sampling. *P. multocida* DNA

was not detected in common eiders in Ungava Bay, nor in snow buntings from East Bay Island. These results support the hypothesis that apparently healthy wild birds can be a reservoir for the disease in the Canadian Arctic. We also examined environmental (temperature), temporal (year, date) and demographic (sex, mass) factors associated with *P. multocida* infection in common eiders. Prevalence of *P. multocida* infection in eiders increased with cooler average temperatures in early spring, potentially pointing to an important link between disease and weather. Pond water and sediment were collected from East Bay Island over five years (n = 748), and near Cape Dorset over two years (n = 138) and Ungava Bay in one year (n = 215). In all years on East Bay Island, *P. multocida* DNA was detected in ponds both before (20/25 pond-sampling events, 80%) and after avian cholera outbreaks began (22/39 pond-sampling events, 56.4%) each year. Ponds were more likely to be positive in May and June, before outbreaks began. *P. multocida* was isolated from 1/347 of environmental samples cultured from East Bay Island. Although avian cholera has never been confirmed on south Baffin Island, 45.5% of ponds sampled near Cape Dorset were positive for *P. multocida* DNA. 56.5% of ponds around Ungava Bay were positive, including ponds from islands that were sites of previous (2006, 2011) avian cholera outbreaks. The role of environmental transmission of *P. multocida* on East Bay Island remains unclear, however it is possible that freshwater ponds play a role in transmission, particularly once an outbreak is initiated.

2.2 Introduction

Pathogens and the diseases they cause are functionally important components of ecosystems and have a variety of short- and long-term effects on their hosts. Currently, globalization, human-altered ecosystems, and climatic anomalies have contributed to a world that is undergoing rapid and often striking ecological changes. These can have a marked effect

on host-parasite relationships (Altizer et. al., 2006, Epstein, 2002). Within the context of these environmental changes, evidence that infectious pathogens play a role in the population dynamics of wildlife is mounting (Thompson et al., 2010, Frick et al., 2010). Increasingly, infectious and non-infectious diseases are recognized as causes of, or contributing factors to, marked declines or local extinctions of wildlife species, including bats (Frick et al., 2010), amphibians (Lips et al., 2006), and birds (Robinson et al., 2010, Oaks et al., 2004). In many cases, little is understood about the ecology of these diseases, in part because of the challenges inherent in studying the ecology of wildlife populations, evaluating exposure to and infection with pathogens, and accurately assessing mortality in wildlife (Wobeser, 2006).

The effects of an infectious disease on a population may be dependent on a variety of factors specific to the host, the pathogen, or the environment (Wobeser, 2006). For example, information about transmission dynamics, pathogen virulence, host density and behaviour, and climatic variables may all help determine the role of disease in population dynamics of the host over both the short and long-term. Effective conservation strategies require information about factors that regulate population dynamics; thus, knowledge of the infectious diseases present in these populations is essential to the development of conservation goals and management strategies (Walker et al., 2007).

A key element for understanding the ecology of infectious diseases is the identification of the pathogen reservoir (Haydon et al., 2002). Haydon et al., (2002) developed a definition for reservoirs that can be applied to wildlife disease. Essential to Haydon et al.'s definition is the concept of a target population; "the population of concern or interest," since the reservoir definition revolves around this population. A reservoir can be defined as "one or more epidemiologically connected populations or environments in which the pathogen can be

permanently maintained and from which infection is transmitted to the defined target population" (Haydon et al., 2002). Using this definition, the reservoir may include the target population species, vector species, the environment, and/or non-target populations. This constitutes a connected "maintenance community" in which the pathogen persists as long as the critical community size is preserved.

Avian cholera, a bacterial disease caused by *Pasteurella multocida*, is a significant cause of mortality in numerous species of waterfowl (Blanchong et al., 2006c; Samuel et al., 2007). Infection can result in acute death, killing birds in excellent body condition so rapidly that they may be found with crops full of food or still sitting on their clutch (Samuel et al., 2007). Outbreaks in waterfowl are often associated with large aggregations of birds, resulting in rapid and large-scale mortality in multiple species (Samuel et al., 2007). In North America, major outbreaks often occur when high densities of waterfowl gather on wintering sites (Blanchong et al., 2006c) or during summer among colonial-nesting species (Wobeser, 1992). During outbreaks, *P. multocida* is most likely transmitted through direct bird to bird contact, ingestion of water or vegetation contaminated with *P. multocida*, and/or inhalation of contaminated water (Wobeser 1992).

East Bay Island, Nunavut, Canada, supports the largest breeding colony of northern common eider ducks (*Somateria mollissima borealis*) in the eastern Canadian Arctic (Buttler, 2011) (Figure 2-1). Since 2005, eiders on East Bay Island have experienced annual avian cholera outbreaks during the summer breeding season (Buttler, 2009). In some years, survival of both adult females and ducklings had been substantially reduced (Descamps et al., 2009, 2011), and the number of breeding pairs in 2011 was at the lowest since population surveys began in 2001 (Descamps et al., 2009; Buttler, 2009). Avian cholera outbreaks on East Bay Island have

continued to occur annually, although the mortality rate due to the disease has decreased since 2005 (Iverson, 2015). Although common eiders compose the majority of cases of avian cholera on East Bay Island, carcasses of other avian species have also been found during the outbreaks, albeit sporadically and at low numbers, including herring gulls (*Larus argentatus*), snow buntings (*Plectrophenax nivalis*), Canada geese (*Branta canadensis*), and Brant geese (*Branta bernicla*) (Buttler, 2009). Lesser snow geese (*Chen caerulescens caerulescens*) and Ross's geese (*Chen rossii*) do not nest on East Bay Island, but small numbers of these species land on the island in early summer as they make their way to their breeding colony on Southampton Island, approximately 4 km northwest of East Bay Island (Buttler, 2009). Outbreaks of avian cholera have also been detected in common eider colonies in Nunavik (northern Quebec) in 2004, 2006, and 2011 (Gaston, 2004; Iverson, 2015).

Reservoirs of *P. multocida* for avian cholera outbreaks in wild birds have been the subject of much research in North America (e.g., Korschgen et al. 1978; Botzler 1991, Wobeser 1992; Samuel et al. 1997, Samuel et al. 2004; Samuel et al. 2005; Blanchong et al., 2206a; 2006b; 2006c). Two main theories, which are not mutually exclusive, are 1) that *P. multocida* persists within the soil, water, or other environmental elements in wetlands or on breeding colonies, serving as a reservoir for susceptible birds, or 2) that one or more avian species can act as carriers of the bacteria, and shed *P. multocida* (likely through oronasal secretions or feces), resulting in disease outbreaks (Wobeser 1992; Samuel et al. 1997; Samuel et al. 2005). Apparently healthy wild birds carrying *P. multocida* have been detected in several studies (Titche, 1979; Wobeser, 1992) although the virulence of these isolates was not determined. Further evidence for carrier birds was found when a virulent serotype 1 *P. multocida* isolate was recovered from a live apparently healthy snow goose from the Northwest Territories, Canada

(Samuel et al., 1997), and from apparently healthy snow geese and Ross's geese in locations with frequent avian cholera outbreaks (Samuel et al., 2005). In an experimental study, mallards that were inoculated with *P. multocida* and mallards in contact with inoculated birds shed virulent *P.multocida* (Samuel et al., 2003). *P. multocida* was also recovered from an oropharyngeal swab collected from an eider in Maine, although the isolate was not examined for virulence (Kroschgen et al., 1978). The importance of apparently healthy carrier birds in domestic poultry (fowl cholera) has been well documented (Christensen and Bisgaard, 2000; Mbuthia et al., 2008). In contrast, evidence for persistence of viable *P. multocida* in wetlands for extended periods of time is lacking (Samuel et al., 2004). Blanchong et al., (2006a) found that *P. multocida* could be recovered from wetland samples during an outbreak up to 7 weeks after an avian cholera outbreak had begun, but that viable isolates did not persist year round.

Despite the importance of avian cholera for waterfowl populations in North America, little is known about the ecology of this disease in common eiders in northern Canada, and nothing is known about the reservoir of the disease or where eiders are initially exposed before outbreaks begin. The recent emergence of avian cholera in the eastern Canadian Arctic provided an opportunity to investigate possible reservoirs of *P. multocida* for avian cholera outbreaks affecting common eiders. Here, I identified potential reservoirs of *P. multocida* for avian cholera outbreaks in the eastern Canadian Arctic in the environment and within migratory bird species, and also examined ecological factors such as temperature that may influence carrier eiders and the proportion of contaminated pond samples. In this study, a bird was considered a potential carrier of *P. multocida* if it was apparently healthy at the time of sampling and positive for *P. multocida* DNA in either an oral or cloacal swab.

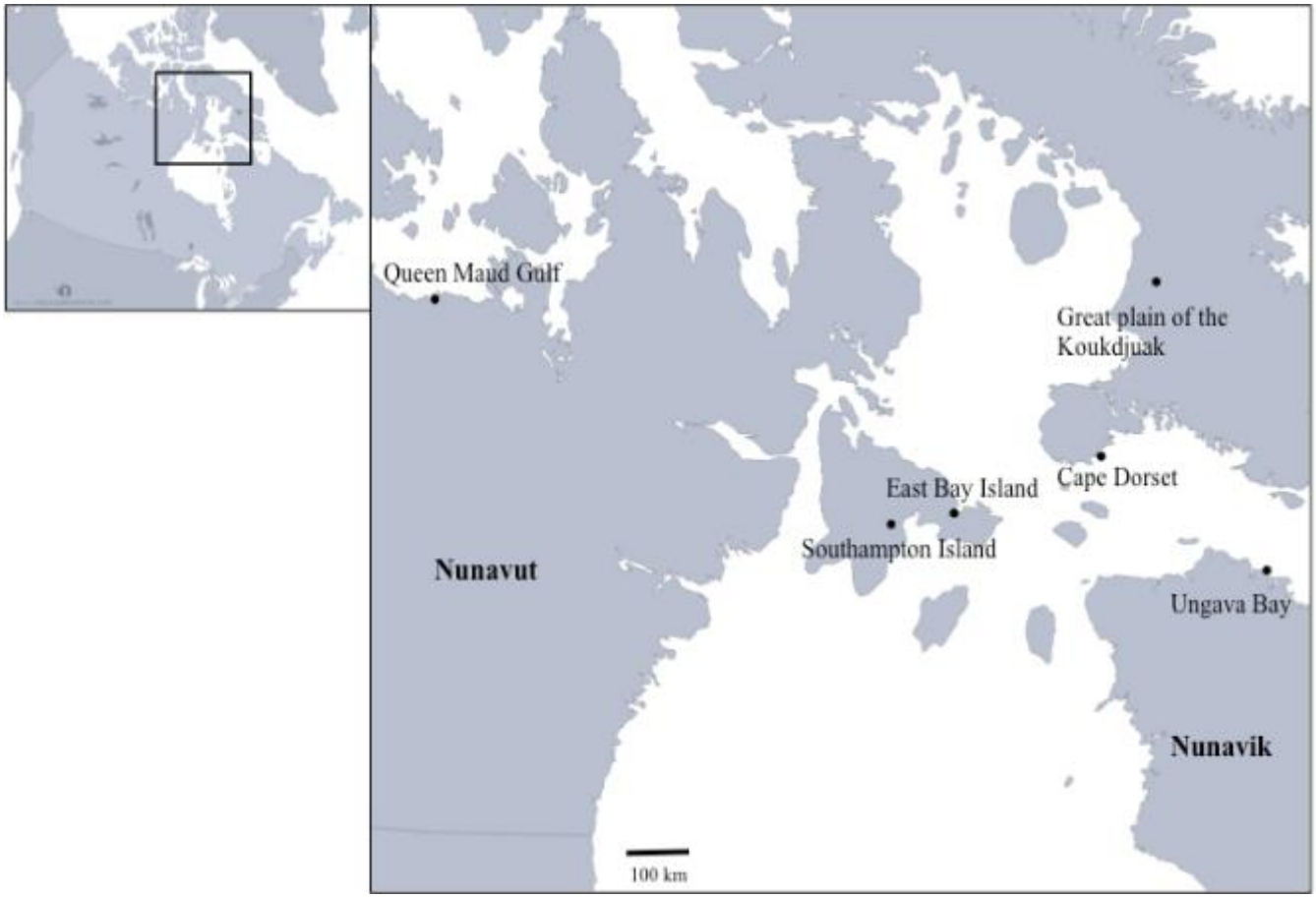

Figure 2-1. Map of the eastern Canadian Arctic showing locations of sample collections from wild migratory birds and environment, from 2007-2011.

2.3 Materials and Methods

2.3.1 Avian sample collection

Eiders

Between 2007 and 2011, oral and cloacal swab samples were collected from 2560 live common eiders and 52 live king eiders from East Bay Island (64°020N, 81°470W) in the East Bay Migratory Bird Sanctuary, Nunavut, Canada (Table 2-1). Pre-breeding eiders (n=2234) were captured using large mist nets early in the breeding season (June), prior to and during the onset of avian cholera outbreaks (Buttler, 2009). Female eiders were also captured at the end of the breeding season (during avian cholera outbreaks; n=326) as they left the island with their ducklings and were funnelled into wire pens set up on beaches of the island (Descamps et al., 2011). Eiders were banded with a metal band (United States Geological Survey) and 2 colored alphanumeric Darvic bands (Pro-Touch, Saskatoon, Canada) (Buttler et al., 2011). Nesting female common eiders were monitored throughout the breeding season from eight observation blinds strategically located within the colony. Observation blinds allowed monitoring of over 90% of the eider nesting area (Buttler et al., 2011) while minimizing disturbance to the colony. Swab samples were collected with sterile polyester-tipped applicators (Puritan Medical Products Co., Guilford, ME, USA) and stored individually in 2 mL polypropylene cryogenic vials (Thermo Scientific Inc., Rochester, NY, USA) containing 1 mL of sterile Trypticase Soy Broth with 15 % v/v glycerol.

In July 2010 and 2011, oral and cloacal swabs were collected from 22 nesting female common eiders captured by bow net, and fresh fecal samples were also collected from the nests of 131 female common eiders on 16 colonies along the coast of south Baffin Island near Cape Dorset, Nunavut, Canada (64° 015N, 76° 017W) (see Iverson 2015). Fecal samples were

collected using a sterile swab from nest cups or eggs when female eiders were flushed from the nest by researchers walking on the colony. Each fecal sample was placed into a cryovial containing 1 mL of Trypticase Soy Broth with 15 % v/v glycerol, and frozen following the protocol described above. In May 2011, oral and cloacal samples were collected from 200 pre-breeding common eiders hunter-harvested near Cape Dorset, Nunavut Canada (Figure 2-1). In July 2011, 281 samples of fresh fecal material from nesting female common eiders were collected from 23 eider colonies in Ungava Bay, Nunavik, Canada (61° 051N, 72° 090W) (see Iverson 2015 and Table 2-1). Common eider colony survey and carcass collection in Ungava Bay are described elsewhere (Iverson, 2015).

Snow geese

In July 2010 and July and August 2011, oral and cloacal swabs were collected from 1310 live lesser snow geese and 210 live Ross's geese captured by helicopter drives in collaboration with the Canadian Wildlife Service (CWS) during annual waterfowl banding operations, on Southampton Island, Nunavut, Canada (64° 030N, 84° 020N), the Great Plain of Koukdjuaq, Baffin Island (66° 022N, 72° 025W), and Queen Maud Gulf Migratory Bird Sanctuary, Northwest Territories (67° 044N, 100° 046W) (Table 2-1, Figure 2-1). Oral and cloacal samples were also collected from 379 lesser snow geese and seven Ross's geese from East Bay Island in 2008, 2009 and 2011, and 36 lesser snow geese in Cape Dorset in 2011 harvested by Inuit (Table 2-1).

Herring gulls

In July and August of 2007, 2008, 2010 and 2011, oral and cloacal samples were collected from 63 apparently healthy herring gulls from East Bay Island. Gulls were trapped

mid-summer as part of ongoing gull research (Allard et al., 2006), using box traps set over nests which were triggered when the bird returned to the nest (Allard et al. 2006) (Table 2-1).

Snow buntings

In June 2010 and June 2011, oral and cloacal samples were collected from 45 live snow buntings from East Bay Island. Buntings were trapped on the island using walk in traps baited with seed (Table 2-1) (Guindre-Parker et al., 2013).

Table 2-1. Species and locations of birds sampled for *P. multocida* from 2007-2011 in the eastern Canadian Arctic.

Species	Location	Year	Males	Females	Unknown	Total birds
COEI	East Bay Island[1]	2007	176	299	--	475
		2008	183	355	--	538
		2009	186	194	--	380
		2010	210	240	--	450
		2011	197	257	--	454
	Cape Dorset	2010	0	35[2]	--	35
		2011	104	199[2]	--	303
	Ungava Bay	2011	0	281[2]	--	281
Total COEI						2916
KIEI	East Bay Island	2009	3	3	--	6
		2010	4	3	--	7
		2011	21	18	--	39
	Cape Dorset	2011	10	0	--	10
Total KIEI						62
SNGO	East Bay Island	2008	--	--	196	196
		2009	--	--	92	92
		2011	41	40	6	87
	Southampton Island	2010	193	204	1	398
		2011	198	199	--	397
	Cape Dorset	2011	8	6	22	36
	Great Plains	2011	158	151	--	309
	Queen Maud Gulf	2011	100	106	--	206
Total SNGO						1721
ROGO	East Bay Island	2008	--	--	1	1
		2011	3	3	--	6
	Southampton Island	2010	0	1	--	1
		2011	1	0	--	1
	Queen Maud Gulf	2011	108	100	--	208
Total ROGO						217
HERG	East Bay Island	2007	--	--	1	1
		2008	--	--	8	8
		2010	--	--	36	36
		2011	--	--	18	18
Total HERG						63
SNBU	East Bay Island	2010	6	14	2	22
		2011	9	10	4	23
Total SNBU						45
		Total	1919	2395	387	4701

[1]East Bay Island common eiders includes both pre- and post-breeding birds that were sampled. Of the pre-breeding birds, only those that were included in the statistical analyses are included in the total numbers in this table.
[2]Includes number of fecal samples collected from nests in addition to swabs from female birds. COEI = common eider, HERG = herring gull, KIEI = king eider, ROGO = Ross's goose, SNBU = snow bunting, SNGO = lesser snow goose

2.3.2 Environmental samples

Between 2007 and 2011, 3-10 samples were collected from each of three or four freshwater ponds multiple times each year prior to and during outbreaks on East Bay Island (Table 2-2; n = 691 water samples and n = 57 sediment samples). Soil samples (n = 53) were also collected from randomly chosen eider nest cups on East Bay Island in 2011. Pond water samples (n = 193) were collected from eider colonies near Cape Dorset, Nunavut, on 13 islands from July 7-19, 2010, and on eight islands from July 8-25, 2011. In 2011 pond water samples (n = 215) were also collected from eider colonies in Ungava Bay, Nunavik (see Iverson, 2015).

On East Bay Island, early in the season while ponds were frozen and covered with snow (May to June), a shovel, which was disinfected between ponds (Virkon, Vetoquinol, Lavaltrie, Quebec, Canada) was used to scrape away snow and ice to allow access to pond ice and sediment. Approximately 15 mL of pond water, ice or sediment were collected using a sterile plastic screw-top container (Falcon tubes, Corning Life Sciences, Tewksbury MA, USA). For water and slush samples, the sample was shaken and 4 mL were removed with a sterile plastic pipette and added to 1 mL of sterile Trypticase Soy Broth with 75% (v/v) glycerol in a 5 mL polypropylene cryogenic vials (Thermo Scientific Inc., Rochester, NY). For sediment samples, a sterile polypropylene swab was used to mix the sample and to collect approximately 0.5 grams and transfer the sample to a 5 mL polypropylene cryovial containing 4 mL of sterile Trypticase Soy Broth with 15% (v/v) glycerol, and shaken to mix.

2.3.3 Sample shipment and storage

All avian and environmental samples were stored frozen at -196 °C in a cryoshipper (model SC 20/12V, MVE Vapour Shipper, MVE Bio-Medical Division, Chart Industries, Inc., Burnsville, MN) for transport to Université de Montréal (St. Hyacinthe, QC) for samples

collected from 2007-2009, and to the University of Saskatchewan (Saskatoon, Saskatchewan) for samples collected from 2010-2011. Samples shipped to Université de Montréal were later shipped in cryoshippers to the Center for Microbial Genetics and Genomics (MGGen), Northern Arizona University (Flagstaff, Arizona) for analysis. Samples were stored at -80°C until analysis.

Table 2-2. Water and sediment samples collected from freshwater ponds on East Bay Island, Nunavut, from 2007-2011. Multiple samples were collected each time a pond was sampled (referred to as a sampling event). Each pond was sampled during multiple sampling events throughout the season (June to August), thus the total number of pond-sampling events refers to the total number of sampling events for all ponds combined throughout the field season.

Year	Date range	# ponds	# sampling events per pond	Total # pond-sampling events	# samples
2007	June 8 - July 29	4	2 - 4	12	169
2008	June 7 - Aug 4	3	4 - 6	15	149
2009	June 7 - Aug 2	3	3 - 4	11	137
2010	June 5 - July 28	4	1 - 5	12	138
2011	June 5 - July 20	3	4 - 5	14	155
Total				64	748

2.3.4 Avian oral and cloacal swab samples: PCR analysis

All oral and cloacal swab samples were tested using a real-time Taqman PCR assay, based on a published protocol (Corney et al. 2007) that targets a portion of the 16S rRNA gene in *P. multocida*. For samples collected in 2007-2009, DNA was extracted at MGGen using methods previously reported in Legagneux et al. (2014), except for 162 samples which were extracted using Qiagen kits following manufacturer's guidelines (Qiagen Inc, Valencia, CA, USA). For samples collected in 2010-2011, samples were extracted at the University of Saskatchewan Microbiology Laboratory using the same protocol described in Legagneux et al. (2014) with the exception that 10 µL of TSB glycerol from each sample was added to 80 µL of 5% Chelex solution.

P. multocida DNA was amplified using the modifications previously described in Legagneux et al. (2014) for the 2007-2009 samples. For the 2010-2011 samples, similar modifications were used, except DNA was amplified in 25 µL PCR mixtures containing 2 µL of template DNA, 12.5 µL mastermix (iQ Supermix, Biorad, Mississauga, Ontario, Canada), 10 pmol of the forward and reverse primers (Sigma-Aldrich, Oakville, Ontario, Canada), 0.5 µL of TaqMan probe at 10 µM (Applied Biosystems, Life Technologies Inc, Grand Island, New York, USA), and 5.5 µL of ultrapure water. Also, a Biorad iQ5 real-time PCR machine (Applied Biosystems, Grand Island, New York, USA) was used to analyse the 2010-2011 samples, using the same cycling protocols (40 cycles of 50°C for 2 min, 95°C for 10 min, 95°C for 15 s and 60°C for 1 min). All PCR plates in both labs were run with a no template control (NTC) and positive controls. At MGGen (2007-2009 samples), a 2ng/µL solution of *P. multocida* DNA was used as a positive control. At UofS (2010-2011 samples), two positive controls were used (a *P. multocida* colony pick extracted using Chelex as described above and 2 µL of *P. multocida*

plasmid solution). The isolate used for the colony pick was isolated from a dead common eider from East Bay Island in 2008. For the plasmid solution used as a positive control, the portion of the 16S rRNA gene in *P. multocida* was amplified from genomic DNA, using the PCR primers as previously described (Corney et al., 2007). The 70 bp product was ligated into pGEM-T Easy (Invitrogen) and used to transform *E. coli* JM109 cells (Invitrogen). Single transformants were isolated and the resulting vectors sequenced to confirm identity.

For the 2007-2009 samples analysed at MGGen we tested the limits and sensitivity of the assay using serial twelve-fold dilutions, starting at a concentration of 1 ng/µL *P. multocida* DNA. The detection limit of the assay was $1x10^{-8}$ ng per reaction. For PCR assays run at the University of Saskatchewan we tested the limits and sensitivity of the assay using serial ten-fold dilutions of the sequence of *P. multocida* DNA in *E. coli* vectors as decribed above. The dilution series was started at a concentration of $4.23x10^{10}$ copies of the amplicon/µL. The detection limit of the assay was 4.23 amplicons/µL. In both labs, DNA concentrations were quantified by UV spectroscopy by NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). A sample was classified as positive if the Ct value was 38 or less (Corney et al. 2007).

2.3.5 Avian oral and cloacal swab samples: Bacterial culture

In order to recover *P. multocida* isolates from any potential carrier birds for further characterization, we attempted to culture all PCR-positive avian swabs sampled in 2010-2011. Thawed samples were held at 4°C during the PCR assay and were cultured immediately following assay completion, except when the assay was run overnight when the samples were cultured the next morning. The swab sample in broth was vortexed for 10 seconds, and immediately a 25µL loop of the sample broth was streaked onto a 5% blood agar plate (Becton, Dickson and Company, Mississauga, Ontario, Canada). The plate was incubated at 37°C in

aerobic conditions for 18-24 hours. The plate was then examined for evidence of colonies consistent with *P. multocida*. Suspect colonies were picked using a sterile toothpick or sterile pipette tip and confirmed as *P. multocida* using the PCR assay as described above. Any colonies that were confirmed or suspect *P. multocida* based on PCR results were subsequently streaked onto 5% blood agar and incubated at 37°C in aerobic conditions for 18-24 hours. If pure growth was achieved, a colony from the plate was tested with the PCR assay for *P. multocida*. *P. multocida* isolates were incubated at 37°C in TSB glycerol broth overnight then frozen at -80°C. A pilot study using inoculation of mice to recover *P. multocida* from PCR positive samples was also attempted, and did not improve recovery rates of PCR-positive samples (Appendix B).

2.3.6 Environmental sample analysis: PCR analysis and bacterial culture

All pond water, sediment, and nest material samples (n = 455) were tested using the real-time PCR assay (Corney et al., 2007) with modifications as described earlier for avian samples. DNA was extracted from vortexed water and sediment samples using the Chelex protocol described above using either 6 µL or 10 µL of sample in 60 µL of 5% Chelex.

Samples collected in 2010-2011 included pond water, sediment and nest soil samples from East Bay Island, Cape Dorset, and Ungava Bay eider colonies (total n = 754). Bacterial culture was also attempted on these samples prior to PCR analysis, using the following methods. Samples were thawed to room temperature, and vortexed for 10 seconds. Immediately, 100 µL of sample was pipetted onto a 5% blood agar plate and spread evenly on the agar surface. The plate was incubated at 37°C in aerobic conditions for 24 hours. After 24 hours, the plate was examined for evidence of colonies that were consistent with *P. multocida*. Suspect colonies were picked using a sterile toothpick or sterile loop and streaked on a 5% blood agar plate and incubated for 24 hours under aerobic conditions. If the suspect colony produced a pure isolate, colonies were

confirmed as *P. multocida* using the PCR assay as described above. A pilot study testing the use of selective media (Moore et al., 1994) to recover *P. multocida* from water and sediment samples was conducted, but was found to be less sensitive than blood agar alone (see Appendix C). Following culture, all 2010-11 environmental samples were analyzed directly for the presence of *P. multocida* DNA using modifications to the real-time PCR assay as described earlier for 2010-2011 avian swab samples.

2.3.7 Statistical analyses

Descriptive statistics were calculated to estimate overall apparent prevalence for each species by location and year. For common eiders at East Bay Island, prevalence was also calculated for prebreeding males and females, and for post-breeding females with ducklings at the end of the breeding season. Impacts of annual population density (number of eider pairs per hectare on East Bay Island; Iverson, 2015) on prevalence of infection in common eiders each breeding season were examined using linear regression in R (R Development Core Team, 2014). Correlations examined relationships between prevalence of infection in birds and proportion of positive environmental samples found each year. I also examined correlations between PCR positive eiders and and mean spring temperature (mean temperature for a three week period beginning seven days prior to arrival of the first eider to the colony each year), and between annual proportion of positive environmental samples and mean seasonal temperature (average air temperature for 71 days beginning May 30 and ending August 8 of each year).

To examine ecological and demographic effects on PCR status, data from pre-breeding common eiders on East Bay Island from 2007-2011 (n = 1971) were analysed with logistic models with a binomial distribution using a logit link function based on maximum likelihood estimation. We were able to combine PCR results from the two laboratories for these analyses as

there was no significant difference in the Ct values of a serial dilution assay between the two labs (Section 2.7.1, supplemental information). Individual birds with missing data on sex, mass, or PCR results were excluded from this analysis. We examined the effects of pre-breeding body condition (standardized mass in grams), sex (male or female), number of days from outbreak start date (date when first eider carcass was detected), and mean spring air temperature (mean temperature for a three week period beginning seven days prior to arrival of the first eider to the colony each year), on a binomial outcome (PCR positive or negative). Year was included in the models as a random effect. For eider ducks, body mass alone has been shown to be a better predictor of condition at this time of year than mass corrected for body size (Descamps et al., 2010; Descamps et al., 2011). Thus body mass was used as our measure of body condition. The number of days to the start date of an outbreak was included in the model as a continuous variable (recoded as number of days a birds was captured and sampled before or after the outbreak start date). Air temperature data was obtained from the Environment Canada weather station at Coral Harbour (64°11'N, 83°21'W; 68 km from East Bay), because spring temperature has been shown to affect timing of laying in eider ducks at this colony (Love et al., 2010). Eider ducks were considered PCR positive if either the cloacal swab, the oral swab, or both were positive. Eiders from East Bay Island with PCR results for only one swab were excluded from statistical analysis.

Model selection was carried out using the Akaike information criterion corrected for small sample size (AICc, Anderson 2008). Global models were built which included biologically relevant variables that had an initial association with the outcome variable in a univariate model and were not correlated with other variables (i.e., $R^2 < 0.4$) (Murray and

Conner, 2009). If models were within 2 AICc values of each other, the more parsimonious model was chosen as the best supported model.

PCR data from pond water and sediment samples from East Bay Island were collated so that pond sampling events were considered positive for *P. multocida* DNA if at least one sample collected during a sampling event was positive (n = 64). PCR results for pond sampling events were analysed using logistic models with a binomial distribution using a logit link function based on maximum likelihood estimation. Explanatory variables included in the models were mean seasonal air temperature (average air temperature for 71 days beginning May 30 and ending August 8 of each year), number of days to outbreak start date (continuous date variable recoded as number of days before or after the outbreak start date that a sample was collected), and a variable 'pond-year' was included as a random effect to account for multiple sampling events for each pond within each year, and multiple years. Year was also included as a fixed effect. Model selection was carried out as described above.

2.4 Results

2.4.1 East Bay Island – Common Eiders

Between 2007-2011, 2560 common eiders were captured, banded, and sampled on East Bay Island. Of these birds, 2234 were prebreeding, and 326 were post-breeding birds. Of the prebreeding birds, 263 were excluded from statistical analysis due to missing data, thus final analyses were conducted on 1971 prebreeding common eider ducks. Avian cholera outbreaks occurred each year, and the first dead eider(s) were observed on 1 July 2007, 24 June 2008, 30 June 2009, 27 June 2010, and 5 July 2011.

Overall, 4.8% of prebreeding eiders from East Bay Island sampled between 2007 and 2011 were PCR positive for *P. multocida* DNA from an oral swab, cloacal swab, or both swabs

(Tables 2-3 and 2-4). The highest prevalence was in 2010 with 9.5% of birds infected, and lowest was in 2009 with 0.8% infected (Table 2-3). Based on the best supported model, average annual spring temperature was the most important variable for predicting whether an eider was PCR positive for *P. multocida* (β= -2.18, SE = 0.74, P = 0.003) (Table 2-5 and 2-6). In years with cooler mean spring temperatures, prebreeding eiders were more likely to test positive for *P. multocida* (Figure 2-2). The model with the lowest AICc also included date in relation to outbreak onset (Table 2-5), however, date was not considered informative in this model as it did not improve AICc by more than 2 units (β= -0.037, SE = 0.023, P = 0.11). Sex (see Table 2-4), body condition, and interaction terms were excluded from the final models because they were not informative when examined alone (supplementary data, Table S2-2). There was no relationship between proportion of PCR-positive eiders and eider population density (β= 0.03, SE = 0.05, P = 0.51). Infection in pre-breeding eiders was weakly correlated with positive pond samples collected both before and after each year (R^2 = 0.62, P = 0.11; Figure 2-4).

Between 2007 and 2011, 326 post-breeding female common eiders were trapped in wire funnel traps as they attempted to leave East Bay Island with their ducklings (Table 2-4). In all years, these eiders were trapped and sampled after the annual avian cholera outbreak was well underway. Overall, 47 (14.4%) post-breeding eiders were PCR positive for *P. multocida* DNA from oral and/or cloacal swabs (Table 2-4). Although there was no influence of date in relation to outbreak onset among prebreeding eiders, post-breeding females with ducklings at the end of the breeding season were 3.0 times more likely to be infected compared to prebreeding females sampled at the beginning of the breeding season prior to and at the beginning of outbreaks (95% CI = 2.0-4.5, P < 0.0001; Table 2-4). Excluding 2009 (with n =1), the highest proportion of PCR positive post-breeding birds occurred in 2010 (24.4%) and 2008 (20.7%), and the lowest

proportion was found in 2007 (1.3%) (Table 2-4). Thus, a relatively high proportion of PCR positive eiders were found leaving the colony after their ducklings had hatched. Of the 47 PCR positive post-breeding female eiders, five of the eiders were subsequently found dead on the island due to avian cholera, either in the same year that they were captured or in a subsequent year. Five other eiders were also captured alive in subsequent years, indicating that the infection was not fatal in these birds.

Table 2-3. Proportion of wild birds PCR positive for *P. multocida* across the eastern Canadian Arctic from 2007-2011. Common eiders from East Bay Island, Nunavut, are all pre-breeding birds.

Species	Location	Year	# positive oral samples/n (%, 95% CI)	# positive cloacal samples/n (%, 95 % CI)	Overall # positive birds/n **	Overall % positive [95 % CI]
COEI	EBI	2007	0/395 (0%)	29/396 (7.3%, 4.7, 9.9)	29/396	7.3% [4.7, 9.9]
		2008	5/388 (1.3%, 0.2, 2.4)	7/389 (1.8%, 0.5, 3.1)	12/388	3.1% [1.4, 4.8]
		2009	3/380 (0.8%, -0.1, 1.7)	0/379 (0%)	3/380	0.8% [-0.1, 1.7]
		2010	13/409 (3.2%, 1.5, 4.9)	26/409 (6.4%, 4.0, 8.8)	39/409	9.5% [6.7, 12.3]
		2011	9/400 (2.3%, 0.8, 3.8)	6/400 (1.5%, 0.3, 2.7)	14/400	3.5% [1.7, 5.3]
	CD	2010	0/22 (0%)	0/22 (0%)	0/22	0%
		2010*	NA	0/15 (0%)	0/15	0%
		2011	1/187 (0.5%, -0.5, 1.5)	4/187 (2.1%, 0.0, 4.2)	5/187	2.7% [0.4, 5.0]
		2011*	NA	16/116 (13.8%, 7.5, 20.0)	16/116	13.8% [7.5, 20.1]
	UB	2011*	NA	0/281 (0%)	0/281	0%
KIEI	EBI	2009	0/6 (0%)	0/6 (0%)	0/6	0%
		2010	0/7 (0%)	1/7 (14.3%, -11.6, 40.2)	1/7	14.3% [-11.6, 40.2]
		2011	0/39(0%)	2/39 (5.1%, -1.8, 12.0)	2/39	5.1% [-1.8, 12.0]
	CD	2011	0/10 (0%)	0/10 (0%)	0/10	0%
SNGO	EBI	2008	7/195 (3.6%, 1.0, 6.2)	4/192 (2.1%, 0.1, 4.1)	10/196	5.1% [2.0, 8.2]
		2009	4/90 (4.4%, 0.2, 8.6)	4/88 (4.5%, 0.2, 8.8)	8/92	8.7% [2.9, 14.5]
		2011	0/87 (0%)	1/87 (1.5%, -1.1, 4.1)	1/87	1.1% [-1.1, 3.3]
	SHI	2010	7/398 (1.8%, 0.5, 3.1)	16/398 (4.0%, 2.1, 5.9)	23/398	6.0% [3.7, 8.3]
		2011	5/397 (1.3%, 0.2, 2.4)	3/397 (0.8%, -0.1, 1.7)	8/397	2.0% [0.6, 3.4]
	CD	2011	4/36 (11.1%, 0.8, 21.4)	0/36 (0%)	4/36	11.1% [0.8, 21.4]
	GPK	2011	8/309 (2.6%, 0.8, 4.4)	2/309 (0.6%, -0.3, 1.5)	10/309	3.2% [1.2, 5.2]
	QMG	2011	4/206 (1.9%, 0.0, 3.8)	2/206 (1.0%, -0.4, 2.4)	6/206	2.9% [0.6, 5.2]
ROGO	EBI	2008	0/1 (0%)	0/1 (0%)	0/1	0%
		2011	0/6 (0%)	0/6 (0%)	0/6	0%
	SHI	2010	1/1 (100%)	0/1 (0%)	1/1	100%
		2011	0/1 (0%)	0/1 (0%)	0/1	0%
	QMG	2011	4/208 (1.9%, 0.0, 3.8)	3/208 (1.4%, -0.2, 3.0)	7/208	3.4% [0.9, 5.9]
HERG	EBI	2007	0/1 (0%)	0/1 (0%)	0/1	0%
		2008	2/7 (28.6%, -4.9, 62.1)	3/8 (37.5%, 3.95, 71.1)	5/8	62.5% [29.0, 96.1]
		2010	14/36 (38.9%, 23.0, 54.8)	3/36 (8.3%, -0.7, 17.3)	16/36	44.4% [28.2, 60.6]
		2011	9/18 (50.0%, 26.9, 73.1)	5/18 (27.8%, 7.1, 48.5)	11/18	61.1% [38.5, 83.6]
SNBU	EBI	2010	0/22 (0%)	0/22 (0%)	0/22	0%
		2011	0/23 (0%)	0/23 (0%)	0/23	0%

** A bird was considered positive if it was PCR positive on either oral or cloacal swab
* Fecal samples collected from common eider nests
COEI = common eider, HERG = herring gull, KIEI = king eider, ROGO = Ross's goose, SNBU = snow bunting, SNGO = lesser snow goose
CD = Cape Dorset, EBI = East Bay Island, GPK = Great Plain of the Koukdjuak, QMG = Queen Maud Gulf, SHI = Southampton Island, UB = Ungava Bay

Table 2-4. Prevalence of *P. multocida* infection (based on PCR testing) in prebreeding male and female common eiders and post-breeding female eiders sampled each year on East Bay Island, Nunavut from 2007-2011. Prebreeding eiders were sampled during and at the beginning of avian cholera outbreaks, while post-breeding females (with ducklings) were sampled well after avian cholera outbreaks had begun, at the end of the breeding season.

	Prebreeding males		**Prebreeding females**		**Post-breeding females**	
Year	**# eiders sampled**	**# PCR positive (%, 95% CI)**	**# eiders sampled**	**# PCR positive (%, 95% CI)**	**# eiders sampled**	**PCR positive (%, 95% CI)**
2007	176	9 (5.1%, 1.9, 8.4)	219	19 (8.7%, 5.0, 12.4)	80	1 (1.3%, -1.2, 3.8)
2008	183	3 (1.6%, -0.2, 3.4)	205	8 (3.9%, 1.3, 6.6)	150	31 (20.7%, 14.2, 27.2)
2009	186	1 (0.5%, -0.5, 1.5)	193	1 (0.5%, -0.5, 1.5)	1	1 (100%)
2010	210	22 (10.5%, 6.4, 14.7)	199	17 (8.5%, 4.6, 12.4)	41	10 (24.4%, 11.25, 37.6)
2011	197	4 (2.0%, 0.1, 4.0)	203	10 (4.9%, 1.9, 7.9)	54	4 (7.4%, 0.4, 14.4)
Total	952	39 (4.1%, -2.1, 10.3)	1019	55 (5.4%, -0.6, 11.4)	326	47 (14.4%, 4.4, 24.4)

Table 2-5. Binary logistic regression models explored to explain variation in *P. multocida* DNA detection in common eiders from East Bay Island, Nunavut from 2007-2011 (n = 1971). Year was included as a random effect in all models.

Model - fixed terms	Number of parameters	AICc
Temperature + Date to outbreak	3	725.2
Temperature	2	725.8
Null	1	727.7
Date to outbreak	2	728.5

Date to outbreak = number of days before or after outbreak start date eachyear, Temperature = average air temperature for three weeks beginning seven days before the first eider was caught each year.

Table 2-6. Summary of best supported model to explain variation in *P. multocida* DNA detection in pre-breeding common eiders from East Bay Island, Nunavut, from 2007-2011 (n= 1971).

Variables	**β**	**SE**	**β 95% CI**	**P-value**
Temperature	-2.18	0.74	-3.6, -0.7	0.003
Date to outbreak	-0.04	0.02	-0.08, 0	0.1

β= coefficient estimate, SE = standard error

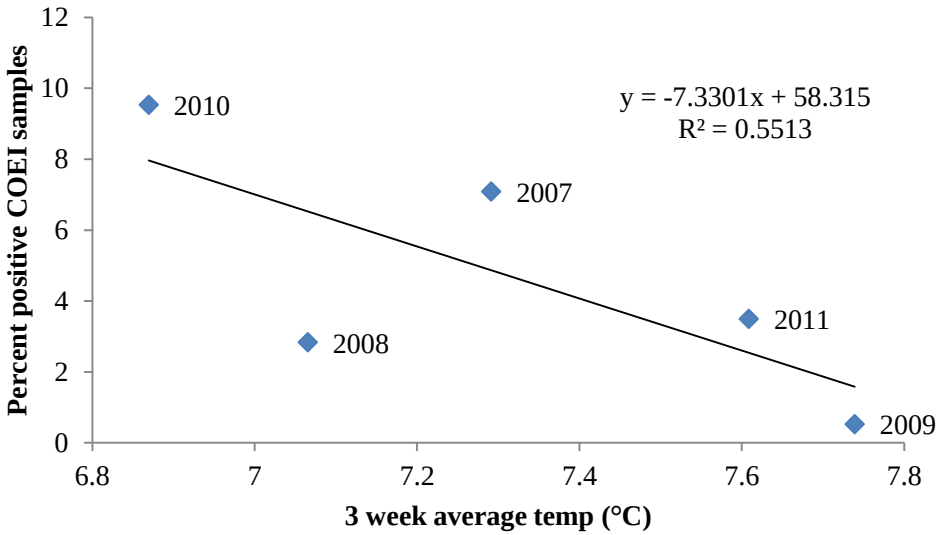

Figure 2-2. Correlation between *P. multocida*-positive pre-breeding common eiders on East Bay Island, Nunavut, and average air temperature during a 3 week period beginning one week before the arrival of the first common eider on the breeding colony, from 2007-2011.

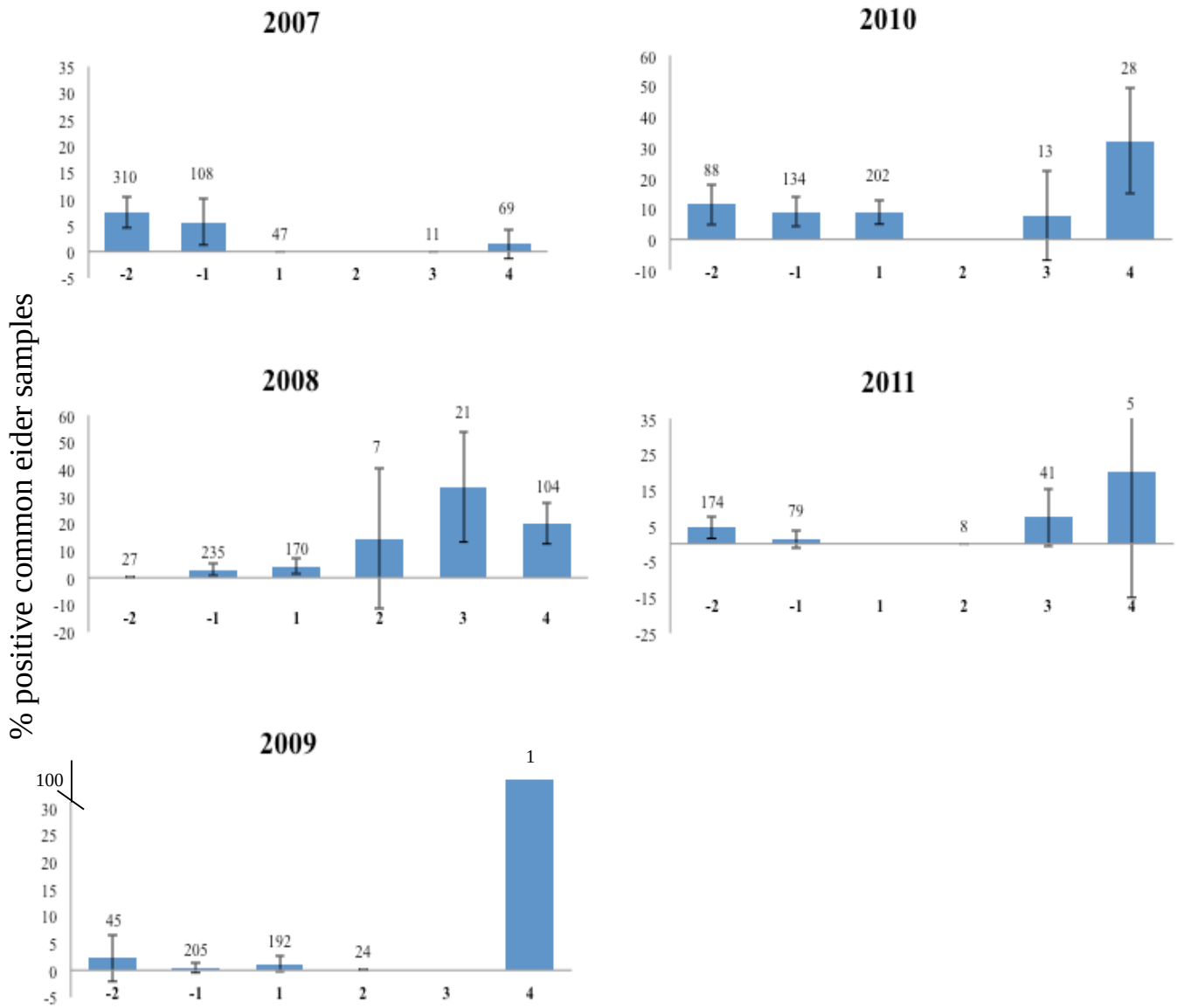

Figure 2-3. Weekly prevalence of *P. multocida*-positive pre-breeding and post-breeding common eiders on East Bay Island, Nunavut, in relation to onset of avian cholera outbreaks, from 2007 to 2011, with 95% confidence intervals. Sample sizes are shown above each bar.

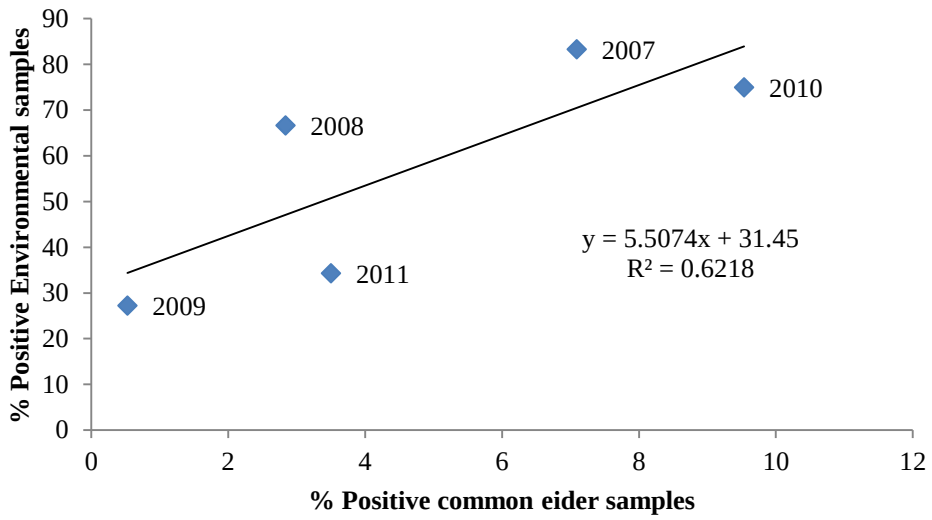

Figure 2-4. Correlation between prevalence of *P. multocida*-positive pre-breeding common eiders on East Bay Island, Nunavut, and proportion of PCR positive ponds on East Bay Island, during the breeding season from 2007-2011. Correlation indicated a weakly positive association (P = 0.11).

2.4.2 Common eiders in other locations and king eiders at East Bay Island and other locations

While all breeding female common eiders sampled near Cape Dorset, Nunavut, tested negative for *P. multocida* in 2010 (Table 2-3), 2.5% of eiders tested positive in 2011, and 13.8% of fecal samples tested positive (Table 2-3). All fecal samples collected from from colonies around Ungava Bay, Nunavik, in 2011 tested negative (Table 2-3).

Of the king eiders tested on East Bay Island in 2009-11, 3/52 (5.8%) tested PCR positive on oral and/or cloacal swabs (Table 2-3). All positive birds were sampled before the outbreaks began in the years they were sampled (Table 2-3). All king eiders sampled in Cape Dorset in 2011 tested negative (Table 2-3).

2.4.3 Lesser snow geese and Ross's geese

From 2008-2011, between 1.1 and 8.7% of sampled lesser snow geese (overall 6.3%) tested positive for *P. multocida* on East Bay Island (Table 2-3). No Ross's geese sampled on East Bay Island tested positive (Table 2-3). Between 2.0 and 6.0% (overall 3.9%) of lesser snow geese sampled from Southampton Island in 2010-2011 tested PCR positive, and 11.1% were positive at Cape Dorset in 2011 (Table 2-3). In 2011, 3.4% of Ross's geese and 2.9% of lesser snow geese from Queen Maud Gulf tested positive, while 3.2% of lesser snow geese from the Great Plain of the Koukdjuaq tested positive (Table 2-3).

2.4.4 Other species on East Bay Island- herring gulls and snow buntings

In all years, herring gulls were sampled after avian cholera outbreaks had begun. Overall, 32/63 (50.8%) of herring gulls tested were PCR positive on oral and/or cloacal swabs (Table 2-3). In general, there was a higher percentage of positive oral swabs compared to cloacal swabs (e.g., 38.9% vs 8.3% in 2010 and 50.0% vs 27.8% in 2011; Table 2-3). All snow buntings were sampled before avian cholera outbreaks had begun, and none tested positive (Table 2-3).

P. multocida was not isolated from any PCR positive avian swab samples from live or harvested apparently healthy birds, including from East Bay Island eiders sampled prior to the beginning of the annual avian cholera outbreaks.

2.4.5 East Bay Island environmental samples

From 2007 to 2011, 80% of pond sampling events (n = 25) were positive for *P. multocida* DNA before the avian cholera outbreak began, and 56.4% of pond sampling sessions were positive following outbreak initiation (n = 39; Table 2-7). Based on the highest ranked model, date of sample collection in relation to outbreak start date (β = -0.05, SE = 0.02, P = 0.02) and year (β= -3.14, SE = 1.11, P = 0.005) were important predictor variables (Table 2-8 and 2-9). Both pond water and sediment samples were more likely to test positive before avian cholera outbreaks began each year (Figure 2-5). This relationship appeared to be driven by 2010 and 2011, as it was not observed in the other years of the study (Figure 2-5, Table 2-7). Furthermore, the decline in the proportion of positive samples appeared to be most prominent prior to outbreaks ramping up (particularly in 2010-11), with a slight increase in the proportion of positive samples once outbreaks began (Figure 2-5). There was no relationship between average seasonal air temperature and the likelihood of detecting *P. multocida* DNA in water or sediment samples (Table 2-8, Figure 2-6, R^2 = 0.1, P = 0.60). Of the 347 pond water, sediment and nest soil samples cultured, *P. multocida* colonies were grown from one water sample collected in July 2010, after the avian cholera outbreak was underway. Of the 53 nest soil samples collected in 2011, 13 (24.5%) were positive for *P. multocida* DNA.

Table 2-7. Summary of *P. multocida* PCR results from pond and sediment samples collected from freshwater ponds on East Bay Island, Nunavut, from 2007-2011. Table shows total number of samples collected during sampling sessions before and after the annual avian cholera outbreak began each year.

Year	Pre- or post-outbreak	# samples	# positive samples (%)	# pond-sampling events	# positive pond-sampling events (%, 95% CI)
2007	Pre	50	10 (20.0)	2	2 (100%)
	Post	119	15 (12.6)	10	8 (80%, 55.2, 104.7)
2008	Pre	29	5 (17.2)	3	3 (100%)
	Post	120	29 (24.2)	12	7 (58.3%, 30.4, 86.2)
2009	Pre	58	5 (8.6)	3	1 (33.3%, -20.0-86.6)
	Post	79	5 (6.3)	8	2 (25%, -5.0, 55.0)
2010	Pre	62	51 (82.3)	7	7 (100%)
	Post	76	3 (3.9)	5	2 (40%, -2.94, 83.0)
2011	Pre	117	33 (28.2)	10	7 (70%, 41.6, 98.4)
	Post	38	4 (10.5)	4	3 (75%, 32.6, 117.4)

Table 2-8. Binary logistic regression models explored to explain variation in *P. multocida* DNA detection in water and sediment samples from freshwater ponds on East Bay Island, Nunavut from 2007-2011 (n = 64). Pond-year was used as a random effect in all models.

Model - fixed terms	Number of parameters	AICc
Year + Date to outbreak	6	82.5
Date to outbreak	2	84.8
Date to outbreak + Temperature	3	86.0
Year + Date to outbreak + Year*Date to outbreak	10	86.4
Year	5	86.5
Null	1	86.6
Temperature	2	86.9

Date to outbreak = number of days before or after outbreak start date each year, Temperature = average air temperature of 71 days beginning May 30 of each year

Table 2-9. Summary of best supported model to explain variation in *P. multocida* DNA detection in pond water and sediment samples from East Bay Island, Nunavut, from 2007-2011 (n= 64).

Variable	β	SE	β 95% CI	P-value
Year (ref = 2007)				
2008	-0.43	1.00	-2.4, 1.6	0.67
2009	-3.14	1.11	-5.4, -0.9	0.01
2010	-1.02	1.09	-3.2, 1.2	0.35
2011	-1.75	1.11	-4.0, 0.5	0.12
Date to outbreak	-0.05	0.02	-0.09, -0.01	0.02

β = coefficient estimate, SE = standard error

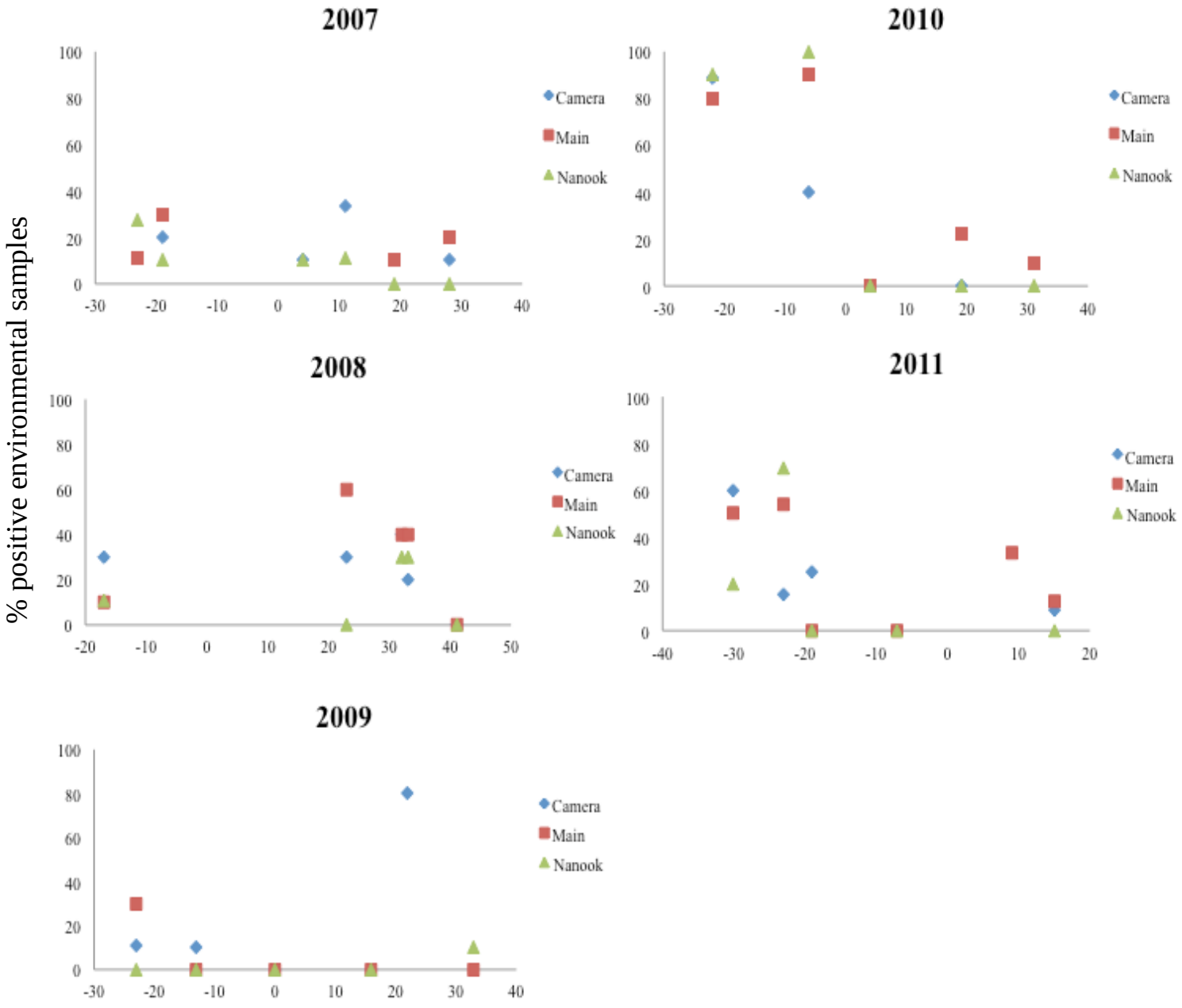

Figure 2-5. Proportion of water and sediment samples that tested positive for *P. multocida* by PCR for each sampling event of freshwater ponds on East Bay Island, Nunavut, in relation to onset of avian cholera outbreaks from 2007 to 2011). Date is shown as days before or after the annual avian cholera outbreak begins each year (day 0).

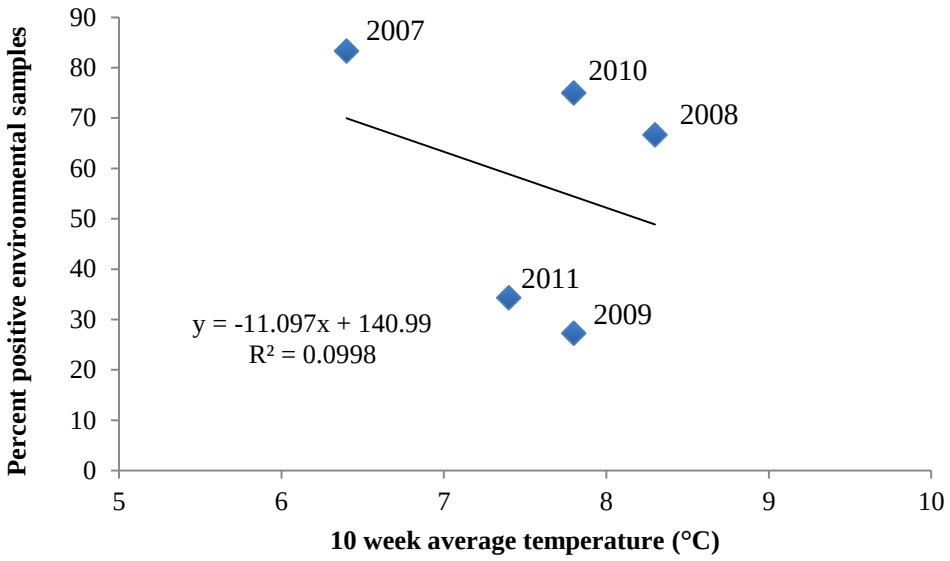

Figure 2-6. Correlation between the proportion ponds that tested positive for *P. multocida* byPCR from East Bay Island, Nunavut in relation to average air temperature for 10 weeks beginning on May 30 each year, from 2007-2011. Correlation was not significant (P = 0.6).

2.4.6 Cape Dorset- environmental samples

In 2010, eight of 138 (5.8%) samples collected on colonies near Cape Dorset were positive for *P. multocida*, and distributed across five of the 11 ponds (45.5%) sampled. Similarly, in 2011 three of 55 (5.5%) samples were positive, and distributed over three of eight ponds sampled (37.5%). No avian cholera outbreaks or large-scale avian mortality events have been reported in this region. *P. multocida* was not cultured from any pond water samples.

2.4.7 Ungava Bay- environmental samples

In 2011, 18/215 (8.3%) of water samples collected from common eider colonies in Ungava Bay were positive for *P. multocida,* and distributed among 13 of the 23 ponds (56.5%) (see Iverson 2015 for details about locations). Of the islands where PCR positive pond samples were collected, one was a location of a confirmed avian cholera outbreak in 2006, three were locations of reported avian mortality (unconfirmed avian cholera outbreaks) in 2006, two had reports of dead common eiders and gull species in 2011, and one was the location of an avian cholera outbreak detected during the colony survey in 2011 (see Iverson 2015). *P. multocida* was not cultured from any pond water samples.

2.5 Discussion

Using a recently developed PCR assay for the detection of *P. multocida* DNA in field samples (Corney et al., 2007), we explored potential avian and environmental reservoirs of *P. multocida*, the causative agent of avian cholera causing large-scale annual mortality in common eiders in the eastern Canadian Arctic in the last decade. We determined that infection with *P. multocida* is common in Arctic nesting common eiders and lesser snow geese at numerous locations across the eastern Canadian Arctic, with prevalences ranging from 0-13.8% for common eiders, and 0-11.7% for snow geese depending on location and year. This provides

further support for the importance of birds that carry and presumably disseminate *P. multocida* in the initiation and spread of avian cholera outbreaks (Vaught et al., 1967; Korschgen et al., 1978; Titche, 1979; Samuel et al., 1997; Samuel et al., 2005). A high proportion of freshwater ponds were also found to be contaminated with *P. multocida* DNA before and during outbreaks.Similar to other studies (Price et al., 1985; Botzler, 1991; Samuel et al., 2004; Lehr et al., 2005), our findings also suggest that the environment is a source of bacteria during avian cholera outbreaks in the eastern Arctic; however, the role of *P. multocida* in initiating outbreaks in the Canadian arctic requires further investigation. .

East Bay Island has been the site of annual avian cholera outbreaks since 2005 (Buttler 2009; Descamps et al., 2009), which strongly suggests that there is a reservoir of the bacteria resulting in exposure and infection of eiders nesting on the colony each year. From 2007 to 2011, 4.8% (0.8-9.5%) of common eiders sampled on arrival to their breeding colony at East Bay Island were found to be carrying *P. multocida* DNA prior to and at the start of outbreaks each year, and 14.4% were found to be infected at the end of the breeding season during colony departure. Although we were not able to culture live isolates from eiders arriving at East Bay Island before the breeding season, this finding indicates that a small proportion of eiders are arriving on the colony prior to outbreaks carrying *P. multocida* each year. A larger proportion leave the island infected with the pathogen. A previous study on common eiders (*Somateria mollissima dresseri*) experiencing sporadic avian cholera outbreaks in Maine also detected *P. multocida* (using culture techniques) from low numbers of live birds sampled before and during the outbreaks (Korschgen et al., 1978).

Exploration of environmental and demographic variables on the probability of infection indicated that common eiders were more likely to test positive for *P. multocida* in cooler springs,

when average spring temperatures were lower. Although few studies have examined the role of environmental temperature on carrier status of *P. multocida,* Muhairwa et al., (2001) looked at carrier chickens and domestic ducks in cool, warm and hot zones in Tanzania, and found that there were more carrier ducks in the warm zone as compared to the cool or hot zones. Climatic variables such as temperature can play important roles in pathogen survival (Stallknecht et al., 1990), transmission dynamics, and disease emergence (Kutz et al., 2013) in addition to affecting host behaviour, ecology, and exposure to pathogens (Parmesan, 2006). Although warmer spring temperatures may lead to eiders breeding earlier, and breeding and incubation can be associated with compromised immune function (Bourgeon and Raclot, 2006; Martin et al., 2009), adverse climatic conditions (e.g., colder temperatures in spring) may increase stress and reduce immune function (Martin et al., 2009). This could possibly lead to increased carriage and/or shedding of *P. multocida*. Climatic variability may have direct effects on individual physiology, as well as act through more complex pathways such as influences on food availability, energetics (Love et al., 2010) or carry-over effects (Legagneux et al., 2013; Harms et al., 2015). Air temperature in June is correlated with mean laying date of eiders on East Bay Island, and is also predictive of ice-free conditions in East Bay (Love et al., 2013). Furthermore, ice-free conditions are strongly correlated with laying and hatching dates (Love et al., 2013). These important relationships between temperature and reproductive timing may indicate that with cooler tempertures during the early breeding season, eiders delay egg laying. Local foraging is important to eiders that arrive earlier in the spring to the breeding colony (Mosbech et al., 2006), so in cooler years birds may spend an increased amount of time feeding in groups near the breeding colony prior to laying eggs. This may provide an opportunitiy for eiders to co-mingle and share pathogens such as *P. multocida* before nesting on the breeding colony, and might be a mechanism by which

increased numbers of birds are positive for *P. multocida* in cooler years. Although the mechanism linking cooler temperatures with carriage of *P. multocida* DNA in eiders at East Bay is unknown, this is the first study showing an effect of temperature on wild birds that may be carrying *P. multocida*.

Female common eiders captured in duckling traps as they were leaving the island with their ducklings were 3 times more likely to be infected (14.4%) compared to eiders sampled during the pre-breeding period in early June (4.8%). This was expected, since the timing of colony departure of eiders occurs while avian cholera outbreaks are well underway, and thus the probability of exposure at that time is higher. This finding raises the question of whether these birds act as carriers and return to the colony the following year, still infected with *P. multocida*. Of the 47 positive eiders captured during colony departure, 10 were resighted, of which five were found dead due to avian cholera in the same or following year, and five were captured alive in subsequent years, indicating that infection was not fatal in all of these birds. These results provide evidence that birds may leave the island shedding *P. multocida,* and may return alive to the colony in subsequent years. Further work investigating whether birds return to the island still carrying bacteria is required.

P. multocida DNA was also detected in samples from common eiders harvested or nesting near Cape Dorset. Although there are no reports of avian cholera outbreaks or large-scale avian mortality on eider colonies around Cape Dorset, the detection of *P. multocida* in eiders and pond water samples (see later in this discussion) may suggest that eiders from these locations have been exposed to the bacteria and are able to shed it into nearby ponds, and/or that the disease has gone undetected on some colonies. In contrast, *P. multocida* DNA was not found in

fecal samples from common eider colonies in Ungava Bay, near sites where avian cholera outbreaks had occurred in 2004, 2006 and 2011. .

We detected *P. multocida* DNA in live or harvested apparently healthy geese across the eastern Canadian Arctic, with prevalences ranging from 1.1 to 8.7% and up to 11.1% in lesser snow geese from East Bay Island and Cape Dorset, respectively, and up to 3.4% in Ross's geese at the Queen Maud Gulf Migratory Bird Sanctuary. These prevalences are similar or higher than those previously reported in geese in the Playa Lake region in Texas (0-4%), and on Banks Island, Northwest Territories (0.7%) obtained by culture of oral, cloacal, and tissue swabs (Samuel et al., 1997; Samuel et al., 2005). There is considerable evidence that snow geese play an important role in carrying and transmitting *P. multocida* to other susceptible birds in North America (Samuel et al., 1997). Blanchong et al. (2006c) found that mortality of other waterfowl species was correlated with lesser snow goose mortality during avian cholera outbreaks in Nebraska, suggesting a link between lesser snow geese and avian cholera outbreaks. Although no mortality of lesser snow geese or Ross's geese due to avian cholera has been documented near East Bay Island or in Nunavut, healthy carrier birds may be shedding the bacteria during brief stop-overs on East Bay Island in early spring, or contaminating nearby environments accessed by eiders. This may partly explain the high proportion of positive pond samples prior to eiders arriving on the colony in some years (see below). Samuel et al., (1997, 2005) also found *P. multocida* carrier geese at wintering and Arctic breeding sites, thus geese en route to their breeding grounds may be a potentially important source of disease outbreaks and spread (Samuel et al., 2005).

Herring gulls are an important component of the East Bay Island ecosystem, and play a key role as a scavengers. During 2008-2011, there was a high prevalence of PCR positive herring

gulls sampled during outbreaks on East Bay Island (from 44.4% in 2010 to 62.5% in 2009). As scavengers of eider carcasses, gulls on East Bay Island were exposed to *P. multocida* as they ingested tissue from carcasses that had died of the disease. This is further supported by the fact that gulls were more likely to have positive oral swabs, compared to cloacal swabs. Small numbers of herring gulls died during avian cholera outbreaks on East Bay Island (n = 4) (I. Buttler, C., Soos, and H.G. Gilchrist, unpubl. data), and lesions in these birds were consistent with chronic avian cholera, including pericarditis, airsaculitis, peritonitis, and poor body condition (Canadian Wildlife Health Cooperative, unpubl. data). The ability of gull species to act as carriers of *P. multocida* has not been investigated but has been proposed (Botzler, 1991), and probably warrants further study, particularly considering that genotyping techniques have shown that *P. multocida* from East Bay Island is genetically similar to isolates recovered from the 2007 outbreak in pelagic gulls in Newfoundland (Chapter 4). In other studies, kelp gulls have been proposed as carriers initiating outbreaks in Antarctica (Leotta et al., 2006). Further work on the role of gulls in initiating or perpetuating avian cholera outbreaks seems warranted.

Wetland environments (water, sediment, and vegetation) become contaminated with *P. multocida* during an avian cholera outbreak, and environmental exposure to *P. multocida* is an important route of transmission, perpetuating outbreaks in wild birds (Price et al. 1985; Botzler, 1991; Samuel et al., 2004; Lehr et al., 2005). Laboratory studies have also shown that under certain conditions, *P. multocida* can remain viable in water for over one year (Bredy and Botzler, 1989). However, several field studies have concluded that it is unlikely that sites of previous outbreaks serve as a year-round source of *P. multocida* (Samuel et al. 2004; Lehr et al. 2005; Blanchong et al. 2006b), and cannot maintain the pathogen without re-introduction of *P. multocida* from avian hosts.

On East Bay Island, the three main ponds we sampled are the only sources of freshwater consistently present throughout the breeding season (i.e., they do not, or rarely, dry out during the summer), and nesting female eiders are observed to take drink breaks at these ponds (Bottitta et al., 2003; Buttler, 2009). A relatively high proportion of pond sampling events on East Bay Island were positive for *P. multocida* DNA throughout the entire breeding season. Model results indicated that, overall, the proportion of positive pond samples declined during the season. We expected the opposite trend since *P. multocida* should accumulate within water and sediment from increasing numbers of sick and dead birds during outbreaks (Price and Brand, 1984; Botzler, 1991; Samuel et al., 2003; Blanchong et al, 2006a; 2006b). On closer examination, the declining trend was most evident prior to the onset of outbreaks each year (Figure 2-5), after which there appeared to be an increasing trend in most years once outbreaks began (Figure 2-5). This trend was particularly noticeable in 2010 and 2011, in which the highest levels of contamination in the environment occurred even prior to ice melt. There was a subsequent sharp decline in the proportion of positive samples prior to outbreak onset (Figure 2-5). Once outbreaks began in those years, there was a small increase in positive pond samples even though avian cholera mortality rates were relatively low in those years (Iverson, 2015). 2008 had the highest proportions of positive pond samples following outbreak onset (Figure 2-5), as it was the year with the highest eider mortality rate during this study (>1400 carcasses, Iverson, 2015). This resulted in substantial contamination of ponds with *P. multocida*.

In all years, moderate to high proportions of samples were positive for *P. multocida* at the beginning of the season prior to snow melt and the arrival of eiders (Figure 2-5). It is possible that evaporation of pond water during fall prior to freezing in winter resulted in higher concentrations of *P. multocida* DNA in ponds early in the season prior to spring thaw, compared

to later in the season when ponds thawed and were diluted with snow melt. Since detection of DNA does not indicate whether the bacteria is viable, and we were unable to culture *P. multocida* from PCR positive pond samples prior to outbreaks, we are not able to draw conclusions about the importance of ponds acting as reservoirs on East Bay Island. Nevertheless, ponds on East Bay Island do become contaminated with bacteria during outbreaks, and there appears to be an increase in contamination once outbreaks begin. This provides some support for the environment playing a role in maintaining outbreaks once they have begun. This is further supported by the fact that we were able to culture *P. multocida* from one pond sample collected after the outbreak had begun in 2010. Furthermore, we found a slight correlation between the proportion of positive pond samples and prevalence of infected eiders detected each year during the breeding season. This suggests that increased shedding from infected birds increases the amount of DNA present in ponds, and in turn, increased levels of DNA in ponds results in more healthy birds becoming infected. Thus, ponds on East Bay Island likely play a role in the maintenance of avian cholera outbreaks.

We also collected both pond water and sediment samples from common eider colonies near Cape Dorset, Nunavut, and Ungava Bay, Nunavik. Avian cholera mortality was observed on several eider colonies in the Ungava Bay region in 2004, 2006, and 2011 (Iverson 2015). Positive water samples were found on 13 of 23 islands associated with avian cholera in 2006 and 2011, as well as on islands with no reported prior history of avian cholera. These findings suggest that freshwater ponds can become contaminated with *P. multocida* during outbreaks of avian cholera, and may play a role in transmission of the disease in the Arctic (Samuel et al., 2007). We also detected *P. multocida* DNA in ponds from eider colonies near Cape Dorset, even though avian cholera had never been reported in this area (Iverson, 2015). The proportion of

positive samples per pond on these colonies was much lower than that observed on East Bay Island (data not shown). This may indicate that a small amount of the pathogen can be introduced to new locations, possibly by carrier birds. For example, *P. multocida* was recovered from wetlands in California that were not directly associated with avian cholera outbreaks (Lehr et al. 2005), and no *P. multocida* was detected in wetlands associated with avian cholera mortality. In that study, the authors speculated that birds were acquiring bacteria from wetlands other than the ones that they died on (Lehr et al., 2005). Further investigation would be needed to determine if environmental sampling could be used as an early indicator of, or evidence of previous, disease outbreaks on eider colonies.

We examined the effect of average summer temperature on the presence of *P. multocida* DNA in ponds on East Bay Island. Unlike our findings for prebreeding eiders, we did not find a significant association between average air temperature and the likelihood of detecting *P. multocida* DNA in water or sediment samples. Few studies examining environmental reservoirs have explored the role of seasonal temperature, with the exception of Blanchong et al., (2006b) who found that *P. multocida* was isolated from significantly more wetlands during years with an El Niño event, as compared to non-El Niño years. Warmer temperatures and rainfall associated with El Nino may have led to a wider distribution of birds across wetlands. Temperature has been shown to be an important factor in infectious disease dynamics, particularly in any diseases when the environment plays a role as a reservoir or is important for transmission or pathogen life-cycle (for example see Vezzulli et al., 2010 on cholera; Blackburn and Goodin, 2013 on anthrax; Kutz et al., 2013 on wildlife parasites). As the climate continues to change, further research into the effects of temperature and other environmental variables on the dynamics of infectious disease will be key to our understanding of their ecology and effects.

Our main interest was to identify avian and environmental reservoirs of *P. multocida*, and evaluate the role of these reservoirs in the initiation and maintenance of avian cholera outbreaks. Our findings of PCR positive swab samples from apparently healthy common eiders, geese, and gulls in multiple locations in the eastern Canadian Arctic are very suggestive that some birds may be transporting and transmitting *P. multocida*. We also provide evidence that ponds likely play a role in maintaining outbreaks once they have begun. We have shown that PCR can be used to detect very small amounts of *P. multocida* DNA in oral and cloacal samples from live, apparently healthy wild birds and ponds across the eastern Canadian Arctic. These findings further our understanding of avian cholera ecology in northern Canada, and open the door for future studies on this disease in the north. However, additional work to isolate and characterize viable *P. multocida* from carrier birds and environmental samples will be needed to further our knowledge of avian cholera disease ecology.

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2.7 Supplementary information for Chapter 2

2.7.1. Comparison of assay results for P. multocida real-time PCR from two laboratories

To enable us to combine PCR results from MGGen and University of Saskatchewan Microbiology laboratory, we compared PCR techniques using a dilution series of *P. multocida* DNA. Four *P. multocida* isolates from dead birds were used; a glaucous gull from Newfoundland from 2007 (serotype 1), a common eider from East Bay Island from 2006 (serotype 1), a common eider from Nunvavik from 2006 (serotype 3x4x12) and a common eider from East Bay Island from 2007 (serotype 3x4). DNA was extracted using the Chelex extraction protocol described in Legagneux et al., (2014). A 7-step dilution series of DNA from each isolate was made from 1ng/µl to $1x10^{-7}$ ng/µl and run in triplicate using the modified PCR assay protocols at both laboratories (Table S2-1), with the following modifications. At MGGen, *P. multocida* DNA was amplified in 10 µL PCR mixtures containing 1 µL of template DNA, 3 µL of molecular-grade water, 5 µL mastermix (TaqMan Universal Mastermix, Invitrogen, Grand Island, New York, USA), 0.5 µL forward and reverse primer at 10 µM (Sigma-Aldrich, Oakville, Ontario, Canada), 0.1 µL of TaqMan probe at 10 µM (Applied Biosystems, Life Technologies Inc, Grand Island, New York, USA), and 0.02 µL Taq (Platinum Taq, Invitrogen, Grand Island, New York, USA) (Table S2-1). The Students t-test (Microsoft Excel version 14.5.1, 2011) was used to compare the average Ct values between laboratories for the four isolates separately and correlation coefficients (R^2) for these data were calculated. Average Ct values for each of the dilutions for four different *P. multocida* isolates between the two labs showed excellent correlation (R^2 = 1.0, 1.0, 1.0, 0.98). There was no significant difference between the average Ct values from each lab for any of the isolates (P = 0.99, 0.75, 0.90, 0.87). Thus, we were able to combine datasets obtained from the two laboratories in our subsequent analyses.

Table S2-1. Cycle threshold (Ct) results (mean±SD) from real-time PCR assays for *P. multocida* conducted at two labs. Assays were run in triplicate at both laboratories using the same dilution series of *P. multocida* DNA, see text for details about *P. multocida* isolates.

DNA dilution (ng/µL)	*P. multocida* 001		*P. multocida* 002		*P. multocida* 003		*P. multocida* 004	
	MGGen	U of S	MGGen	U of S	MGGen	U of S	MGGen	U of S
1	11.7±0.3	7.9±0.5	9.5±0.5	8.3±0.7	10.5±0.5	11.4±0.3	14.7±0.3	12.3±5.8
$1x10^{-1}$	14.6±0.3	13.8±0.3	12.3±0.4	13.3±0.5	14.1±0.4	14.1±0.8	16.5±0.4	18.1±3.1
$1x10^{-2}$	17.9±0.1	18.3±0.1	16.3±0.4	18.4±0.5	17.9±0.7	19.0±0.8	19.4±0.5	20.7±2.2
$1x10^{-3}$	21.5±0.4	21.1±0.4	20.2±0.4	21.2±0.8	21.6±0.4	21.6±0.9	22.8±0.3	25.1±2.3
$1x10^{-4}$	24.8±0.6	24.9±0.2	23.5±0.3	24.6±1.0	24.3±0.6	24.2±0.4	26.1±0.5	27.7±0.9
$1x10^{-5}$	27.5±0.6	28.2±0.9	26.0±0.2	27.9±1.3	26.6±0.7	27.9±0.2	29.5±0.2	30.2±1.2
$1x10^{-6}$	30.4±0.5	32.0±0.7	29.4±0.2	31.6±1.9	29.1±0.5	29.9±0.7	33.0±0.2	33.1±1.1
$1x10^{-7}$	33.0±0.8	35.9±1.6	32.2±0.8	35.0±2.5	32.3±0.4	32.3±0.8	35.6±0.6	35.6±0.5

Table S2-2. Logistic models explored to explain variation in *P. multocida* DNA detection in pre-breeding common eiders from East Bay Island, Nunavut from 2007-2011 (n = 1971). Non-informative variables are in italics.

Model - fixed terms	Model - random term	Number of parameters	AICc
Mean_Temp + *Date_cont*	Year	3	725
Mean_Temp	Year	2	726.2
Sex + Mean_Temp	Year	3	726.2
Mass + Mean_Temp	Year	3	727.2
null	Year	1	727.7
Mass	Year	2	727.7
Sex	Year	2	727.7
Mass + Sex + Mean_Temp	Year	4	728.1
Date_cont	Year	2	728.5
Sex + Date_cont	Year	3	728.5
Mass + *Sex* + Mean_Temp + *Date_cont* + *Mass*Sex*	Year	6	728.6
Mass + Sex	Year	3	729.1
Mass + Date_cont	Year	3	729.1
*Mass + Sex + Mean_Temp + Mass*Sex*	Year	5	730.1
Mass + Sex + Date_cont	Year	4	730.3
*Mass + Sex + Mass*Sex*	Year	4	730.8
*Mass + Sex + Date_cont + Mass*Sex*	Year	5	732.2

Mean_temp = average temperate for 7 days prior to the date a bird was captured, Date_cont = date before of after the start date of the outbreak

CHAPTER 3
MOLECULAR EPIDEMIOLOGY OF AVIAN CHOLERA IN THE EASTERN CANADIAN ARCTIC

Key words: avian cholera, *Pasteurella multocida*, repetitive element PCR, multilocus sequence typing, genetic diversity

3.1 ABSTRACT

Avian cholera, caused by infection with *Pasteurella multocida*, is an important infectious disease of wild birds in North America. Avian cholera emerged in the eastern Canadian Arctic in 2004, resulting in annual outbreaks on the largest breeding colony of northern common eiders (*Somateria mollissima borealis*) in Canada and sporadic outbreaks in eider colonies in Northern Quebec (Nunavik). We investigated the potential origins of *P. multocida*-causing avian cholera in Arctic eider colonies, and explored the local molecular epidemiology of the disease on East Bay Island. We compared eastern Arctic isolates of *P. multocida* to those collected from wild birds across Canada and from birds and wetlands in the central flyway in the United States using repetitive extragenic palindromic-PCR (REP-PCR) (n = 297) and multi-locus sequence typing (MLST) (n = 269). Both MLST and REP-PCR detected a low degree of genetic diversity among isolates, with genotype correlated to somatic serotype. Although serotype 1 isolates were found to be mostly homogenous by REP-PCR, MLST revealed that isolates from East Bay Island were distinct from *P. multocida* from eider colonies in the St. Lawrence Estuary and from isolates from the US central flyway, but were indistinguishable from isolates from a pelagic avian cholera outbreak that occurred on the Canadian east coast. Serotype 4 and 3x4 isolates from eiders from both East Bay Island and Nunavik had indistinguishable sequence types, indicating possible transmission of isolates among eider colonies in the eastern Arctic. Both MLST and

REP-PCR detected genetic differences in isolates cultured from apparently healthy live birds in Quebec compared to birds that had died from avian cholera, suggesting that carrier birds may carry different strains of the bacteria than those causing disease. Finally, analysis of MLST results revealed that *P. multocida* isolates in Canada form only three genetically distinct populations, suggesting that *P. multocida* in wild birds forms clonal populations.

3.2 Introduction

Emerging infectious diseases are diseases that have recently increased in incidence or geographic range, moved into new host populations, are newly discovered, or are caused by newly-evolved pathogens (Daszak et al., 2001). Emergence of new diseases in wildlife can be caused by novel pathogens that have recently invaded a wildlife population, pathogens that are spreading in their host population due to new external factors, or pathogens that have emerged as a result of a combination of factors, such as movement of a novel pathogen into a population that is rendered more susceptible to disease due to external factors (Dobson and Foufopoulos, 2001). Avian cholera, a disease caused by infection with *Pasteurella multocida*, was first detected in wild birds in North America in the 1940s, and spread rapidly throughout the continent in the 1970s (Samuel et al., 2007). Avian cholera is a significant disease in numerous species of waterfowl in North America since it can result in large-scale mortality, affect many species, and cause annual outbreaks (Blanchong et al., 2006a; 2006b; Samuel et al., 2007). In North America, major outbreaks occur when high densities of waterfowl gather on wintering sites (Blanchong et al., 2006a) or during summer in colonial-nesting species of waterfowl (Wobeser, 1992).

Despite the fact that avian cholera has been an endemic disease in Canada for several decades (Wobeser et al., 1979), it has only recently been documented in the eastern Canadian Arctic (Descamps et al., 2009). Since it was first confirmed in 2005, avian cholera has resulted in

annual mortality at the largest northern common eider (*Somateria mollissima borealis*) breeding colony in Canada (East Bay Island, Nunavut, hereafter East Bay Island, 64°02'N, 81° 47' W), with mortality rates of breeding females ranging from as high as 36.3% in 2006 and 26.9% in 2008, to less than 5% in recent years (Descamps et al., 2012; Iverson 2015). Subsequent impacts on the size of the breeding colony has led to increasing concern about the vulnerability of the population given that the species is long-lived with a low annual reproductive rate, and the East Bay Island population is subject to other stochastic events in a changing Arctic environment (Descamps et al., 2012). Mortality has also been detected and confirmed in other bird species during outbreaks, including herring gulls (*Larus argentatus*), black guillemots (*Cepphus grylle*), brant geese (*Branta bernicla*), Canada geese (*Branta canadensis*), and snow buntings (*Plectrophenax nivalis*) (Buttler, 2009), as well as common eiders in other locations in the eastern Canadian arctic, including colonies along the west coast of Ungava Bay, Nunavik, in 2004 (Gaston, 2004), 2006 and 2011 (Iverson, 2015). Prior to 2004, avian cholera outbreaks had been detected in snow goose colonies in Northwest Territories (Samuel et al., 1999), and greater white-fronted geese in Alaska (Samuel et al., 2005), but had never been diagnosed in the eastern Canadian Arctic (Henri et al., 2010). Serotypes of *P. multocida* isolated from East Bay island outbreaks since 2005 have varied among years, and have included serotypes 1, 3, 4, and 3x4 (Buttler, 2009; Soos, C., unpubl. data). Furthermore, multiple serotypes have also been identified within outbreaks, which suggests that multiple unrelated strains of *P. multocida* have been involved in these mortality events.

The recent emergence of annual outbreaks in the eastern Canadian Arctic causing extensive mortality of common eiders, and the identification of multiple serotypes of *P. multocida* on East Bay Island raises questions about the ultimate origins and relatedness of the

bacterial strains causing this disease. Understanding possible origins and factors involved in the emergence of this disease in the eastern Arctic is important not only for its potential impact on conservation, but also because it may have negative impacts on northern residents, given that eiders are an important source of food, eggs, and down for subsistence hunters in Nunavut and Greenland (Henri et al., 2010).

Potential sources of *P. multocida* include wild carrier birds (Samuel et al., 2003) such as lesser snow geese, which have been shown to be apparently healthy carriers of *P. multocida* (Samuel et al., 1997), and migrate from the United States to nesting sites in the Canadian Arctic. While snow geese do not nest on East Bay Island, small numbers make short stopovers on the colony in the spring each year, prior to the arrival of eiders (Buttler, 2009), and large numbers nest within the East Bay Migratory Bird Sanctuary approximately 4 km away. Other possible sources include avian cholera outbreaks occurring in southern Canada such as along the east coast or within the St. Lawrence estuary in Quebec. Some common eiders from East Bay Island are known to migrate and overwinter in eastern Canada (Mosbech et al., 2006) and may overlap spatially with other eiders as well as snow geese and gull species. Carrier birds or environmental sources from previous outbreaks in wintering areas may spread the bacteria to eiders that eventually return to East Bay, subsequently acting as sources of the disease.

P. multocida is a Gram-negative, non-motile, non-spore forming, facultative anaerobic bacterium that traditionally has been characterized using capsular serotyping (Carter, 1955; Rimler and Rhoades, 1987) and somatic serotyping (Heddleston et al., 1972). Although serotyping is useful for broadly characterizing *P. multocida*, these techniques have relatively low discriminatory power for most avian isolates associated with disease (Townsend et al., 1997; Amonsin et al., 2002; Subaaharan et al., 2010), and are not consistent predictors of pathogenicity

(Dziva et al., 2007). Techniques that can distinguish among *P. multocida* isolates of the same serotype and describe the relatedness of isolates are needed to improve our understanding of avian cholera epidemiology and movement patterns in wild birds. Genetic typing or fingerprinting can be used to understand clonal relatedness among isolates, and can provide information regarding the source and routes of infection (Ranjbar et al., 2014). Numerous genotyping techniques have been applied to *P. multocida* isolates from domestic (Ganwardana et al., 2000; Amonsin et al., 2002; Blackall and Miflin, 2000; Shivachandra et al., 2008; Sellyei et al., 2008; Subaaharan et al., 2010) and wild birds (Christensen et al., 1998; Pedersen et al., 2003; Blehert et al., 2008), as well as from a range of other animal species (Townsend et al., 1997; Saxena et al., 2006). Wilson et al., (1995) used DNA fingerprinting with HhaI and HhaII restriction endonucleases to show that somatic serotype 1 *P. multocida* isolates from raptors were distinct from poultry serotype 1 strains, indicating that poultry were an unlikely source of the bacteria for predatory birds. Amplified fragment length polymorphism (AFLP) techniques have detected genetic variation in a diverse group of serotype 1 *P. multocida* strains from wild birds and environmental samples, and found sufficient diversity among isolates to distinguish a range of epidemiological patterns (Blehert et al., 2008). However, AFLP poses some challenges as a genotyping method, including the complexity of the procedure, challenges and expense of testing large numbers of samples, and difficulty with comparing results between labs (reproducibility) (Ranjbar et al., 2014). Genotyping methods that identify highly variable, uncharacterized regions of the genome such as repetitive extragenic palindromic PCR (REP-PCR) are frequently used for local epidemiological studies. REP-PCR has been used in several studies to differentiate *P. multocida* isolates from multiple apparently unrelated sources (Amonsin et al., 2002) and to distinguish isolates collected from multiple outbreaks within a geographical region (Gunwadana

et al., 2002). REP-PCR is a fingerprinting technique based on amplification and detection of highly conserved, repetitive DNA sequences dispersed throughout the genome of prokaryotes (Versalovic et al., 1991; Townsend et al., 1997), and has been shown to have a high index of discrimination when used with *P. multocida* isolates (Amonsin et al., 2002). Multi-locus sequence typing (MLST) determines genetic relatedness among bacterial strains by analysing sequences of seven highly conserved, house-keeping genes for nucleotide substitutions (Subaaharan et al., 2010; Ranjbar et al., 2014). Using only seven loci, many distinct genotypes can be resolved, and unrelated isolates are highly unlikely to share a similar allelic profile by chance (Feil and Spratt, 2001). However, the slow accumulation of variation may lead to a lack of discriminatory power between epidemiologically unrelated strains and may not be as useful to study the movement of strains within an outbreak or examine epidemic spread within a short time frame (Melles et al., 2007). Despite some disadvantages, including that MLST is relatively expensive and time consuming, sequence typing can provide invaluable information about long term or global epidemiology of a group of organisms (Maiden et al., 1998). Hence, depending on epidemiological question being asked, applying a combination of genotyping techniques to a group of isolates may be the best approach to differentiating and understanding relationships among isolates.

Although avian cholera has been present in wild birds in North America since the 1940s, little is understood about the relationship of outbreaks occurring in different hosts or in different locations. The objectives of our study were to investigate the origins of *P. multocida* causing avian cholera outbreaks in the eastern Canadian Arctic; and explore the local epidemiology of avian cholera outbreaks on East Bay Island by using REP-PCR and MLST to compare genotypes of isolates collected from each annual outbreak, and isolates collected from wild birds over the

past 17 years from various locations throughout North America. Results from this study will contribute to our understanding of distribution, movement, and host-pathogen interactions of *P. multocida* genotypes causing avian cholera in North America.

3.3 Materials and Methods

3.3.1 P. multocida isolates from East Bay Island and other eastern arctic sites

Tissue samples were collected from common eiders, herring gulls, brant geese, and snow buntings found dead on East Bay Island during annual avian cholera outbreaks from 2005-2011 (n = 183) (see Harms et al., 2015) and from dead eiders and a dead Great Black-backed gull found on colonies in Nunavik in 2004, 2006, and 2011 (n = 11) (Table 3-1). Samples were collected in the field from a subset of dead eiders, and remaining birds were frozen at -20ºC and shipped to the Canadian Wildlife Health Cooperative (CWHC) based at the University of Saskatchewan, Saskatoon, SK, or the Université de Montréal, St-Hyacinthe, QC. Portions of liver, kidney, spleen, brain, and lung were collected and placed in 2 mL polypropylene cryogenic vials (Thermo Scientific Inc., Rochester, NY). Oral, cloacal, and bone marrow samples were collected using sterile polyester-tipped applicators (Puritan Medical Products Co., Guilford, ME) and stored individually in 2 mL polypropylene cryogenic vials. All samples were stored frozen at -196 °C in a cryoshipper (model SC 20/12V, MVE Vapour Shipper, MVE Bio-Medical Division, Chart Industries, Inc., Burnsville, MN) for transport to the (University of Saskatchewan, Saskatoon, SK, Canada. Samples were stored at -80°C until analysis.

3.3.2 P. multocida isolates collected or archived from other outbreaks in Canada and the US

In addition to samples from the eastern Canadian Arctic, isolates of *P. multocida* were collected from numerous locations throughout Canada from 1994 to 2009, and from select locations in the United States from 1996 to 2002 (Table 3-1). These isolates included 68 samples

from previous outbreaks in Canada and the US, 20 from apparently healthy birds, and 7 from environmental samples during outbreaks in the US (Table 3-1). Isolates from Newfoundland, Canada, were collected from the first outbreak of avian cholera detected in a pelagic environment in Atlantic Canada, from December 2006 to March 2007. Mortality consisted primarily of gull species, approximately 300-400km off the coast of Newfoundland, in St. John's, Newfoundland and along coastal Labrador (Whitney, H., et al., unpubl. data). Carcasses were shipped to CWHC in PEI and the Animal Health Laboratory, St. John's, Newfoundland, and processed immediately. *P. multocida* isolates from avian cholera outbreaks in the St. Lawrence estuary, Quebec, and various locations in Saskatchewan (see Table 3-1) were obtained from the CWHC. Isolates from samples processed at the CWHC in Quebec (Universite de Montreal) included those cultured from live and dead birds in outbreaks from East Bay Island (2005-2009), Quebec (2004-2009), Manitoba (2002), Saskatchewan (2000-2007), Nunavik (2004, 2006), and Northwest Territories (2002). Isolates from samples processed at the CWHC in Prince Edward Island (Atlantic Veterinary College) were those cultured from dead birds in the outbreak in Newfoundland (2007), as described below. These isolates were lyophilized following Matejtschuk (2007) and stored at room temperature until shipped to the University of Saskatchewan for DNA extraction. *P. multocida* isolates from snow geese from California were collected in 1999 (n = 1) and 2002 (n = 3) and three isolates from snow geese from Nebraska were collected in 2002. These seven isolates were collected from unidentified locations within these states, the outbreak status is unknown, and it is not known if the birds were live or dead when the sample were collected. These isolates were stored at -80ºC at the University of Saskatchewan. All other archived *P. multocida* isolates from the US and three from the NWT were shipped from the University of Wisconsin to the Center for Microbial Genetics and

Genomics, Flagstaff, Arizona, US, frozen on dry ice. Archived isolates from water or sediment samples from wetlands in Nebraska (n = 7) were also obtained, and had been collected, isolated, and stored as described in Samuel et al. (2003). Isolates from snow geese from Texas, and Colorado and Kansas (n = 6) were recovered from shot apparently healthy snow geese, isolates from a snow goose (n = 1), a northern pintail (n = 1) and greater white-fronted geese (n = 2) were recovered from found dead birds on Nebraska wetlands and isolates from snow geese of unknown status from California (n = 4). Isolates from snow geese (n = 2) were cultured from dead birds on Banks Island, Northwest Territories in 1995, and an isolate from a live snow goose on Banks Island (n = 1) was cultured in 1994. Isolates were collected from multiple tissues from the same bird for some birds from East Bay Island (n = 22), for the analyses only one isolate per bird was included, except for seven eiders from East Bay Island collected in 2010 and 2011 (see REP-PCR results). Isolates (n = 289) from all birds and environmental samples were included in the REP-PCR analysis, and a subset of these (n = 269) were also analysed by MLST (Table 3-1).

Table 3-1. Summary of *P. multocida* isolates cultured from wild birds.

Province/ Territory/ State	Location	Year	Species	Number of Isolates	Type of sample	Live or dead?	Serotypes
Nunavut	EBI	2005	COEI, HERG, BRGO, SNBU	13	lung, skin, unknown	dead	1
		2006	COEI, HERG	19	liver, unknown	dead	1; 3x4
		2007	COEI	39	lung, kidney, bone marrow, liver, spleen	dead	4; 3x4; 4x10x12x14
		2008	COEI, SNBU	47	unknown, oral swab	dead (28)/live (19)	1; 3; 4; 3x4
		2009	COEI	28	lung, kidney, lung, cloacal swab, oral swab	dead	1; 3x4
		2010	COEI	7	lung, brain, kidney, cloacal swab, oral swab	dead	4 (based on genotyping)
		2011	COEI	38	kidney, liver, lung, bone marrow, brain, spleen, oral swab, cloacal swab	dead	4 (based on genotyping)
Nunavik	Kuujjuaq	2004	COEI	1	unknown	dead	3x4
	Kuujjuaq, Kangirsuk	2006	COEI, GBBG	7	bone marrow, unknown	dead	3x4
		2011	COEI	3	kidney	dead	4 (based on genotyping); 3 (based on genotyping)
Quebec	Ile aux Pommes/Iles Bicquette	2002	COEI	2	Unknown	dead	1
	Unknown	2004	COEI	1	Unknown	dead	1
	Pot-du-Phare/Bature aux Alouettes/Ile aux Oeufs/Ile Bicquette	2005	COEI	7	unknown, oral swab	dead (3)/live (4)	1; 3x12; 4x7x12

	Ile aux Pommes/Iles Bicquette	2006	COEI	5	bone marrow, oral swab	dead (4)/live (1)	1; 14
	Ile Bicquette/ Gros-pot	2008	COEI	7	oral swab	live	3; 12; 4x7x12x15
	Ile Blanche/Ile aux Pommes/Ile Bicquette/Gros-pot	2009	COEI	9	oral swab	live	3; 4; 12; 3x4; 4x7x12
California	Unknown	UNK	SNGO	4	unknown	dead	4 (based on genotyping)
Colorado	Unknown	2002	SNGO	1	cloacal swab	live	1
Kansas	Unknown	2001	SNGO	2	eye swab	live	3; 3x4
Manitoba	Churchill	2002	HERG	1	l	dead	1
Nebraska	Rainwater Basin	1996-1999	Environment, SNGO, NOPI, LWFG	14	water, mud, liver	dead	1; 4 (based on genotyping)
Texas	Unknown	2001, 2002	SNGO	3	oral swab, nasal swab	live	1; 3x12
Newfoundland	St. John's, Flemish Pass, Flemish Cap, Grand Banks	2007	GBBG, GLGU, ICGU, HERG, CORA, ICGU, BLKI	19	lung, heart, liver	dead	1
Northwest Territories	Banks Island	1994, 1995, 2002	SNGO	4	bone marrow, unknown	dead	1; 3; 3x12
SK	Dore Lake, Sovereign, Kazan, Milden	2000, 2001, 2005-2007	DCCO, SNGO, COGE, AWPE, CORA	16	liver, spleen, lung, oral swab	dead (14), live (2)	1; 12

AWPE = American white pelican, BLKI = black-legged kittiwake, BRGO = Brant goose, COEI = common eider, COGE = common golden eye, CORA = common raven, DCCO = double crested cormorant, GBBG = great black backed gull, GLGU = glaucous gull, HERG = herring gull, ICGU = Iceland gull, KIEI = king eider, LWFG = lesser white front goose, NOPI = northern pintail, ROGO = Ross's goose, SNBU = snow bunting, SNGO = lesser snow goose

3.3.3 Bacteriology

Intact bird carcasses collected from East Bay Island and Nunavik were examined grossly for lesions consistent with avian cholera, except in the case where only bones or severely scavenged carcasses were available (CWHC database; Buttler, 2009; Harms, N.J., unpubl. data). For carcasses shipped whole from East Bay Island, carcasses were thawed, swab samples were collected from the tissues listed previously and immediately plated onto 5% blood agar and incubated at 37 °C for 18–24 hours. For tissues or swab samples sent from the field, samples were thawed and plated onto 5% blood agar and incubated at 37°C for 18 to 24 hours. Plates were examined for colonies with morphology and Gram stain consistent with *P. multocida*. These colonies were re-plated on blood agar and incubated for 18–24 hours at 37 °C. If a pure culture was obtained, several colonies were picked and confirmed as *P. multocida* using a real-time PCR assay (Corney et al., 2007) with modifications as described in Chapter 2 of this thesis.

For Newfoundland samples, fresh samples from gull carcasses were cultured on both blood agar and MacConkey II Agar (BD, Mississauga, Canada) at 35ºC for 24–48 hours. Bacterial colonies were identified by selective growth, colony morphology, Gram stain and biochemical characteristics. Isolates were identified based on a combination of catalase, oxidase (Dryslide; BD, Mississauga, Canada), indole (Kovac's) and sugar fermentation (TSI Slant; Oxoid. Nepean, Canada) activity.

All isolates shipped lyophilized from the CWHC in Quebec or Prince Edward Island were reconstituted by adding 500 or 1000 µL of sterile physiological saline solution to the isolate and gently agitating the vial, then immediately plating the solution onto 5% blood agar and incubating it for 18–24 hours at 37°C. Archived isolates stored frozen at the University of Saskatchewan were thawed and plated on blood agar as described above.

3.3.4 Somatic Serotyping

All isolates except those collected from East Bay Island and Nunavik colonies in 2010 and 2011 were serotyped by the agarose gel precipitin test (Heddleston et al.,1972) performed at the USGS National Wildlife Health Center, Madison, Wisconsin, USA.

3.3.5 DNA extraction

For all REP-PCR reactions, with the exception of the 20 isolates from Nebraska, Texas, Colorado, and Northwest Territories, *P. multocida* genomic DNA was extracted using a modified salting out procedure (Martin-Platero et al., 2007), with the following modifications. For each *P. multocida* isolate, a sterile pipette tip was used to collect 2-3 isolated colonies from a pure culture and transfer the colonies to 100 µL of TES buffer (10%w/v sucrose, 25mM Tris-HCl (ph 8.0), 10 mM EDTA, 10 mg/ml lysozyme, 100 u/ml mutanolysin, 40 µg/ml RNase) and incubated at 37°C for 30 minutes, or colonies were incubated in brain-heart infusion (BHI) agar for 18 hours at 37°C, and 1–1.5 mL of BHI agar was pelleted, and then resuspended in 100 µL of TES buffer. The quality of DNA extractions was assessed by spectrophotometric measurements at 260 and 280 nm, and samples with an A260/A280 ratio of 1.8–2.0 used for analysis (Katyal et al. 2013). Extracted DNA was stored at -80°C in TE buffer (1 mM EDTA and 10 mM Tris–HCl at pH 7.4). A subsample of the DNA was shipped to the Center for Microbial Genetics and Genomics on dry ice for MLST analysis. For 17 isolates from the US and 3 from Northwest Territories, *P. multocida* genomic DNA was extracted at the Center for Microbial Genetics and Genomics from broth cultures using a commercial Qiagen DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) following the manufacturer's instructions for Gram-negative bacteria.

3.3.6 REP-PCR protocol and analysis

REP-PCR was performed as described by Gunawardana et al., (2000) with the following modifications. Amplification reactions were performed in a final volume of 25 µL, containing 10 µM of each primer (REP1R-IDt 3' -CGGNCTACNGCNGCNNNN-5' and REP2-IDt 5'-NCGNCTTATCNGGCCTAC-3'), 10 µM of dNTPs, 10x PCR buffer, 50 mM $MgCl_2$, 0.15 µL of Platinum Taq DNA polymerase (Life Technologies, Burlington, Ontario, Canada), 15.35 µL of ultrapure water, and 2 µL of template DNA (approximately 30-50 ng/µL). A no-template negative control was included in every assay. A subset of isolates (n = 151) were included in the assay in duplicate. PCR was performed using thermocycler conditions as described by Gunwardana et al., (2000). Amplified products were resolved by electrophoresis in 2% agarose gel containing ethidium bromide at 100V and 450 mA for 150 minutes. Three ladders (Gene ruler 1 kb DNA ladder, Life Technologies, Burlington, Ontario, Canada) were used as size markers for each gel. DNA fragments were examined by UV transillumination and photographed (AlphaImager, Protein Simple, San Jose, California, USA) (Figure 3-1). Gel images were analysed using GelCompare II software (version 5.1, Applied Maths, Austin, Texas, USA). A binary data matrix was built on the presence or absence of any fragment appearing within any isolate, between approximately 300 and 3700 bp. Only distinct and prominent bands were included. The program automatically computed the similarity for each pair of fingerprints on the bases of band positions with the Dice coefficient. A cut off value of 0.90 was used so that pairs of isolates with a similarity coefficient of 0.90 or greater were considered similar and not distinguishable. Cluster analysis was performed using the unweighted pair group method with arithmetic linkages (UPGMA).

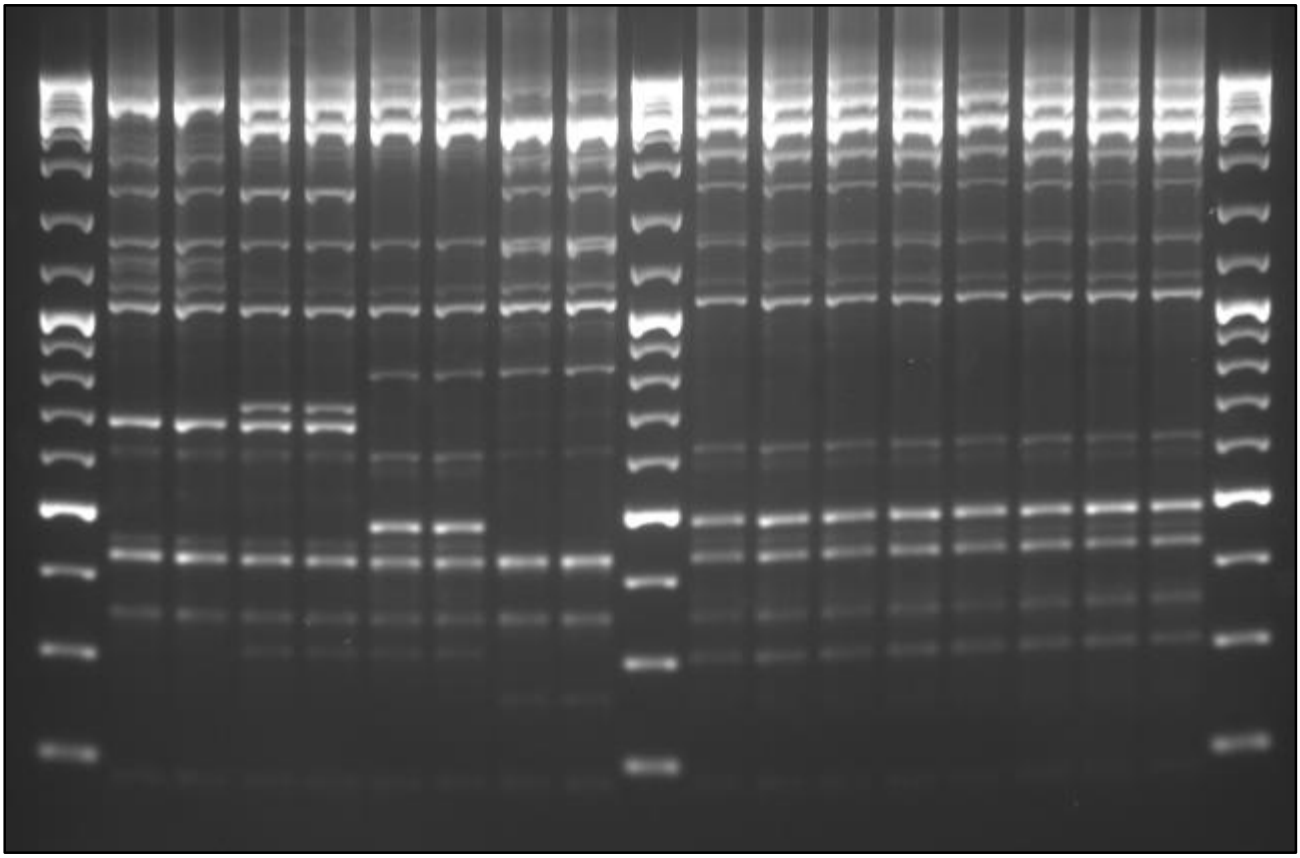

Figure 3-1. A photograph of a representative gel showing REP-PCR profiles generated for some *P. multocida* isolates used in this study. Lanes 1-19 contain: Gene ruler 1 kb DNA ladder, Pm 273, Pm 273, Pm 089, Pm 089, Pm 697, Pm 697, Pm 041, Pm 041, Gene ruler 1 kb DNA ladder, Pm 373, Pm 373, Pm 808, Pm 808, Pm 374, Pm 374, Pm 360 Pm 360, Gene ruler 1 kb DNA ladder.

3.3.7 MLST protocol and analysis

The MLST scheme used in this study is based on Subaaharan et al., (2010). In each 10 µL polymerase chain reaction, 1 µL *P. multocida* DNA was added to 9 µL PCR mix. Amplifications were performed with the PCR mix of 10x PCR buffer without $MgCl_2$, varying $MgCl_2$ concentrations by marker (5.1 mM for adk, est, pgi, pmi, and zwf; 4.5 mM for mdh and gdh), 0.8 mM dNTP mix, 0.1 U/ µL Platinum *Taq* DNA Polymerase (Life Technologies, Carlsbad, CA), 0.2 uM of each primer, and molecular grade H_2O and was used with the following thermocycling conditions: one cycle of 5 min at 94°C for initial denaturation, 35 cycles of 30 s at 94°C for denaturation, 30 s of varying annealing temperatures by marker (62°C for adk, est, mdh, 65°C for pmi, and 69°C for gdh, pgi, zwf), 1 min at 72°C for extension, and one cycle of 5 min at 72°C for final extension. The resultant PCR products were visualized on agarose gels. PCR product was purified using the QIAquick spin column-based PCR Purification Kit or plate-based 96 PCR Purification Kit (Qiagen) according to manufacturer's instructions, with 30 µL and 60 µL elutions, respectively.

Sequencing of purified PCR product was performed on an Applied Biosystems 3130 Genetic Analyzer with BigDye® Terminator v3.1 chemistry (Life Technologies). Raw sequence data were edited and aligned using Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, MI). Sequences were assigned an allele and sequence-type according to the *Pasteurella multocida* MLST database (www.pubmlst.org/pmultocida) following the RIRDC scheme, with novel alleles confirmed with sequence data for both DNA strands by the database curator, P. Blackall. Population genetic structure was determined using program *Structure* v2.3.4, a Bayesian clustering algorithm, in which we evaluated K values (number of discrete populations) from 1 to 10 with each K value repeated for 10 iterations. The burnin value was set at 20,000 for 100,000

reps. The true K value was determined using the Evanno method (Evanno et al., 2005) within the program *Structure Harvester* (Earl and vonHoldt, 2012).

3.4 Results

3.4.1 REP-PCR

Cluster analysis assigned the 285 isolates from 282 birds (four isolates from one bird were included) and seven wetland water samples into 22 distinct fingerprint patterns (n =292) using a 90% similarity cut off value. The 22 fingerprint patterns could be further grouped into five distinct clusters, each of which were closely associated with one or more serotypes. A dendrogram showng the relationships among all *P. multocida* isolates (n= 292) is shown in Figure 3-2. The minimum percent similarity among all isolates was 62.2%. Cluster I contained predominantly serotypes 3 and 4x7, which were mainly isolated from live birds. Cluster II contained mainly isolates of serotypes 3x4 and 4, clusters III and IV contained isolates with serotypes 3 and 3x12, and cluster V contained serotype 1 isolates (Figure 3-2).

Three separate dendrograms for each group of serotypes (serotype 1, serotypes 3x4 and 4, and serotypes 3 and remaining serotypes) were also created (data not shown). A total of 297 isolates from 282 birds and seven environmental samples were used to create these dendrograms (two isolates from six birds and three isolates from one bird were included) (Table 3-1). Serotype 1 isolates (n = 86) formed three profiles, each containing at least two isolates. The largest profile contained 81 isolates, including those from East Bay Island from 2005, 2006, 2008 and 2009, isolates from Saskatchewan from 2000, 2001, 2005-2007, Quebec isolates from 2002, 2004 and 2006, isolates from Newfoundland from 2007, and several isolates from US outbreaks (nine isolates from Rainwater Basin, Nebraska from 1997-1999, and one isolate from Texas collected in 2001).

Isolates with serotypes 4 and 3x4 (n = 183) clustered together but formed seven distinct profiles. At a 75% similarity cut-off level, however, all isolates were grouped into one profile (data not shown). *P. multocida* cultured from birds on East Bay Island and Nunavik colonies in 2010 and 2011 were not serotyped using the agarose gel precipitin test. However, all samples from these birds clustered with other 4 and 3x4 isolates, suggesting that they shared the same serotype. The largest profile contained 89 isolates, and included isolates from East Bay Island from 2006-2011, and isolates from Nunavik outbreaks from 2004, 2006, and 2011.

Serotype 3 isolates clustered with a diverse group of serotypes (total n = 28) including 3x12, 12, 4x7x12, 4x7x12x15, 4x10x12, and 4x10x12x14. These isolates formed 8 distinct profiles. Serotype 3 isolates from East Bay Island collected in 2008 formed a separate profile from those collected from eiders in Quebec.

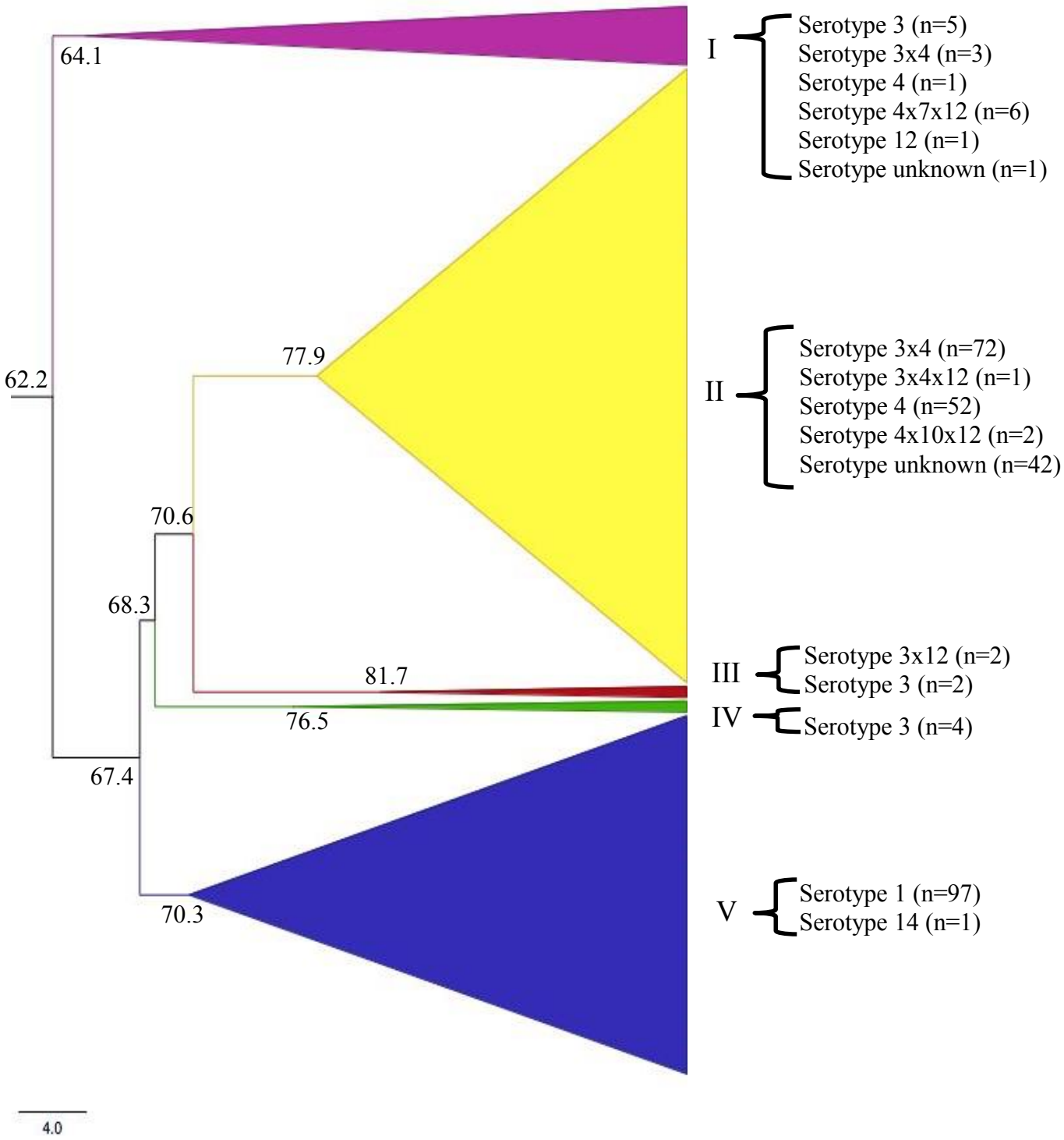

Figure 3-2. UPGMA clustering of REP-PCR fingerprints of all *Pasteurella multocida* isolates. Fingerprints have been collapsed at the last common node for each cluster. Numbers on the branches indicate percent similarity.

3.4.2 MLST

MLST data were explored using two methods. Based on the DNA sequences of each locus, isolates were assigned to a sequence type. Sequence types were then assigned to a clonal complex based on their degree of similarity to other isolates; clonal complexes contain sequence types that share identical sequences for at least 5 of the 7 loci. MLST data were also analysed using the program *Structure* (Pritchard et al., 2000) and the software Structure Harvester (Earl and vonHoldt, 2012). *Structure* uses a Bayesian approach and uses genetic data to determine the most likely number of "populations" (K), given the data. The program implements a model-based clustering algorithm for inferring population structure using genotype data consisting of unlinked markers (Pritchard et al., 2000). Each population may be a clonal lineage, formed through recombination and/or mutation. Analysis of *P. multocida* isolates found that the highest likelihood was observed for K=3 (Figure 3-3). The three populations were closely associated with the serotype of the isolates. Population 1 included all isolates with serotype 1 isolates (n = 88), population 2 included all isolates with serotype 3 as well as many of the non-serotype 1, 3x4, and 4 isolates (n = 30), and population 3 included all serotype 4 and 3x4 (n = 151).

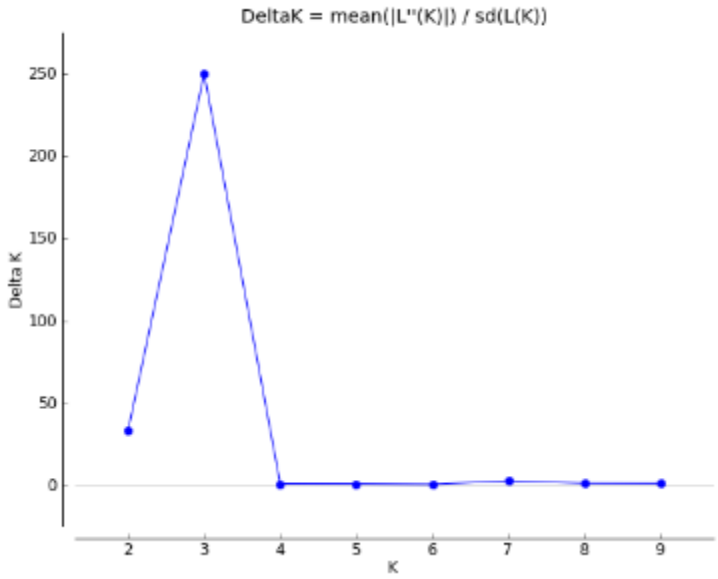

Figure 3-3. Summary of the MLST results for *P. multocida* isolates from wild birds in North America collected between 1994-2011 (n= 269) using the program *Structure*. This plot indicates the presence of three populations (K= 3) that best fit the data. Population (K), mean (L), standard deviation (sd).

A total of 15 sequence types (STs) were recognized within the 269 *P. multocida* strains included in this analysis, which were assigned to seven clonal complexes (CCs) (Table 3-2, Figure 3-4). CCs are assigned when isolates share at least five identical loci. Similar to the REP-PCR results, isolates of the same serotype appeared to cluster within CCs. Further exploration of STs within the CCs revealed additional information about the relatedness among isolates included in the same CC (Figure 3-5). On East Bay Island, more than one ST was isolated in four of the outbreaks between 2005-2011, and in 2008, the outbreak included isolates of five different STs. The largest CC (CC 224) contained 151 isolates and included two STs (Figure 3-5b); ST 224 (n = 150) and ST 227 (n = 1). Serotypes included in this CC included 3x4, 4, 3x4x12, 4x10x12x14, and 36 samples from East Bay Island collected in 2010 and 2011 with unknown serotype. Although these latter 36 isolates were not serotyped, the genotyping data from both REP-PCR and MLST indicates that they are serotype 4 or 3x4. With the exception of one isolate from East Bay Island assigned to ST 227, all isolates from East Bay Island and Nunavik within this CC were genetically indistinguishable using MLST. The majority of serotype 1 isolates fell within CC 61 (n = 68; Figure 3-5e), with fewer isolates in CC 158 (n = 20; Figure 3-5a) and CC 74 (n = 2; Figure 3-5f). Of the serotype 1 isolates, the majority of isolates from Saskatchewan, East Bay Island, and most isolates from Quebec and Newfoundland were in one CC, however MLST revealed that these isolates were not identical. Isolates from Quebec were assigned to a different sequence type (CC 61: ST 60 and CC 158: ST 279) than those from East Bay Island (CC 61: ST 158) (Figure 3-5a and e). Most isolates from Saskatchewan and Newfoundland were indistinguishable from East Bay isolates. A single isolate from a snow goose in Nebraska and five isolates from snow geese collected in California were also assigned to CC 61: ST 158 (Figure 3-5e). These six isolates are of unknown serotype and the date and precise location of

collection are unknown. Interestingly, 4 of 19 Newfoundland isolates were assigned to a different CC and ST (CC 158: ST 278; Figure 3-5a). Serotype 1 isolates collected from birds and water samples from Nebraska and Colorado (n = 11) were included in CC 158, and shared a ST with a single serotype 1 isolate recovered from East Bay Island in 2008. Finally, two isolates from live, apparently healthy comorants collected prior to an avian cholera outbreak in Saskatchewan in 2006 were assigned to an entirely different clonal complex and sequence type (CC 74; ST 74) from other serotype 1 isolates. One of these isolates was assigned to serotype 12 while the other was assigned to serotype 1. The fact that these two isolates are genetically identical strongly suggests that at least one was not assigned to the correct serotype.

Twelve isolates with a range of serotypes including 3, 4, 4x7x12, 4x7x12x15, and 3x4, were founds in CC9: ST 226 (Figure 3-5g). All isolates were recovered from live common eiders from Quebec colonies from 2005, 2008 and 2009. CC 225: ST 225 contained two 3x12 isolates from a Quebec eider colony in 2005. The remaining serotype 3 (n = 8), 3x12 (n = 2), 12 (n = 3) and unknown serotype (n = 1) isolates were not assigned to a CC, and were grouped into seven different STs. Seven isolates from live common eiders from Quebec colonies were grouped into three STs and two isolates from dead birds from East Bay Island were assigned to separate STs. An unserotyped isolate from Nunavik in 2011 was genetically indistinguishable from a serotype 3 isolate from East Bay Island collected in 2008, and two serotype 12 isolates cultured from live common eiders in Quebec in 2008 and 2009 shared an ST (268) with a serotype 3x12 isolate from a snow goose from Banks Island, Northwest Territories in 1995, and a serotype 3x12 isolate cultured from a live snow goose from Texas in 2002. The remaining two serotype 3 isolates (one from a Banks Island snow goose from 1995 and one from an environmental sample

from a wetland in Nebraska in 1996) were each assigned to STs not shared with any other isolates.

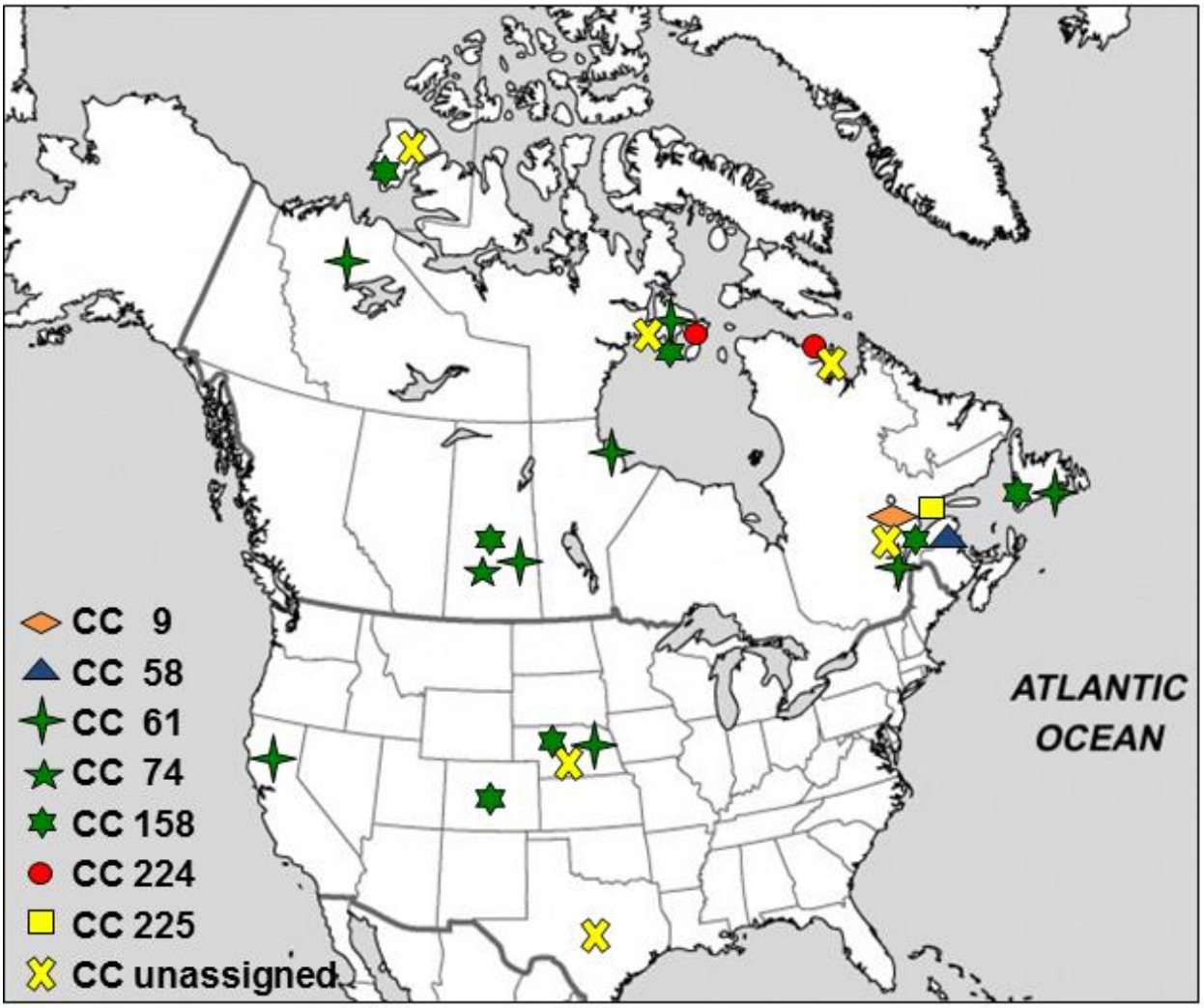

Figure 3-4. Map of North America showing the clonal complexes (CC) of *P. multocida* isolates from wild birds (n = 269) collected from 1994-2011, analysed with multilocus sequence typing. Colour indicates *P. multocida* serotype within each CC: green – 1; red – 4, 3x4, 3x4x12, 4x10x12x14; yellow – 3, 3x12, blue – 14; orange – 3, 4, 3x4, 4x7x12, 4x7x12x15; Map from: blank_map_directory/all_of_north_america.txt. Last modified: 2015/01/16 06:25 by Hobelhouse

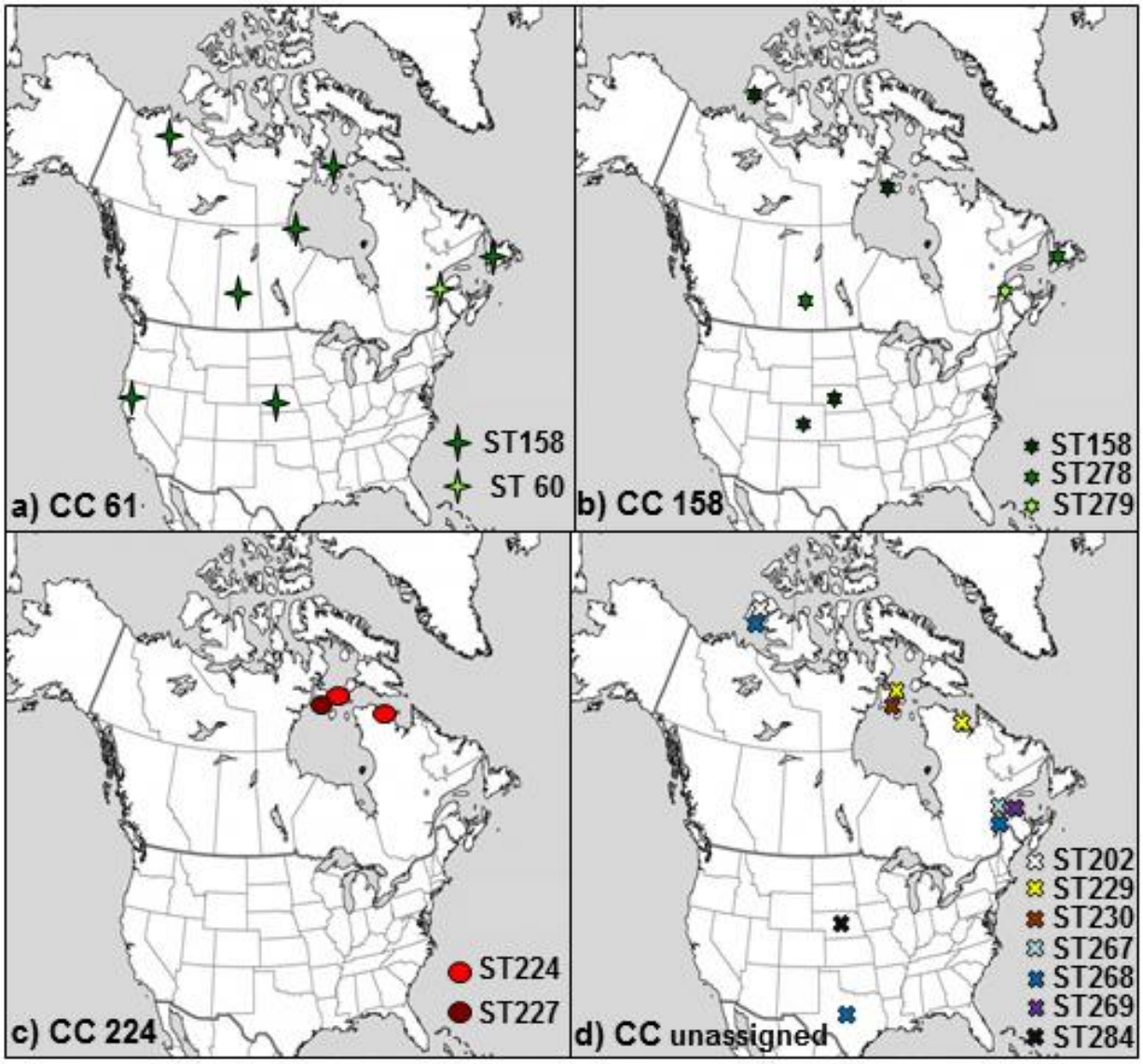

Figure 3-5. Distribution of sequence types (ST) and clonal complexes (CC) of *P. multocida* isolates from wild birds (n = 269) collected from 1994-2011, analysed with multilocus sequence typing. Colour indicates *P. multocida* serotype within each ST: green – 1; red – 4, 3x4, 3x4x12, 4x10x12x14. STs in d) include 3, 12, 3x12, and not assigned.
Map from: blank_map_directory/all_of_north_america.txt. Last modified: 2015/01/16 06:25 by Hobelhouse

Table 3-2. Serotypes, sequence types (ST) and clonal complexes (CC) of *P. multocida* isolated from wild birds in North America collected between 1994-2011 (n = 269), demonstrating the relationship between *P. multocida* serotype and genotype. STs and CCs were assigned by multilocus sequence typing of isolates.

Clonal complex	Sequence type	Number of isolates	Serotypes of isolates included in CC and ST
9	226	11	3, 4, 4x7x12, 4x7x12x15, 3x4
58	58	1	14
61	60	8	1
61	158	60	1
74	74	2	1
158	278	5	1
158	279	2	1
158	158	13	1
224	224	150	3x4, 4, 3x4x12, 4x10x12x14, not assigned
224	227	1	4
225	225	2	3x12
Not assigned	229	2	3, not assigned
Not assigned	230	1	3
Not assigned	267	1	3
Not assigned	268	5	3x12; 12
Not assigned	269	3	3
Not assigned	202	1	3
Not assigned	284	1	3

3.5 Discussion

Avian cholera is considered one of the most important diseases of wild birds, and evidence suggests that wild carrier birds are the most likely source of bacteria for initiating outbreaks in new locations (Samuel et al., 1997; Samuel et al., 2003a). The emergence of avian cholera in the eastern Canadian Arctic within the past decade prompted an investigation of the ecology and molecular epidemiology of *P. multocida* causing avian cholera outbreaks in this region. Using REP-PCR and MLST genotyping techniques, we found a low degree of genetic diversity among *P. multocida* isolates from wild birds across Canada and the United States, and identified three likely clonal populations of the pathogen. Genotyping also allowing us to examine potential origins of the bacteria found in the Eastern Canadian Arctic, and identify genetic similarities between *P. multocida* from the eastern Canadian Arctic and isolates from the east coast of Canada.

REP-PCR analysis grouped 296 isolates into 22 different profiles, however, this appears to be a lower level of diversity than what has been detected in some groups of *P. multocida* isolates previously using the same genotyping method (Shivachandra et al., 2008). Conversely, Gunwardana et al. (2000) found considerable homogeneity in a group of isolates from fowl cholera outbreaks in Vietnam, and speculated that perhaps these outbreaks were caused by an especially stable *P. multocida* genotype. In Denmark, *P. multocida* isolates from avian cholera outbreaks that affected a range of wild birds species over several years were found to be identical using pulsed field gel electrophoresis (PFGE) (Pedersen et al., 2003).

Genetic variation in bacterial populations can arise from recombination and mutation (Spratt, 2004). Some genes, such as those involved in pathogenicity or transmission may be strongly affected by a variety of biological and ecological factors such as host specificity

(Amonsin et al., 2002), host immune function (Spratt, 2004), and adaptation to diverse environments (Earl et al., 2007). High levels of recombination may result in short-term evolutionary change (Feil and Spratt, 2001) that can be detected by some genotyping techniques such as REP-PCR. However, detecting changes in these genes may be less useful for defining population structure within a bacterial population (Spratt, 2004). Housekeeping genes are highly conserved and variation in these genes accumulates slowly. The genetic variation in these conserved genes can be detected within a bacterial population by techniques such as MLST, which provides a high level of discrimination. Mutational events occurring over time in a bacterial population result in the "fittest" clones increasing in frequency with eventual loss of less fit genotypes (Spratt, 2004).

In our study, REP-PCR profiles of serotype 1 isolates were genetically distinct from isolates of other serotypes. Of the 86 serotype 1 isolates studied, 81 formed a single cluster that included isolates from eiders from the St. Lawrence Estuary from outbreaks occurring in 2002, 2004, 2005 and 2006, East Bay Island outbreaks from 2005, 2006, 2008, 2009, all isolates from Saskatchewan and Newfoundland, isolates from two live snow geese from Texas and Northwest Territories, two isolates cultured from dead snow geese from Northwest Territories and northern Manitoba, and nine isolates from Nebraskan wetlands from the late 1990s. Two serotype 1 isolates from Quebec colonies from 2005 and 2006 were unrelated to the largest cluster, and three isolates from Texas, Colorado (live snow geese) and Nebraska (dead northern pintail) formed a second cluster. MLST results were slightly more revealing, as they showed that serotype 1 isolates mainly fell into two main clonal complexes. Clonal complex 61 included isolates from East Bay Island and Quebec, however, the Quebec isolates were of a different sequence type than East Bay isolates. Most isolates from Newfoundland and Saskatchewan and a

few isolates from Nebraska and California were indistinguishable from those from East Bay. It is interesting that there were two sequence types of *P. multocida* isolates found within the 2007 avian cholera outbreak in Newfoundland. This suggests two possibilities; either mutations arose in the original strain during the outbreak, or the outbreak was caused by two separate but closely related isolates. Since housekeeping genes are highly conserved and genetic variation is slow to accumulate (Melles et al., 2007), it is more likely that this outbreak involved two strains. The different strains were apparently not associated with a particular host species, with both strains isolated from multiple gull species. Interestingly, isolates from Newfoundland assigned to sequence type CC158: ST278 were distinct from East Bay Island isolates, while isolates assigned to the other sequence type (CC61: ST 158) were identical to isolates from East Bay.

On East Bay Island, multiple strains of *P. multocida* were found in 4 of 7 annual avian cholera outbreaks. Remarkably, in 2008, isolates belonging to five different sequence types were isolated from eiders that died during the outbreak. This suggests that the isolates involved in the outbreaks were not closely related and very likely represented multiple introductions of *P. multocida* on East Bay Island. This is unusual compared to other avian cholera studies, which have found that a single strain or genetic variant was responsible for an outbreak (Christensen et al., 1998; Pedersen et al., 2003).

Although REP-PCR showed very little genetic variation in the serotype 1 isolates, MLST was able to identify several key differences. Namely, that while closely related, most isolates from Quebec were genetically different than isolates from East Bay, and no isolates from East Bay were included in a clonal complex than included some isolates from Newfoundland, and isolates from Saskatchewan, Quebec, and United States. This may indicate that serotype 1 isolates causing disease in the eastern Canadian Arctic may have originated in southern Canada,

but possibly less likely to have originated with eiders in the St. Lawrence Estuary, or with birds in migratory stop over sites in the United States. One possible source of *P. multocida* for the outbreak in the eastern Arctic is migrating carrier birds such as lesser snow geese moving north from their wintering grounds. Samuel et al., (2005) found that apparently healthy wild birds may act as carriers of *P. multocida* and have the potential to move bacteria to other locations. While our results do not rule out this possibility, the difference in genotypes of the isolates suggest it is less likely in the case of East Bay outbreaks. Identical genotypes were found in snow geese from California and eiders on East Bay, which is unexpected since there is likely little overlap between eiders migrating from eastern Canada to the Arctic and snow geese along the Pacific flyway. Unfortunately, the isolates from California are from an undocumented location and collection date, making it difficult to draw further conclusions. Finally, most of the isolates from the avian cholera outbreak that occurred in 2007 in Newfoundland, Canada, were identical to those from East Bay. Mosbech et al., (2006) found that a proportion of common eiders from East Bay winter along the east coast of Canada, in close proximity to the location of the 2007 outbreak. This is a potential link between avian cholera outbreaks, and suggests that isolates may be moving between the eastern Arctic and the east coast of North America. Although several eiders migrating to southern Canada from East Bay overwintered on Anticosti Island in the outlet of the St. Lawrence River (Mosbech et al., 2006), there is no evidence linking them to other eider colonies on islands within the St. Lawrence where avian cholera has occurred. This may be reflected in the fact that sequence typing shows genetic differences between the isolates from Quebec eiders and those from East Bay.

MLST of serotype 4 and 3x4 isolates resulted in one main cluster which included 138 isolates from East Bay from 2006-2011 and 10 isolates Nunavik from 2004, 2006 and 2011. The

fact that these isolates are indistinguishable may be additional evidence that *P. multocida* strains may move between northern locations, possibly by eiders serving as carriers. As noted above, three isolates from live common eiders in Quebec serotyped as 4 and 3x4 were assigned to a clonal complex and sequence type shared with other isolates from live birds in Quebec, further highlighting the ability of genotyping to provide a more precise understanding of isolate relatedness than serotype alone. Analysis of the REP-PCR results for serotype 4 and 3x4 isolates, including 11 isolates with serotypes that demonstrated a cross reaction with serotype 4 (e.g. 4x7x12), found nine clusters, with two large profiles that included 168 of the isolates. The largest profile contained 132 isolates, with 127 from East Bay from 2006-2011, and 7 isolates from Nunavik from 2004 and 2006. The other large profile contained 32 isolates, with 30 East Bay isolates from 2007-2011, and 2 isolates from Nunavik from 2006 and 2011. The snow goose isolates from California and Nebraska of unknown serotype formed two separate clusters that did not include other isolates. Isolates from live eiders from Quebec colonies were assigned to two profiles that were distinct from all other 4 and 3x4 isolates.

With both MLST and REP-PCR, serotype 3 isolates clustered with a range of other less common serotypes, and showed the most genetic diversity compared to serotypes 1, 4, and 3x4. Furthermore, our results showed that serotype 3 isolates from wild birds have a more widespread distribution than has been previously reported. REP-PCR results showed in several cases that serotype 12 isolates were identical to serotype 3 isolates, and many of the isolates with a serotype cross that included 12 were also similar or identical to serotype 3 isolates. Isolates in this group were from very diverse locations, hosts, and dates, such that isolates from live eiders from Quebec were clustered with samples from Nebraskan wetlands, and live snow geese from Texas and Kansas. Intriguingly, this group of isolates includes nearly all of the isolates cultured

from live, apparently healthy birds. Furthermore, two isolates from live, apparently healthy cormorants collected prior to an avian cholera outbreak in Saskatchewan in 2006 were genetically disctinct from other *P. multocida* collected during the outbreak in 2006. They were also distinct from isolates collected from birds that died during avian cholera outbreaks in other years in Saskatchewan.

Data from both MLST and REP-PCR indicates that isolates cultured from live birds are consistently genetically distinct from those from dead birds found at similar geographical locations. This suggests that isolates from carrier birds may be genetically distinct from those strains associated with mortality in avian cholera outbreaks. Using AFLP to study the relationship of a parental *P. multocida* isolates with isolates shed by inoculated mallards throughout the course of a disease trial, Blehert et al., (2008) detected a decrease in the correlation between the parental isolate and subsequence isolates cultured throughout the trial from live, shedding birds. This finding also supports the notion that carrier birds that survive an outbreak may carry a genetic variant of the original disease causing isolate. This finding requires further investigation, but may be an important feature when studying reservoirs for avian cholera.

Isolates identified as serotype 3 were assigned to seven different sequence types, and only one clonal complex was identified, which included two isolates from live eiders from Quebec in 2008. Nine other isolates also belonged to the same clonal complex and sequence type; these isolates were cultured from live eiders from Quebec in 2005, 2008, and 2009. Interestingly, these isolates had been serotyped as 4 (n = 1), 3x4 (n = 2), 4x7x12 (n = 3) and 4x7x12x15 (n = 3). An isolate of unknown serotype from Nunavik in 2011 had a sequence type identical to an isolate

obtained from East Bay in 2008, providing further evidence that there is likely movement of isolates among northern locations.

Our results showed a relationship between the genotype of a *P. multocida* isolate and its somatic serotype. Other genotyping studies of *P. multocida* have found little or no relationship between somatic serotype and genotype (e.g. Wilson et al., 1995; Amonsin et al., 2002; Saxena et al., 2006; Singh et al., 2013). Somatic antigens are probably related to adaptive traits and are under strong evolutionary pressure, thus can be poor indicators of genetic similarity (Amonsin et al., 2002). Some evidence of the relationship between genetic and phenotypic characterization of *P. multocida* was evident in our study, since different serotypes appeared to cluster together in both MLST and REP-PCR. However, the genotyping techniques were able to further distinguish among isolates of the same serotype, and provide information on the relatedness of isolates associated with avian cholera outbreaks occurring in different years and in different locations in North America. In a similar study, Blehert et al., (2008) examined the genetic diversity of serotype 1 isolates from wild birds and environmental samples using amplified fragment length polymorphism (AFLP), and found sufficient diversity among isolates to distinguish a range of epidemiological patterns.

Previous genotyping studies using a wide range of techniques have indicated that avian strains of *P. multocida* are extremely genetically diverse (Davies et al., 2003). For example, Bisgaard et al. (2013) found 67 different sequence types in a MLST analysis of 116 P. multocida isolates, and 47 different STs were from avian isolates alone. In a study of *P. multocida* genotypes associated with fowl cholera outbreaks, in domestic poultry, 13 different STs were associated with disease on six farms (Singh et al., 2013). Ribotyping of 69 *P. multocida* isolates (64 of which were of avian origin) showed considerable genetic diversity with isolates assigned

to six clusters (Petersen et al., 2001). Analysis of our MLST results indicate that across Canada and likely across North America, *P. multocida* isolates associated with disease in wild birds can be assigned to three main populations, and members of each population are associated with diverse geographical locations and host species and are relatively stable over time. This strongly suggests that despite the genetic variation detected by visual examination of MLST sequence types and REP-PCR banding patterns, *P. multocida* circulating in wild birds in North America is clonal, with the same, or closely related, strains causing disease in numerous species and locations continent-wide. An alternative interpretation of our findings may be that MLST is not able to detect sufficient genetic diversity within *P. multocida*. However, MLST has been used to detect genetic diversity in several previous molecular epidemiological studies of *P. multocida* (Subhaaran et al., 2010, Singh et al., 2013, Bisgaard et al., 2013) and both MLST and REP-PCR results from our study show similar results. Serotype 1 *P. multocida* isolates have generally been associated with avian cholera occurring along the Pacific, Central and Mississippi flyways, while serotypes 3 and 4 seem to predominate in outbreaks occurring along the Atlantic flyway (Samuel et al., 2007). In our study, serotype 1 isolates were responsible for an outbreak occurring on the east coast of Canada, and serotype 1, 3, and 4 isolates caused outbreaks in the eastern Arctic in eiders that migrate along the Atlantic flyway (Mosbech et al., 2006). Our results suggest that there are not flyway specific *P. multocida* lineages or strains, but that there is genetic exchange among *P. multocida* throughout the continent. The most genetic diversity appeared to be in isolates from eastern Canada. The east coast and St. Lawrence Estuary may be an important region for the exchange of *P. multocida* among infected or shedding wild birds, a finding which probably warrants further investigation. In Denmark, a single *P. multocida* clone caused two separate outbreaks and affected multiple wild avian species (Christensen et al., 1998), and was

also responsible for avian cholera outbreaks in Denmark occurring over a seven year period (Pedersen et al., 2003). Christensen et al., (1998) speculated that this could be attributed to spread by wild birds, which is also a likely scenario in North America due to the presence of apparently healthy carrier birds (Samuel et al., 2005), and the substantial geographic distances between outbreaks in some cases. Sellyei et al., (2008) also found that some *P. multocida* clones were recovered from a range of avian species, substantiating the possibility of cross-infection between various host species. DNA HhaI HhaII restriction endonucleases were used to compare genetic fingerprints of *P. multocida* isolated from water and sediment samples from wetland sites of avian cholera outbreaks in the United States (Samuel et al., 2003b). In that study, *P. multocida* isolates with similar fingerprints were widely distributed throughout the western United States (Samuel et al., 2003b).

REP-PCR is relatively inexpensive, rapid, and simple to perform, has a high index of discrimination, is comparable to methods such as pulsed field gel electrophoresis (PFGE), and superior to others such as REA and ribotyping (Gunwardana et al., 2000) when used to genotype *P. multocida*. A disadvantage of REP-PCR is the inability to share isolate profiles between laboratories. MLST compares nucleotide substitutions in the DNA sequences of alleles at seven housekeeping genes to differentiate related isolates (Ranjbar et al., 2014), and sequence results can be easily compared among laboratories or with other data from an Internet database. MLST is a highly discriminatory method because it detects the nucleotide polymorphisms within a gene and can provide evidence for the role of recombination (replacement of a small portion of a chromosome with that from another bacterial isolate) within a population (Feil and Spratt, 2001). Although it was beyond the scope of this study, MLST data from our study could be compared to sequences in an Internet database, to further explore the relatedness of *P. multocida* isolates.

In conclusion, both REP-PCR and MLST were useful for genotyping *P. multocida* and determining potential origins of the pathogen in the eastern Canadian Arctic. The techniques provided complimentary information on the relatedness among isolates. Several genetic variants of *P. multocida* were responsible for avian cholera outbreaks emerging in eider colonies in the eastern Canadian Arctic, with involvement of multiple strains in annual outbreaks in some years. Isolates from the eastern Arctic were genetically similar to isolates from Atlantic Canada and Saskatchewan, but were distinct from isolates from outbreaks occurring along the central flyway in the United States, and from isolates causing outbreaks in eiders in Quebec. Sequence typing data from this group of isolates indicated that, overall, there is low genetic variation in *P. multocida* from wild birds in North America; recombination and overlap of isolates over time has resulted in three distinct populations responsible for avian cholera in North America.

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CHAPTER 4
FEATHER CORTICOSTERONE REVEALS EFFECT OF MOULTING CONDITIONS IN THE AUTUMN ON SUBSEQUENT REPRODUCTIVE OUTPUT AND SURVIVAL IN AN ARCTIC MIGRATORY BIRD

Harms, N.J., Legagneux P., Gilchrist, H.G., Bety J., Love O.P., Forbes M.R., Bortolotti G.R., Soos, C., 2015. Proc. R. Soc. B. 282 (1800), 20142085, by permission of the Royal Society.

Key Words: Carry-over effects, moult, feather corticosterone, reproductive success, avian cholera, Common eider

4.1 Co-authorship statement

Both P. Legagneux and I contributed equally to this chapter. My contributions to chapter 4 included developing the research questions with P. Legagneux and C. Soos, participating in the field work for collection of the common eider data in 2010 and 2011, and supervising or carrying out sample collection specific to my thesis project (e.g. feather collection). I coordinated the organization and analysis for eider samples and data collected between 2007-2009, and wrote the majority of the drafts and incorporated comments from co-authors and reviewers on most of the sections in this chapter except for the description of statistical analyses. P. Legagneux conducted all of the statistical analyses and interpretation of results.

This manuscript has been reformatted from the original published version for inclusion in this thesis.

4.2 ABSTRACT

For birds, unpredictable environments during the energetically stressful times of moulting and breeding are expected to have negative fitness effects. Detecting those effects however, might be difficult if individuals modulate their physiology and/or behaviours in ways to minimize short-term fitness costs. Corticosterone in feathers (CORTf) is thought to provide information on total baseline and stress-induced CORT levels at moulting, and is an integrated measure of hypothalamic–pituitary–adrenal (HPA) activity during the time feathers are grown. We predicted that CORTf levels in northern common eider females would relate to subsequent body condition, reproductive success and survival, in a population of eiders nesting in the eastern Canadian Arctic during a capricious period marked by annual avian cholera outbreaks. We collected CORTf data from feathers grown during previous moult in autumn and data on phenology of subsequent reproduction and survival for 242 eider females, over five years. Using path analyses, we detected a direct relationship between CORTf and arrival date and body condition the following year. CORTf also had negative indirect relationships with both eider reproductive success and survival of eiders during an avian cholera outbreak. This indirect effect was dramatic with a reduction of ~30% in subsequent survival of eiders during an avian cholera outbreak when mean CORTf increased by 1 standard deviation. This study highlights the importance of events or processes occurring during moult on subsequent expression of life history traits and relation to individual fitness, and shows that information from non-destructive sampling of individuals can track carry-over effects across seasons.

4.3 Introduction

To cope with unpredictable environments, individuals can modulate their physiology and behaviour to minimize short-term fitness costs. Although environmental factors can influence individual condition and fitness directly over the short term, they might be more likely to result in indirect consequences (carry-over effects) later in life (Newton et al., 2006; Harrison et al., 2011). Carry-over effects (COEs) are defined as events or processes that occur in one season and that can affect an individual's performance in a subsequent period (Harrison et al., 2011). COEs on the state of individuals can have important repercussions by magnifying or reducing population regulatory processes (Norris, 2005; Norris and Taylor, 2006). For migratory species, obtaining relevant metrics of individual state outside the breeding period is challenging, which makes identifying linkages between conditions experienced at specific locations or habitats and phases of the life cycle also challenging, more so due to the difficulty in tracking migrants across seasons and locations (Harrison et al., 2011).

Studies of migratory bird species provide examples of COEs. In earlier studies, arrival date on the breeding grounds was shown to be related to factors that occurred prior to the breeding season such as use of high quality versus marginal wintering habitats (Norris et al., 2004; Runge and Marra, 2005; Studds and Marra, 2005). The importance of spring body condition on reproduction is another example of a COE reported in several income-capital breeders (Alisauskas, 2002; Bety et al., 2003; Klaassen et al., 2006; Sedinger et al., 2011). Recently, an experimental manipulation of greater snow geese (*Anser caerulescens*) showed that stressful events (captivity) during migration affected subsequent reproduction (Legagneux et al., 2012). Despite these examples, COEs are understudied, particularly in the context of novel environmental challenges such as climate change, food web disruption, or emerging infectious diseases. Such factors have the potential to either magnify or ameliorate COEs making detection

and the subsequent study of COEs and their impacts context-dependent. Also important in the study of COEs is the ease of measurement and reliability of indices by which conditions experienced 'earlier on' are assessed and tracked.

In birds, corticosterone (CORT) is the primary glucocorticoid released by activation of the hypothalamic-pituitary-adrenal (HPA) axis in response to a stressor (Romero et al., 2005). CORT is responsible for mediating allostasis and promoting foraging and gluconeogenesis (McEwan et al., 2003); high levels of CORT can be a consequence of exposure to a threat or a result of increasing requirements for energy, movement (locomotion) and/or metabolism (Romero, 2004; Angelier et al., 2010; Fokidis et al., 2011). The stress response and quantification of CORT in natural populations have become important components of many studies in ecology, physiology and conservation biology (Wasser et al., 1997; Romero, 2002; Wikelski and Cooke, 2006; Blas et al., 2007; Husak and Moore, 2008).

Stress hormone levels have been linked to body condition in several species, although the mechanisms are not clear (Husak and Moore, 2008). In upland geese (*Chloephaga picta leucoptera*), individuals with higher fecal CORT levels had decreased body condition (Gladbach et al., 2011). Experimentally, tree swallow (*Tachycineta bicolor*) nestlings that received CORT implants showed reduced growth rates compared to controls (Fairhurst et al., 2012). Since increased CORT may affect body condition, CORT levels may be linked to arrival date and reproductive success in subsequent seasons.

In birds, CORT is deposited into feathers during growth so that the amount of CORT measured in a feather can provide an index of an individual's HPA activity during the growth of that feather, and provides an integrated measure of CORT (Fairhurst et al., 2013; Bortolotti et al., 2008; Fairhurst et al., 2012). Previous studies have demonstrated the utility of measuring CORT

in feathers and have shown that feather CORT (CORTf) is related to parental efficiency (Fairhurst et al., 2012), social signals (Bortolotti et al., 2009), nest microclimate (Fairhurst et al., 2012), egg mass (Kouwenberg et al., 2013), cost of reproduction (Crossin et al., 2013) and possibly climatic conditions (Legagneux et al., 2013). Thus, CORTf has the potential to be used as a reliable index to study COEs of events occurring during the moulting period on the subsequent breeding period. In Anatidae, including northern common eiders (*Somateria mollissima borealis*), all flight feathers are moulted simultaneously once a year in late summer, after breeding. A feather collected in spring, prior to breeding, could thus provide an indication of the energetic demands experienced by the moulting birds approximately nine months earlier.

In addition to increasing energetic and catabolic costs, elevated CORT can also alter feather quality. Elevated levels of CORT in passerines during moult can affect the rate of feather growth (Romero et al., 2005; Fairhurst et al., 2013) and experimental increases in CORT resulted in a decline in feather quality (Lattin et al., 2011). If eiders with higher CORTf levels have diminished feather quality, this may lower their flight or foraging efficiency during the over-wintering period and result in negative COEs into the breeding season. In common eiders, CORT measured in feathers likely reflects responses to environmental conditions experienced by birds during moult (Legagneux et al., 2013) and potentially be used as a metric to study COEs of responses to climatic conditions on subsequent reproduction and survival many months later. Furthermore, it may also be used as a tool to examine the relationship between glucocorticoid responses during the molting period and infectious diseases.

Glucocorticoid levels can affect susceptibility to disease in many species, usually through effects on immune function (Bourgeon and Raclot, 2006) and energy metabolism. Exposure to chronically elevated CORT levels may decrease immune function (McEwan et al., 1997) and

increase susceptibility to disease, and even acute stress has been shown to affect survival of eiders in the face of an infectious disease outbreak (Buttler et al., 2011). Avian cholera (caused by the bacterium *Pasteurella multocida*) has been a cause of massive annual adult mortality in common eiders nesting at our study site in the low Arctic since 2005 (Buttler et al., 2011; Descamps et al., 2009). Female eiders do not eat during the approximately 26 day incubation period. Egg laying and incubation are energetically demanding activities that may reduce immune function and future fecundity (Hanssen et al., 2005). Large clutch sizes in eiders are associated with lower survival of female eiders in the face of severe avian cholera outbreaks (Descamps et al., 2009). Prior to 2005, avian cholera had not been documented in this population of eiders (Buttler et al., 2011), and avian cholera in northern common eiders in the eastern Canadian arctic has previously only been documented in northern Quebec (Canadian Wildlife Health Cooperative, S. Iverson, N.J. Harms, unpubl. data).

Breeding success of common eiders is strongly influenced by body condition at time of breeding and by timing of migration (Descamps et al., 2011). Here, we expand the previous path analyses of Descamps et al., 2011) by testing whether HPA activity during moult could be carried-over approximately 9 months later to affect the timing of migration and arrival condition, and have direct or indirect links to reproductive success and survival, in the face of avian cholera outbreaks. The unexpected appearance of annual disease outbreaks in our study colony was the impetus behind our investigation into the potential COEs of events occurring during moult on the following breeding season.

4.4 Methods

4.4.1 Study area and field methods

Eiders were captured on Mitivik Island (64°02'N, 81° 47' W) in the East Bay Migratory Bird Sanctuary, Nunavut, Canada, from 2007 to 2011 (Legagneux et al., 2013). Eiders were captured using large mist nets very early in the season when they were flying over the colony; we therefore assumed that capture date was a good proxy of arrival date (Descamps et al., 2010). At capture, body mass was measured using a Pesola scale (± 2.5 g) and one tail feather (second lateral right feather) was plucked from each individual and stored in an envelope in a dark and dry place until laboratory analyses. All eider flight feathers are moulted simultaneously once a year in late summer, after breeding. Commonly, eider tail feathers are grown during moult in August-September, following the breeding season (Mosbech et al., 2006) and prior to fall migration. Eiders from the eastern Canadian Arctic winter along the western coast of Greenland and northeastern coast of Labrador, Canada (Mosbech et al., 2006; Goudie et al., 2000).

Venous blood samples were collected from a subset of female eiders. Blood samples were collected from the tarsal vein within 3 minutes of capture, placed into heparinized vials, and centrifuged to harvest plasma. Plasma samples were stored frozen at -20°C in the field and -80°C in the lab until analysis for CORT. Although the plasma was collected as part of another study, we used the plasma CORT data to examine the relationship between plasma baseline CORT levels and CORTf. Eiders were banded with a metal band (United States Geological Survey) and 2 colored alphanumeric Darvic bands (Pro- Touch, Saskatoon, Canada) (Buttler et al., 2011). All females were also marked with a unique color and shape combination of 2 temporary plastic nasal markers (Juno Inc., Minneapolis, MN) to enable identification of individual birds from a distance. We attached nasal markers with synthetic absorbable suture monofilament (Polydiaxanone suture, 2.0 or 3.0 metric; Ethicon, Markham, Canada), so that nasal tags would be shed prior to fall migration. We restricted the analyses to birds captured

during the prelaying period to compare body condition and avoid any effect of egg laying on body mass. To do so, for each year, we included data only from birds caught before the date at which >2.5% of the population had started laying (Legagneux et al., 2013). Individuals with known laying dates were subsequently added to the dataset if known laying date was later than capture date (with a buffer of 3 days to account for potential error on laying date estimation). Arrival and laying dates were standardized relative to the median (0 – median arrival or laying date in each year). Because body mass alone is a better predictor of condition than mass corrected for body size in this species (Descampe et al., 2011; Descamps et al., 2010), body mass was used as our measure of condition.

In 2011, 69 female eiders received subcutaneous corticosterone or sham implants (Innovative Research of America, Sarasota, Florida) as part of a separate study, and 44 of the implanted birds were included in this study. Such manipulation could have induced changes in eider reproductive outcomes, which could alter our conclusions. We performed all analyses with and without inclusion of eiders captured in 2011 to assess the robustness of our conclusions. Precise information on reproduction (lay date of the first egg, and hatching success- at least one duckling hatched) for all eiders in this study was collected by monitoring nesting birds from eight observation blinds strategically located within the colony. Observation blinds allowed us to monitor over 90% of the eider nesting area (Buttler et al., 2011) while minimizing disturbance to the colony. Final number of ducklings hatched was available for a very limited number of females included in this study. Therefore we did not examine the link between CORTf and number of ducklings.

The island was scanned with spotting scopes twice a day throughout the nesting season to detect nesting females. Females observed ≥2 times at the same nest within 36 hours were

considered to be breeding, and nesting status and fate were monitored twice daily until hatch or nest failure (Buttler et al., 2011). We were able to evaluate the lay date and nesting success of up to 350 (marked and unmarked) females each year (Descamps et al., 2009; McKinnon et al., 2006).

Carcasses of nasal-tagged female eiders that died on their nests were located and collected during daily observation periods, or were recovered at the end of the breeding season when transects spaced one meter apart were walked across the entire island by 5 observers to enumerate dead birds. Following the avian cholera outbreak on East Bay Island in 2005 (Descamps et al., 2009), we assumed that eiders found dead on their nests or at the end of the breeding season died due to avian cholera. A subset of eider carcasses recovered each year were submitted to the Canadian Wildlife Health Cooperative (CWHC) for confirmation of the diagnosis of avian cholera using gross and histopathologic findings and bacteriology (Legagneux et al., 2014). Marked birds that were no longer observed on the colony and not found dead were assumed to have survived an avian cholera outbreak. This assumption is feasible because eiders that abandon their nests leave the colony within 24 hours (McKinnon et al., 2006) and thus are no longer exposed to the disease during the avian cholera outbreak. Furthermore, given that the island is very small (24 ha), we are confident that our methods for monitoring nests and surveying transects are effective at detecting the majority of carcasses on the island.

4.4.2 CORT analysis

CORTf measurements were performed using a previously established protocol (Bortolotti et al., 2008) that includes a methanol-based extraction followed by analysis of the extracts via radioimmunoassay. This method has been previously used for eider feathers (Legagneux et al., 2013) and other avian species (Fairhurst et al., 2012; Fairhurst et al., 2012; Harms et al., 2010;

Fairhurst et al., 2011). In this study, we assessed the efficiency of methanol extraction by including eider feather samples spiked with a small amount (approximately 5000 CPM) of ^{3}H-corticosterone in the extraction. Greater than 92% of the radioactivity was recoverable in the reconstituted samples. Bortolotti et al., (2008) and Bortolotti et al., (2009) showed that CORT is deposited into feathers in a time-dependent fashion; therefore, our values are expressed as a function of feather length (pg/mm). All samples were measured in duplicate and were run randomized and blind. Assay variability was determined as the % coefficient of variation (CV) resulting from repeated measurement of samples spiked with a known amount of CORT in each assay. The average within-assay variation was 5.4% (range 2-10%), and inter-assay variation was 13.7%. Serial dilution of feather extracts from eider feathers produced displacement curves that were parallel to the standard curves. Hormone analyses were performed at the Department of Biology, University of Saskatchewan (Canada).

Baseline plasma CORT was analysed using a previously-validated enzyme-linked immunoassay (EIA; Assay Designs, Ann Arbor, MI, USA; Love and Williams, 2008) run in triplicate at a 1:20 dilution with 1.5% of kit-provided steroid displacement buffer. Each plate was run with a kit-provided standard curve by serially diluting a 200,000 pg/mL CORT standard and a control of laying hen plasma (Sigma-Aldrich Canada, Oakville, Ontario, Canada). Assay plates were read on a plate reader at a wavelength of 405 nM, and the mean inter- and intra-assay coefficient of variation across all plates was 7.17% and 6.22%, respectively.

4.4.3 Path analyses

The importance of HPA activity levels during pre-migratory moulting period on reproduction and survival was assessed using path analysis, a special form of structural equation model (SEM; Shipley, 2000), following Shipley, (2009). The principle of the method is to

specify how the variables are linked together in terms of direct and indirect effects or relationships. Information on CORTf, condition at arrival, arrival date, reproduction (laying date, reproductive success), and survival was available for 242 eider females from 2007-2011 (Table 3-1). Among the 242 females, only 2 females were sampled in two years representing <1 % of the data. The two additional measurements of the same individuals were considered as being independent. We developed our path diagram (Figure 4-1) from a similar analysis that tested a condition-dependent optimization model on the same eider population (Descamps et al., 2011). We expanded the relationships (both direct and indirect) to include CORTf, reproductive success and survival. Arrows in Figure 4-1 indicate relationships between two variables following event chronology (moulting, pre-breeding, breeding). Arrival date and body condition were assessed at the same time so determining causation was not possible. Mortality of female eiders due to avian cholera was detected following nest initiation (Buttler et al., 2011). Therefore, we investigated whether lay date or nest success could affect the survival of eiders during an avian cholera outbreak.

The fit of a generalized multi-level path model was assessed using the concept of d-sep (directional separation) tests (Shipley, 2000). A d-sep test represents a test of the statistical independence between two variables. If two variables are d-separated relative to a set of variables Z in a directed graph, then they are independent conditional on Z in all probability distributions such a graph can represent (see Shipley, 2009 for more details). Shipley, (2000) shows that for each acyclic path model, there is a subset of independence tests referred to as a ‘‘minimum basis set’’ that account for all possible independence relationships (or claims). Model fit is evaluated using a set of (k) mutually independent claims of probabilistic independence that must be true if the structure of the hypothesized path model is correct. The null probabilities (p_i)

from these k tests are used to derive Fisher's C statistic: $C = -2\sum\ln(p_i)$, which follows a chi-squared distribution with 2k degrees of freedom (Shipley, 2000). The null hypothesis is that the proposed correlational structure of the model does not differ from the observed correlational structure in the data, and therefore $P \leq 0.05$ indicates the proposed causal structure is incorrect (Shipley, 2000). To calculate path coefficients, each variable was standardized [(value – average)/SD] such that path coefficients represent standardized partial regression coefficients, or the standard deviation change in y when x is increased or decreased by 1 SD (Shipley, 2000).

Shipley, (2009) showed how the d-sep test can be combined with generalized linear mixed models (GLMM). We followed detailed instructions provided in Shipley, (2009) using the packages nlme and lme4 in R (R Development Core Team, 2013). We used linear mixed models (using terms CORTf, arrival date, body condition, laying date, hatching success, and survival) to regress each variable on its direct causes. A random Year effect was included in each model. The random effect accounted from 0.46 to 5.71 % of the deviance explained depending on the dependent variable considered.

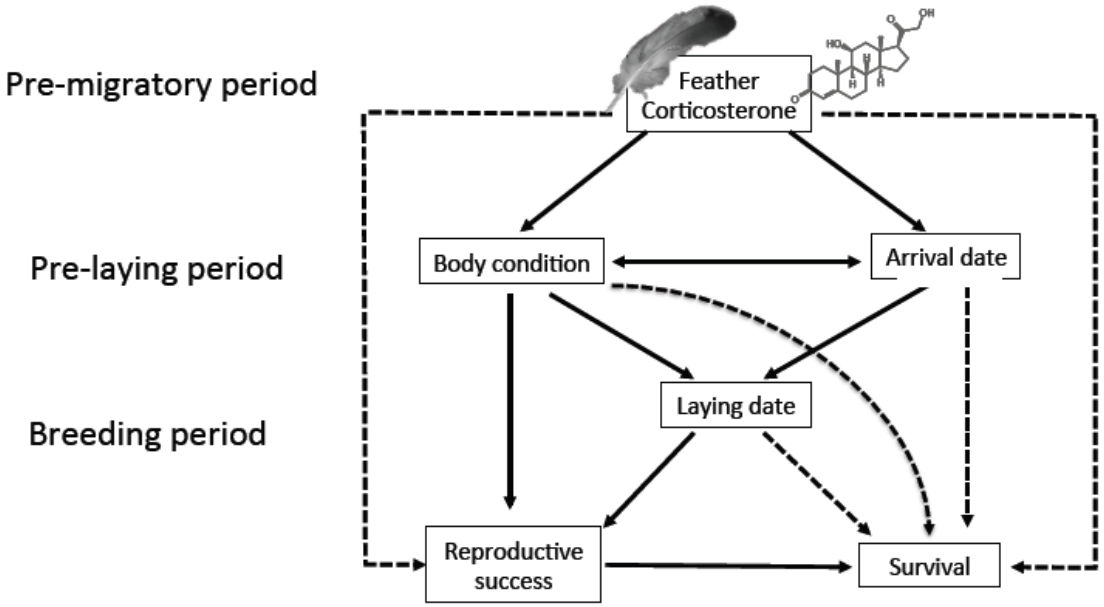

Figure 4-1. The hypothesized causal structure linking energetic management, as represented by CORTf, during molt, arrival state, timing of reproduction and fitness components (reproductive success and survival of an avian cholera outbreak) in the face of an avian cholera outbreak in an eider colony on East Bay Island, Nunavut, Canada. Solid lines indicate the significant paths included in the final best fit model while dotted lines indicate additional or alternative paths that were tested but not found significant. The direction of the arrows indicates predicted effect.

Table 4-1. Description of model variables by year for female northern common eiders captured on East Bay Island, Nunavut, Canada.

Year	N common eiders	CORTf (pg/mm) (mean±SD)	Body condition (grams) (mean±SD)	Julian arrival date (mean±SD)	Julian lay date (mean±SD)	Reproductive success (%)	% eiders survived outbreak
2007	106	5.44±2.00	2191±165	172.7±3.2	183.7±5.2	42.5	83
2008	38	4.33±1.23	2218±132	171.2±3.3	179.9±5.5	44.7	73.7
2009	44	4.05±1.23	2225±167	176.4±4.2	184.8±5.7	20.5	95.5
2010	10	3.58±0.97	2213±164	166.4±4.9	176.6±5.2	50.0	100.0
2011	44	5.82±1.61	2255±206	170.0±2.5	179.8±4.5	50.0	100.0

4.4.4 Ethical treatment of animals

This study adhered to guidelines of the Canadian Council on Animal Care, and all protocols were reviewed and approved by the University Committee on Animal Care and Supply – Animal Research Ethics Board of the University of Saskatchewan (Protocol Number 20100063 to CS), the University Committee on Animal Care of the University of Windsor (Protocol number 11-06 to OL), Environment Canada's Animal Care Committee (Protocol Numbers: EC-PN-07-008 (2007), EC-PN-08-026 to EC-PN-11-026 (2008 to 2011) to HGG).

4.5 Results

The correlational structure of our path model (Fig. 3-2) was consistent with the correlational structure of the data (7 tests of probabilistic independence; Fisher's C_{14} =8.82, P = 0.84; implied independencies did not differ from those observed). The model defined in Figure 4-1 provided a strong fit to the data as indicated by the high P-values (null probabilities) of the goodness-of-fit tests (Table 4-2). Partial regression slope of CORTf was not different from zero in all claims revealing no direct effect of CORTf on lay date, reproductive success or survival. CORTf was significantly different among years ($F_{4,237}$ = 11.43; P<0.001). Arrival date on the breeding colony was positively associated with CORTf (Figure 4-3a; $\beta = 0.26 \pm 0.13$ (SE); t_{236} = 2.07; $P = 0.04$). Lower body condition (body mass) at arrival was related to higher levels of CORTf (Figure 4-3b: $\beta = -22.56 \pm 6.09$; $t_{236} = -3.71$; $P < 0.001$). There was no direct relationship between CORTf and lay date ($t_{236} = 1.50$; $P = 0.14$), reproductive success (z_{236}=-1.38; P=0.17), or survival eiders during the avian cholera outbreak (z_{236}=-0.16; P=0.87). We found that body condition increased over time during the pre-breeding period ($\beta = 6.78 \pm 3.14$; $t_{236} = 2.16$; $P = 0.03$), so that birds arriving later were in better body condition. As expected, arrival date was positively linked to lay date and birds that arrived earlier laid earlier, and eiders that arrived in

better body condition also laid earlier ($\beta = 0.73 \pm 0.09$; $t_{235} = 8.52$; $P < 0.001$ and $\beta = -4.73 \pm 1.75$; $t_{235} = -2.71$; $P = 0.007$ respectively). Later lay date had a direct negative effect on reproductive success, so that birds laying earlier in the season were more likely to hatch at least one egg ($\beta = -0.24 \pm 0.04$; $z_{236} = -6.42$; $P < 0.001$).

Body condition at arrival was positively associated with reproductive success ($\beta = 2.68 \pm 0.90$; $z_{236} = 2.96$; $P = 0.003$) but was not associated with survival. However there was a direct negative relationship between reproductive success and survival ($\beta = 1.29 \pm 0.50$; $z_{242} = 2.59$; $P = 0.01$) where birds that reproduced successfully were more likely to survive. The overall indirect relationships between CORTf and reproductive success and survival were relatively high; the sum of all products of path coefficients for each variable were -0.22 and -0.27 respectively. This indicates that if CORTf increased by 1 SD from its mean, reproductive success was decreased by 0.22 SD and survival of eiders during avian cholera outbreak decreased by 0.27 SD from its own mean. Female eiders captured in 2011 underwent an additional manipulation (subcutaneous CORT implant; see Methods) that was not done in any of the other years. Similar results were found when excluding eiders captured in 2011 from the analyses. The results presented in the manuscript thus included 2011 to maximize sample size. Within same individuals, there was no significant relationship between baseline CORT levels (measured in plasma during the pre-breeding period (O. P. Love, H. Hennin et al. unpub. data) and feather CORT ($F_{1,195} = 0.02$; $P = 0.89$).

Table 4-2. Test of conditional independence implied by the path diagram (Fig. 3-1). (X; Y) | {Z} means that variables X and Y are independent conditional of variable Z (i.e. if Z is held constant, variation in X does not imply variation in Y). The associated mixed model used to test the independence claims are Y ~ Z + X + 1|Year, where Year represents a random effect. The variable whose partial regression includes zero is X1 for all claims. Variables: X1 (CORTf) , X2 (Arrival date), X3 (Body condition), X4 (Laying date), X5 (hatching success), X6 (survival of an avian cholera outbreak).

d-sep claim of independence	Mixed model	Partial regression slope (SE)	Null probability (distribution)
(X1,X4) | {X2,X3}	X4 ~ X1 + X2 + X3 + (1 | Year)	0.072 (0.176)	0.68 (normal)
(X1,X5) | {X4}	X5 ~ X4 + X1 + (1 | Year)	-0.046 (0.090)	0.61 (binomial)
(X2,X5) | {X1,X4}	X5 ~ X4 + X1 +X2 + (1 | Year)	0.014 (0.096)	0.89 (binomial)
(X1,X6) | {X4}	X6 ~ X4 + X1 + (1 | Year)	0.014 (0.039)	0.71 (binomial)
(X2,X6) | {X1,X4}	X6 ~ X4 + X1 +X2 + (1 | Year)	-0.074 (0.069)	0.28 (binomial)
(X3,X6) | {X1,X4}	X6 ~ X4 + X1 + X3 + (1 | Year)	0.010 (0.039)	0.79 (binomial)
(X4,X6) | {X5}	X6 ~ X4 + X5 + (1 | Year)	0.057 (0.046)	0.21 (binomial)

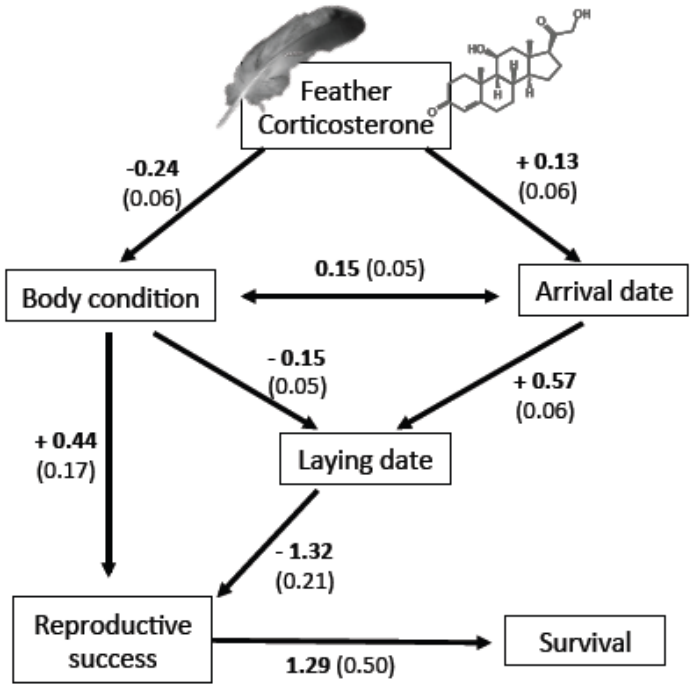

Figure 4-2. Standardized path coefficients in hypothesized structural model. Bold numbers are standardized beta coefficients with S.E in brackets (n = 242).

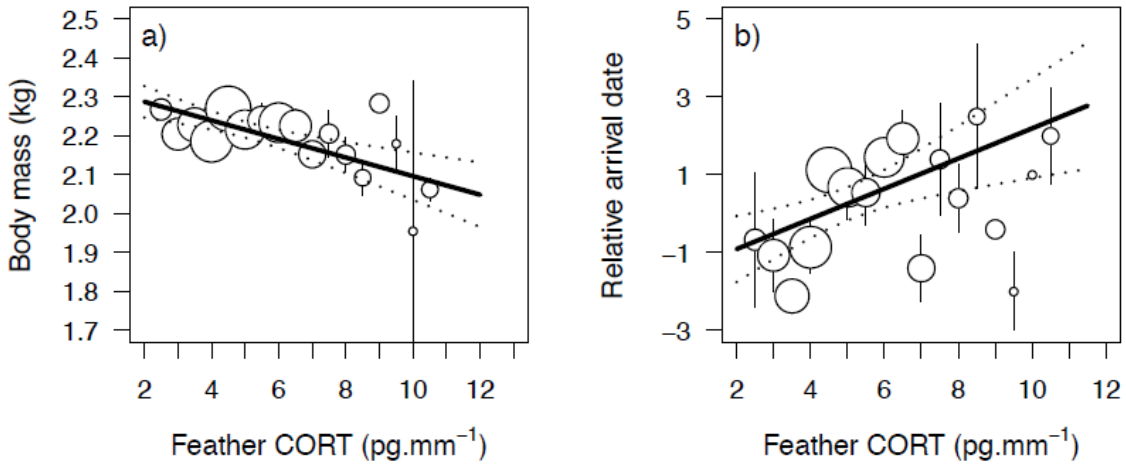

Figure 4-3. Relationship between feather corticosterone (CORTf), arrival date (3a), and body condition (3b) in female eiders. The model presented here controls for the other covariates (either mean arrival date or condition). Mean and standard errors are provided. Dot sizes are proportional to log (n). The fitted mixed linear model (black line) and its confidence interval at 95% (grey line) are shown.

4.6 Discussion

Our results provide evidence that energetic management during the moulting period, reflected by CORT levels in feathers, can be carried-over to the subsequent breeding season and affect reproductive success and survival. Using path analyses we detected a direct relationship between CORTf levels during moult and body condition and arrival date the following year, and an indirect negative relationship between reproductive success and survival. The magnitude of the indirect relationship between CORTf and fitness parameters was important, with a decrease of approximately 0.25 SD from the mean of reproductive success and survival for every increase of 1 SD of CORTf. The importance of arrival date and condition on reproductive success was expected given that the path analysis developed here is an extension of Descamps et al., (2011) who found similar relationships in accordance with the condition-dependent optimization model (Bety et al., 2003; Rowe et al., 1994). As per causal pathways drawn from the optimization model (Descamps et al., 2011; Rowe et al., 1994) birds can adjust their reproductive decisions as a function of their arrival date and body condition at arrival. Our study is unique in showing that both these variables can be significantly related to CORTf levels, which may reflect a level of energetic management experienced during the previous year. Increases in energetic challenges or response to stressors experienced during the time of moult in autumn can ultimately have significant fitness consequences, indirectly affecting both reproduction and survival in the following breeding season.

Another possible explanation is that CORTf values reflect basal CORT levels of individuals regardless of the time period considered. This hypothesis would gain credence if baseline CORT during the breeding or pre-breeding period could predict relative fitness of individuals (Bonier et al., 2009; Cockrem, 2013; Schmid et al., 2013; Narayan et al., 2013).

However, we found no evidence for any relationship between CORTf and pre-breeding basal CORT within individuals, providing little support for this explanation. Furthermore, we also found that CORTf was not repeatable within individuals from year to year, and was influenced more by environmental factors encountered during moult (e.g. temperature) rather than intrinsic measures of quality (e.g. body size) (Legagneux et al., 2013).

While COEs are increasingly reported to influence fitness components (Harrison et al., 2011), measuring such effects at the individual level are rare in the literature, as is their measurement relative to novel environmental challenges. Furthermore, very few studies have reported events occurring during the previous fall with latent effects on subsequent summer reproduction and/or survival. Interestingly, in an Icelandic eider colony, Jónsson et al., (2009) reported negative effects of warm and wet autumns on subsequent clutch size. The authors argued that the effect was probably due to delay in migratory movements and in pair formation. In addition, we recently reported that warmer autumn temperatures were linked to slightly higher CORTf levels, suggesting a physiological cost to increasing temperatures in an arctic environment (Legagneux et al., 2013).

Finally, we documented that survival on the breeding colony was also indirectly related to increasing energetic demands experienced during moult the previous autumn (i.e, CORTf). Carry-over effects on survival or mortality rates from events occurring in autumn have been rarely reported in the literature (Studds and Marra, 2005; Dawson et al., 2000; Mitchell et al., 2011). Recently, Koren et al., (2012) found that high CORTf levels in house sparrows (*Passer domesticus*) were predictive of lower survival over the subsequent winter. Crossin et al., (2013) measured feather CORT in giant petrels (*Macronectes* spp.), which begin moult during the breeding season, and found that variation in CORTf was linked to both current reproductive

success and future reproductive effort. In contrast to the results found in sparrows (Koren et al., 2012), CORTf levels in giant petrels were not related to overwinter survival (Crossin et al., 2013). Female petrels with high CORTf values were, however, successful breeders in the current year but more likely to defer breeding in the next year, suggesting that CORT up-regulation does exact a cost on future effort (Crossin et al., 2013). Even if the mechanism remains unclear, energetic costs associated with maintaining elevated CORT levels have often been reported to explain reduced survival rates (Wikelski et al., 2006; Blas et al., 2007; MacDougall-Shakleton et al., 2009; Hau et al., 2010; Goutte et al., 2010). Since pre-breeding condition and CORTf were related in female eiders, such increased energy expenditure could be involved. However, survival was only indirectly linked to CORTf, through its positive association with breeding success. This agrees with previous studies from our research group (Descamps et al., 2009; Descamps et al., 2012) suggesting that breeding decision, reproductive investment, and the duration of exposure to disease at the nesting colony are key factors explaining survival of eiders facing avian cholera outbreaks.

4.7 Conclusion

Our work emphasizes the importance of determining how events are linked throughout the annual cycle to better understand population dynamics of migratory animals. Our approach also highlights the importance of energetic management challenges outside the breeding period (possibly generated by climatic variability) that can have subsequent carry-over effects on reproduction and survival during outbreaks of avian cholera, an emerging disease in arctic-nesting common eiders. Little is known about the moulting period for many bird species, including eiders (Savard et al., 2011), thus our results shed some light on a relatively unknown stage of the annual cycle. Combining information that can be gained from non-destructively

sampling a single feather, including stable isotopes (Marra et al., 1998; Sorensen et al., 2009; Rushing et al., 2014), coloration (Norris et al., 2004), or physiological analysis such as hormone levels (Koren et al., 2012) can contribute to tracking COEs across seasons. Furthermore, considering both direct and indirect pathways may be required to understand relationships among spatiotemporally distinct events affecting individual fitness.

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CHAPTER 5
GENERAL DISCUSSION AND CONCLUSIONS

5.1 Introduction

The overall aim of this thesis was to investigate the disease dynamics of avian cholera in the eastern Canadian Arctic with a particular focus on East Bay Island, Nunavut, where annual avian cholera outbreaks have affected a population of nesting common eiders (*Somateria mollissima borealis*) since 2005. My work forms part of a larger, ongoing project exploring the ecology of common eiders in the eastern Canadian Arctic and effects of disease on populations of Arctic nesting waterfowl. In this thesis, three aspects of avian cholera ecology in the eastern Canadian Arctic were studied: the detection and ecological correlates of avian and/or environmental reservoirs of *P. multocida*; the genetic variation of *P. multocida* across Canada, including the potential origins of the bacteria causing avian cholera outbreaks in the eastern Arctic; and carry-over effects of past energy expenditure on survival and reproductive success of common eiders experiencing annual avian cholera outbreaks. The importance of avian cholera as a disease of waterfowl populations in North America, particularly in light of the changing ecological conditions and increasing anthropogenic influences in the Arctic, necessitates a better understanding of the disease and its drivers in the north. Addressing disease emergence in wildlife populations is becoming increasingly important, since failure to do so may result in decreased capacity to protect and sustain distribution and population abundance of wildlife, especially threatened or endangered species (Friend et al., 2001). Furthermore, the increasing human population, which leads to intensifying agricultural activities, problems with waste disposal, (over)use of natural resources, and land degradation and fragmentation, among other impacts, is having marked effects on animal populations and on the emergence and transmission

of infectious diseases (Woodford, 2009). For example, Rubin et al., (2013) detected several strains of *Brachyspira* sp., which are well known bacterial pathogens of pigs, in samples from healthy lesser snow geese collected in the eastern Canadian Arctic, approximately 1,500 km north of the nearest pig producing areas. This points to overlap between agriculture and wildlife populations, and suggests that migratory birds are important in the transport and distribution of pathogens along their migratory routes. This conectivity has consequences for the emergence of disease in wildlife and domestic animals.

5.2 Emergence of avian cholera

Avian cholera is one of the most important infectious diseases in North American waterfowl (Blanchong et al., 2006a; Samuel et al., 2007), and continues to occur globally. In the north, the disease has recently occured in the eastern Canadian Arctic causing annual outbreaks over eight years on East Bay Island and sporadic outbreaks in other locations. Even more recently, in 2013, avian cholera was the cause of a multi-species die-off of seabirds in a remote area of the western Arctic (Bodenstein et al., 2015). Avian cholera has also occurred in several species of seabirds in Antarctica (T. Boulinier, pers. comm.; Leotta et al., 2006), an even more remote and unpopulated region than the Arctic. Recognition of these outbreaks occurs because of humans living or working in remote areas, therefore wildlife diseases likely also occur undetected in animal populations in remote areas. As our understanding of disease dynamics, reservoirs, transmission, and genetic variation of pathogens increases, we will be better able to predict and potentially manage disease outbreaks in wildlife.

The appearance and spread of diseases in Arctic-nesting migratory bird populations are potential causes for concern. Wildlife at northern latitudes are likely at higher risk for changing patterns of diseases with a warming climate in part due to higher rates of pathogen prevalence,

changes in disease transmission dynamics, and parasite life cycles (Kutz et al., 2005), as well as range expansion of new hosts and their associated pathogens (Burek et al., 2008; Kutz et al., 2009). Most emerging infectious diseases in humans are zoonoses that often have their origins in wildlife (Jones et al., 2008), and the human health implications of any emerging disease must be evaluated. Avian cholera is not a direct threat to human health, but other avian pathogens such as West Nile virus and avian influenza virus are zoonotic, and many avian pathogens like *P. multocida* and the previously mentioned viruses can also be transmitted to domestic animals. Avian cholera, like other infectious diseases can have significant effects on wildlife health and conservation. Annual large-scale mortality events affecting the eider population on East Bay Island have contributed to significant colony population declines (Descamps et al., 2009; Iverson 2015). Common eiders are long-lived species with low rates of annual reproduction, and population recovery may not be able to keep up with repeated mortality events affecting adult birds (Descamps et al., 2012). The extent of this annual adult mortality may necessitate management of the disease and/or of eider populations. A key mechanism to manage adult mortality in eiders would be to change harvest regulations to reduce mortality due to hunting.. Other management options to reduce the impact of avian cholera outbreaks could include vaccination of succeptible individuals (Price, 1985), burning carcasses of birds that have died of avian cholera (Waller and Underhill, 2010), or destruction of nests to disperse birds from their breeding grounds (Iverson, 2015).

Other avian populations, particularly species at risk, may be affected by changing patterns of multi-host pathogens, which may contribute to extinctions. Many wildlife species in the north including common eiders are important culturally and are depended on as a food resource for First Nations residents. Infectious diseases are one of many factors that may affect

wildlife populations in the Arctic, and they have significant implications for local residents who rely on wildlife for subsistence.

The impact of disease on wildlife populations has potential to result in significant conservation concerns (Thompson et al., 2010; Frick et al., 2010; Daszak et al., 2001). Infectious diseases result from a pathogen (or pathogens) infecting a host and negatively affecting the host's ability to function (Burek et al., 2008). Interaction of factors specific to the host, the pathogen, and the environment that increase the host's exposure or susceptibility to a pathogen, or increase the virulence or transmission rate of a pathogen, will influences the incidence, severity, and extent of a disease in a wildlife population (Burek et al., 2008; Wobeser, 2006). Mechanisms that drive pathogen transmission and maintenance in wildlife populations must be identified and characterized, if management actions are to be taken (Cowled et al., 2012).

5.3 Reservoirs of avian cholera in the eastern Arctic

Effective management of diseases in wildlife relies on understanding determinants of disease in wildlife populations. One of the key elements of infectious diseases is the pathogen reservoir - an environment or populations that can permanently maintain the pathogen and from which the pathogen can be transmitted (Haydon et al., 2002). We aimed to further our understanding of common eiders and other sympatric avian species as carriers of *P. multocida*, and the potential for freshwater ponds on eiders colonies to also act as a reservoir for the bacterium and act as a possible source of the organism for initiating or maintaining avian cholera outbreaks in the eastern Canadian Arctic. We also explored several ecological and demographic factors associated with common eiders on East Bay Island being infected with or "carrying" *P. multocida,* and the ecological factors associated with the variation in prevalence of *P. multocida* in pond samples. To the best of our knowledge, this is the only study that has consistently

sampled birds just prior to and during avian cholera outbreaks each year, to detect *P. multocida* DNA in live, apparently healthy eiders in all 5 years of the study, and to examine patterns and determinants of infection during outbreaks.

P. multocida DNA was found in swab samples from live, apparently healthy, pre-breeding common eiders arriving on the breeding colony in all years of this study. Variation in *P. multocida* DNA carriage in eiders was partially explained by average temperatures during the three week periods in spring during which most of the eiders were captured and sampled. Eiders were more likely to be PCR positive in cooler years. These findings highlight a potential association between avian cholera dynamics and weather.

P. multocida DNA was also detected in swab samples from live and harvested apparently healthy lesser snow geese, Ross's geese, and common and king eiders from several locations across the eastern Canadian Arctic. Lesser snow geese and Ross's geese have been shown previously to act as carriers of virulent *P. multocida* (Samuel et al., 1997; 2005), and there is considerable evidence to show that they play an important role in carrying and transmitting *P. multocida* to susceptible birds (Blanchong et al., 2006a). Our results also point to a small proportion of snow geese and Ross's geese potentially carrying *P. multocida* to their northern breeding sites.

Herring gulls and other gull species play an important role as scavengers in the Arctic ecosystem. Gulls have been well documented to scavenge on carcasses of birds that have died of avian cholera (Buttler, 2009) and are also susceptible to avian cholera (Samuel et al., 2007). We found a high prevalence of PCR positive herring gulls on East Bay Island, sampled after the avian cholera outbreak had begun each year. Although little is known about the role of gull species as carriers of *P. multocida*, this and other studies (Botzler, 1991, Leotta et al., 2006;

Whitney et al., unpubl. data) have proposed that gulls may be important carriers of the bacteria and may transmit it to susceptible birds or shed it into the environment. Further studies on the role of gulls in avian cholera outbreaks are needed.

Wetlands and freshwater ponds have been shown to be an important source of *P. multocida* during avian cholera outbreaks, as they become contaminated with bacteria from infected and dead birds (Botzler, 1991; Samuel et al., 2004; Blanchong et al., 2006b). Research has also shown that wetlands are unlikely to maintain viable *P. multocida* year-round, and thus do not serve as a reservoir for initiating disease outbreaks (Samuel et al., 2004, Lehr et al., 2005, Blanchong et al., 2006b). To determine if this was also the case in an Arctic ecosystem, we collected water and sediment samples from ponds on East Bay Island before and after avian cholera outbreaks began, as well as from ponds near eider colonies at other sites in the eastern Arctic. *P. multocida* DNA was found in ponds on East Bay both before and after avian cholera outbreaks began, but interestingly, ponds were more likely to be PCR positive before the outbreak began. Despite this, there was a declining trend in positive samples prior to the onset of avian cholera outbreaks, followed by an increasing percentage of positive samples in most years after the outbreak began. These findings show that ponds on East Bay Island do become contaminated with *P. multocida*, and provide some support for the environment playing a role in maintaining outbreaks once they have begun. *P. multocida* was isolated from one water sample collected after the outbreak began in 2010, which also supports previous observations that ponds play a role as a source of infection once an outbreak is underway. However, it is not clear whether freshwater ponds play a role in initiating outbreaks, because we were not able to culture viable bacteria from samples collected before outbreaks began.

One limitation of this study was our inability to culture *P. multocida* from PCR positive swab samples collected from birds, and from the majority of environmental samples. Without isolating bacteria from these samples, we were unable to gather additional information about the isolates, such as serotype, genotype, and virulence, and this hampered our ability to fully understand the role that carrier birds and the environment might play in the ecology of avian cholera in the Arctic. Other studies have successfully cultured *P. multocida* from oral and cloacal swabs and other tissues of wild, apparently healthy birds (Samuel et al., 1997; Samuel et al., 2005) and have determined the serotype and virulence of these isolates. However, viable *P. multocida* recovered from live apparently healthy wild birds were often not further characterised in past studies (e.g., Korschgen et al. 1978; Titche et al., 1979; Botzler, 1991).

Unsuccessful attempts to recover *P. multocida* from apparently healthy wild birds have also been reported (e.g., Samuel et al., 1999; Leotta et al., 2006). In our case, there may be several reasons for our inability to culture the bacteria from PCR positive samples, including: very low numbers of bacteria in swab samples from healthy carriers; *P. multocida* may be harboured in tissues other that the oral cavity or cloaca; and samples were frozen for various amounts of time before culturing. Swab samples collected from birds that died of avian cholera on East Bay Island were treated identically to the samples from apparently healthy birds. Interestingly, I was able to culture *P. multocida* from those samples, suggesting that the transit time and method, freezing time, and transport and culture media used were not prohibitive to recovering isolates. However, samples from birds that died of avian cholera contained very high numbers of *P. multocida*, likely increasing the probability of recovering isolates from dead infected birds.

PCR positive avian swab samples were cultured by plating on blood agar in this study. In an attempt to improve our ability to culture *P. multocida*, we conducted a pilot study using inoculation of mice with sample material. Although mouse inoculation has been found to be an excellent method for isolating *P. multocida* from field samples (Muhairwa et al., 2000; 2001), it did not improve our ability to culture isolates and thus was not used for the remainder of the study (see Appendix B). We also conducted a pilot study using selective enrichment media (Moore et al., 1994) to improve our success at culturing environmental samples (Appendix C). Although selective media has been found to improve recovery of *P. multocida* from field samples (Moore et al., 1994), it did not improve our ability to culture isolates compared to using blood agar, and thus was not used for the remainder of the study (see Appendix C). For this study, we used a sensitive and specific PCR assay adapted from Corney et al., (2007) to screen swab samples as an efficient means of testing large numbers of samples. Corney et al., (2007) found that the highest dilution yielding a positive result was 10.7 cfu of *P. multocida* per reaction, but our results showed that the assay could detect as low as 0.2 cfu/reaction (corresponding to a sample containing1 cfu/ul) (data not shown). Samuel et al., (2005) used a PCR assay described by Rocke et al., (2002) to test swab samples from apparently healthy lesser snow geese and Ross's geese but found that it only minimally enhanced their ability to detect *P. multocida* in swab samples.

5.4 *P. multocida* isolates from the eastern Arctic and the east coast share genetic similarities

In addition to the importance of environmental factors in understanding disease, factors associated with the disease-causing agent can influence the ecology of a disease. We used two genotyping techniques to elucidate the source of *P. multocida* causing avian cholera in the eastern Arctic, and to evaluate genetic variation of *P. multocida* from wild birds in Canada.

Both REP-PCR and MLST detected genetic diversity within the *P. multocida* isolates included in this study. REP-PCR found a lower level of diversity in our samples than was detected in some groups of *P. multocida* isolates previously using the same genotyping method (Shivachandra et al. 2008). Gunwardana et al., (2000) also found considerable homogeneity in a group of isolates from fowl cholera outbreaks, suggesting that these outbreaks were caused by an especially stable *P. multocida* genotype. In Denmark, molecular epidemiology of avian cholera outbreaks in different locations and over several years using restriction enzyme analysis and ribotyping found that they were caused by the same strain of *P. multocida*, strongly suggesting spread by migratory birds (Christensen et al. 1998; Eigaard et al., 2006). Further studies also indicated transmission of *P. multocida* strains between wild and domestic birds (Pedersen et al., 2003).

MLST showed that, while isolates from East Bay Island and the St. Lawrence estuary, Quebec, were both in the same clonal complex, the Quebec isolates were of a different sequence type than East Bay isolates. Interestingly, most isolates from an outbreak that occurred in Newfoundland were identical to some of the East Bay Island isolates. Two distinct sequence types of *P. multocida* were found within the 2007 avian cholera outbreak in Newfoundland, which affected mainly gull species. Isolates belonging to one of the sequence types were distinct from East Bay Island isolates, while isolates assigned to the other sequence type were identical to isolates from East Bay. Previous studies have shown that some eiders from East Bay Island winter along the east coast of Canada (Mosbech et al., 2006), in close proximity to the location of the 2007 outbreak in Newfoundland. This suggests a potential transmission link between some of these avian cholera outbreaks; isolates may be moving between the eastern Arctic and the east coast of North America.

Although eiders from East Bay have been also found to overwinter in the outlet of the St. Lawrence River (Mosbech et al., 2006), there is no evidence linking East Bay eiders to other eider colonies on islands within the St. Lawrence estuary where avian cholera has occurred, and sequence typing shows genetic differences between the isolates from Quebec eiders and those from East Bay.

Sequence typing revealed that *P. multocida* isolates from East Bay Island and from outbreaks occurring in other parts of the eastern Arctic (Nunavik) were indistinguishable, which suggests that *P. multocida* strains may move between northern locations, possibly by eiders serving as carriers. Interestingly though, multiple strains of *P. multocida* were found in four of seven annual avian cholera outbreaks on East Bay Island between 2007 and 2011. In 2008, isolates belonging to five different sequence types were isolated from eiders that died during the outbreak. This means the strains involved in the East Bay Island outbreaks were not closely related and very likely represented multiple introductions of *P. multocida* on East Bay Island. Furthermore, nearly all of the isolates cultured from live, apparently healthy birds that were included in this study formed genetically distinct groups according to both REP-PCR and MLST results. These isolates were consistently genetically distinct from those from dead birds found at similar geographical locations, suggesting that isolates from live carrier birds may be different from those strains associated with mortality in avian cholera outbreaks. Using amplified fragment length polymorphism, Blehert et al., (2008) found that the genetic similarity decreased between the genotype of a parental *P. multocida* isolate and subsequence isolates cultured throughout an experimental infection trial using mallards. This finding also supports the notion that carrier birds that survive an outbreak may carry a genetic variant of the original disease causing isolate.

5.5 MLST reveals three genetically distinct *P. multocida* lineages in wild birds

Although previous genotyping studies using a wide range of techniques have indicated that avian strains of *P. multocida* are extremely genetically diverse (Davies et al., 2003), MLST results from this study indicated that, across Canada and likely across North America, *P. multocida* isolates associated with disease in wild birds can be assigned to just three main populations. Isolates from each population were associated with diverse geographical locations and host species, and were relatively stable over time. In our study, *P. multocida* circulating in wild birds in North America was clonal, with the same, or closely related, strains causing disease in numerous species and locations continent-wide. Our results did not support the concept that there are flyway specific lineages of *P. multocida* in North America (reviewed in Samuel et al., 2007), but rather are suggestive that *P. multocida* in wild birds has clonal populations that are found throughout the continent.

5.6 Moulting conditions can cause carry-over effects on reproductive success and survival

The importance of ecological factors to disease occurence and pathogen persistence in wildlife cannot be overstated. Friend et al., (2001) points out that one of the barriers inhibiting adequate management responses to diseases in wild bird populations is a tendency to focus on factors associated with the affected species or the disease-causing agent, and not the environment.

We used feather corticosterone (deposited in feathers during the moulting period) to explore possible effects of energy management and environmental factors occurring during moulting on subsequent reproduction and survival of avian cholera in eiders from East Bay Island (Chapter 3, Harms et al., 2015). Hormones are important mediators between environmental conditions and phenotype, and thus can provide useful information in the study of

life history trade-offs (Wingfield et al., 2008). In breeding female common eiders, corticosterone has been shown to decreased humoral immunity, mediated through its effect on body energy reserves (Bourgeon and Raclot, 2006).

In female eiders nesting on East Bay Island, we found that hypothalamic-pituitary-adrenal activity during the moulting period (as measured by feather corticosterone level) was directly related to both body condition on arrival and arrival date on the breeding colony, with higher feather corticosterone levels detected in birds with lower body condition, and in birds arriving later on the breeding colony. Furthermore, there was an indirect negative relationship between feather corticosterone levels and both reproduction and survival during an avian cholera outbreak in the subsequent breeding season. While other studies have described carry-over effects on body condition and reproductive success (see Table 1 in Harrison et al., 2011), our study is novel in that it demonstrates that energy challenges and possibly response to stressors in one time period can have important effects on an individual eider's ability to survive and reproduce in the face of an infectious disease encountered at a later period.

A few other studies have also described carry over effects on survival using feather hormone levels. For example, Koren et al., (2012) found that higher levels of feather corticosterone in wild house sparrows (*Passer domesticus*) were predictive of decreased survival over the following winter. This study did not specify causes of decreased survival, nor were the sparrows faced with an annual disease outbreak. The indirect link between feather corticosterone and survival in female common eiders is most likely mediated by breeding success. We found an indirect link between feather corticosterone and subsequent reproductive success and we know that eiders with larger clutches are more likely to succumb to avian cholera (Descamps et al., 2009). These findings suggest that survival during an avian cholera outbreak is in part influenced

by reproductive decisions and duration of exposure to the disease at the breeding colony. In a related study, we found a relationship between feather corticosterone levels in common eiders and air temperatures in August and September, months which are just prior to and during the moulting period (Appendix A; Legagneux et al., 2013). Although the mechanisms have yet to be elucidated, these results suggest that climate affects energy management, possibly through effects on food availability and/or quality, or through impacts on thermoregulation (Legagneux et al., 2013). Combining these results, our study highlights a potential link between climate and disease mortality in eiders, and opens the door for future research investigating the relationship between climate variables, climate change and infectious disease impacts on wild birds in the north. This study shows that non-invasive techniques such as measuring corticosterone in feathers can be used to better understand how life-history stages are connected, and the influence of environmental conditions on disease response.

5.7 Future work

This research has improved our understanding of avian cholera dynamics in northern Canada, has shown the usefulness of feather corticosterone concentrations to understand carry-over effects and the potential for climate to influence reproduction and survival, and has demonstrated how *P. multocida* may be circulating in migratory bird populations. This work has also raised numerous new questions about the ecology of avian cholera in the Arctic and in Arctic nesting migratory waterfowl.

Although we were able to demonstrate shedding of *P. multocida* DNA from a range of migratory avian species from across the eastern Arctic (Chapter 2), more work is required to improve recovery of *P. multocida* from carrier birds, and to track migratory and dispersal routes of birds leaving sites of disease outbreaks, in order to further delineate the importance of *P.*

multocida reservoirs in the Arctic. Recovering *P. multocida* isolates from apparently healthy carrier birds is essential for further virulence testing and genotyping of isolates, which provides important information about the pathogen as well as about the ability of various bird species to act as carriers of *P. multocida*. Using common eiders in experimental studies similar to the studies conducted by Samuel et al., (2003) would improve our knowledge of the ability of eiders to act as carrier birds and sources of transmission of *P. multocida*, the rate of seroconversion and duration and protectiveness of circulating antibodies, and aspects of immune function of infected and recovered birds.

Knowledge of seroconversion and duration of immunity in eiders and other species such as herring gulls would be useful to identify avian populations that have been exposed to *P. multocida*, even if a current disease outbreak is not detected (Samuel et al., 2005). Serological data could also be used to model the potential for outbreaks in certain populations. The work described in this thesis showed that three main lineages of *P. multocida* genotypes are responsible for avian cholera in wild birds in North America (Chapter 3). Further genotyping work using full genome sequencing would provide additional information on the rates of genetic shift and genetic drift responsible for the low genetic diversity of *P. multocida* in wild birds. Full genome sequencing would also provide information on virulence factors, shedding light on the wide variation of pathogenicity within and among *P. multocida* genotypes (Johnson et al., 2013). Comparison of genetic variation among isolates from wild birds and domestic birds in North America also may provide further insights into the importance of pathogen transmission between wild and domestic avian species (Pedersen et al., 2003).

Finally, we have shown that environmental conditions in previous seasons can have carry-over effects on both reproduction and survival of eiders in the face of an infectious disease

outbreak (Chapter 4). This work poses new questions about the events and processes experienced by wildlife populations across seasons, and how these may affect aspects of their fitness. Using novel methods such as feather and hair corticosterone (Legagneux et al., 2013; Macbeth et al., 2010), or stable isotopes (Fairhurst et al., 2013), as well as field-based studies resulting in ecological data that can be used to create predictive models (Iverson 2015; Kutz et al., 2013), the role of environment on disease occurrence in wildlife can be further explored.

This thesis describes several important features of the host, agent and environmental dynamics of avian cholera in North America. Profound and intensifying global environmental changes are affecting patterns of disease in humans and animals (Leighton, 2011). Further exploration of infectious wildlife disease dynamics is needed to better predict, manage, and mitigate disease emergence that can threaten human and animal health and species conservation.

5.8 References

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APPENDIX A

DOES FEATHER CORTICOSTERONE REFLECT INDIVIDUAL QUALITY OF EXTERNAL STRESS IN ARCTIC NESTING MIGRATORY BIRDS?

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Co-authorship statement

My contributions to this manuscript included developing the research questions with P. Legagneux and C. Soos, participating in the field work for collection of the common eider data in 2010 and 2011, and supervising or carrying out sample collection specific to my thesis project (e.g. feather collection). I coordinated the organization and analysis for eider samples and data collected between 2007-2009. I contributed to the writing of manuscript drafts and incorporating comments from co-authors and reviewers on previous drafts.

This manuscript has been reformatted from the original published version for inclusion in this thesis.

Keywords: Cost of reproduction, common eider, greater snow geese, repeatability, glucocorticoids, recaptures, temperature, molt

ABSTRACT

The effects of environmental perturbations or stressors on individual states can be carried over to subsequent life stages and ultimately affect survival and reproduction. The concentration of corticosterone (CORT) in feathers is an integrated measure of hypothalamic–pituitary–adrenal activity during the molting period, providing information on the total baseline and stress-induced CORT secreted during the period of feather growth. Common eiders and greater snow geese replace all flight feathers once a year during the pre-basic molt, which occurs following breeding. Thus, CORT contained in feathers of pre-breeding individuals sampled in spring reflects the total CORT secreted during the previous molting event, which may provide insight into the magnitude or extent of stress experienced during this time period. We used data from multiple recaptures to disentangle the contribution of individual quality *vs.* external factors (i.e., breeding investment or environmental conditions) on feather CORT in arctic-nesting waterfowl. Our results revealed no repeatability of feather CORT within individuals of either species. In common eiders, feather CORT was not affected by prior reproductive investment, nor by pre-breeding (spring) body condition prior to the molting period. Individual feather CORT greatly varied according to the year, and August-September temperatures explained most of the annual variation in feather CORT. Understanding mechanisms that affect energetic costs and stress responses during molting will require further studies either using long-term data or experiments. Although our study period encompassed only five years, it nonetheless provides evidence that CORT measured in feathers likely reflects responses to environmental conditions experienced by birds during molt, and could be used as a metric to study carry-over effects.

Introduction

Hormones play an essential role in the study of life-history trade-offs because they link

environmental condition and phenotypic expression (Wingfield et al., 2008). Glucocorticoids are of particular interest when examining how individuals respond to environmental variation, because they are the main mediators of allostasis allowing an organism to cope with both predictable and unpredictable conditions (Saplsky et al., 2000; McEwan et al., 2003; Landys et al., 2006). Corticosterone (CORT), the primary glucocorticoid in birds, has been investigated in numerous studies attempting to identify physiological mechanisms linking individual state to environmental parameters and fitness consequences. Measurement of baseline CORT is difficult in wild populations because CORT levels measured in plasma and feces rise shortly after capture (Romero and Romero, 2002). CORT measured in feathers are not affected by capture when a fully grown feather is collected, and incorporate the amplitude and duration of all CORT secreted during the period of feather growth, and thus represent an integrated measure of both baseline and stress-induced CORT secretion during the molt period (Bortolotti et al., 2008). This technique is relatively recent, and an understanding of the information conveyed by feather CORT in wild populations is only beginning to be unravelled (Fairhurst et al., 2012a; Fairhurst et al., 2012b; Fairhurst et al., 2013).

Immediately following reproduction, migratory birds are in a critical and energetically demanding period when they must accumulate lean tissue and fat for migration and molt (Murphy et al., 1996; Piersma et al., 2011; Vezina et al., 2012). Understanding the mechanisms of carry-over effects of breeding effort on body condition and molt is logistically challenging in migratory species because molt often occurs on distinct locations away from breeding sites, which makes the follow up of individuals almost impossible (Norris et al., 2004; Newton, 2006). Some studies investigated, either empirically or experimentally, how reproductive activities could delay timing of molt (Siikamaki et al, 1994; Hemborg and Merila, 1999; Conklin and

Battley, 2012; Dietz et al, 2013) and potentially induce lean tissue mass reduction (Dawson, 2004), lower insulation (Nilsson and Svensson, 1996), or a decrease in feather quality (Vagasi et al., 2010). Late breeders can adjust their timing by molting their feathers more rapidly than early ones (Dietz et al., 2013). However, there are associated costs based on growing evidence showing that molt speed, and therefore, feather growth rate, influences the quality of ornaments, aerodynamics, and insulation (Dawson et al., 2000; Serra et al., 2007; Griggio et al., 2009; Vagasi et al., 2010; Vagasi et al., 2011; Vagasi et al., 2012). Although less investigated, environmental conditions can also be important drivers of molting date (Dawson, 2005) and determine feather quality (Middleton, 1986).

In some bird species, like Anatidae, all flight feathers molt simultaneously once a year in late summer, after breeding. During this period, birds are flightless and thus potentially more vulnerable to predation, which can represent an additional source of stress compared to birds that molt their feathers sequentially. A feather collected in spring, prior to breeding, could thus provide an indication of the stress responses or energetic demands experienced by the molting birds approximately nine months earlier (Figure 1).

Thus, feather corticosterone in these species can potentially be a very useful tool for studying the role of carry over effects of conditions experienced during the previous molt, on reproduction and survival in the subsequent breeding period.

Using multiple recaptures of the same individuals, our aim was to disentangle the contribution of individual quality *vs.* external factors (Figure 1) on feather CORT in two wild bird species, the common eider *Somateria mollissima* and the greater snow goose *Chen caerulescens*. Although very valuable, intra-individual variation and repeatability in physiological traits are rarely investigated (Williams, 2008) and not yet reported for feather

CORT. If individual quality, here viewed as an intrinsic (i.e. genetic) trait (Bergeron et al., 2011), is a strong determinant of feather CORT, we would expect a high temporal repeatability of relative feather CORT (Angelier et al., 2010; Ouyang et al., 2011).

Alternatively, if CORT in feathers was highly variable among years within the same individual, this would suggest that external factors such as breeding investment or environmental conditions have a stronger influence on CORT levels than intrinsic factors (e.g., individual quality). Secondly, in the common eider, we examined whether the timing of reproduction and breeding investment (breeders vs. non breeders), and pre-breeding mass affect CORT sequestered in feathers during molt immediately following reproduction. Finally, we investigated correlations between environmental conditions (weather conditions and trophic interactions) and feather CORT in common eiders.

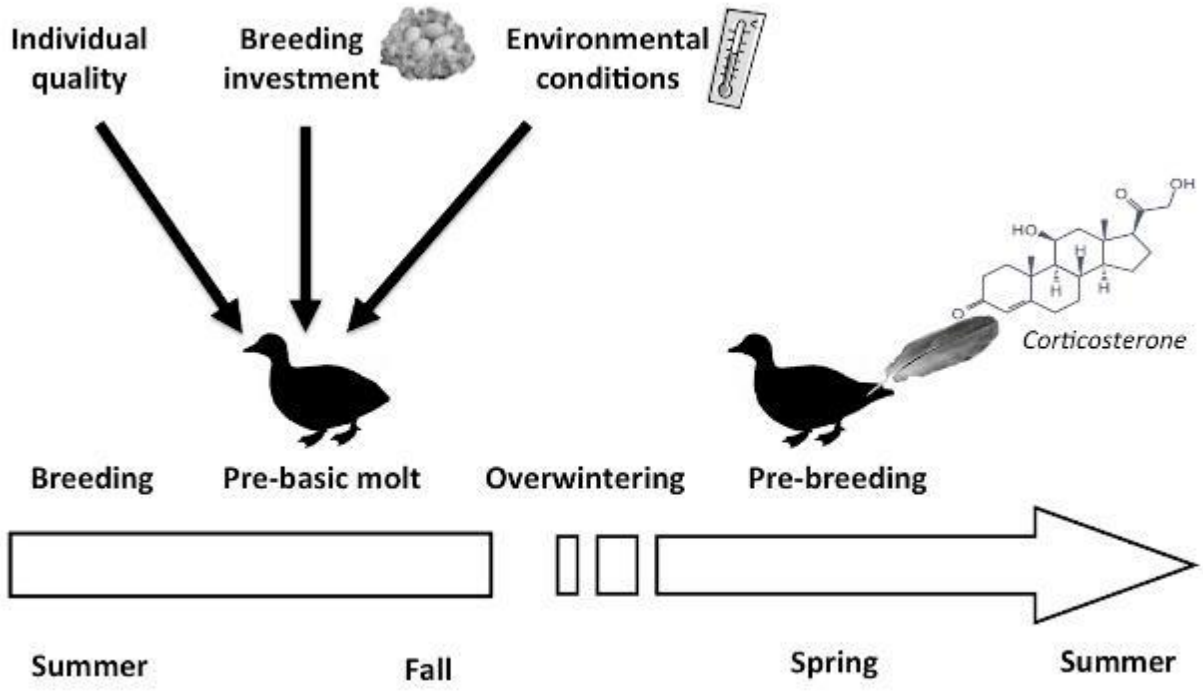

Figure 1. Time scale of partial capital breeders breeding in the High Arctic. From multiple recaptures, we tested whether individual quality, reproductive investment or environmental conditions (black arrows) explained variation in feather CORT from birds captured in spring during the pre-breeding period. The effects of reproductive investment or environmental conditions were investigated only in Common eiders.

Materials and Methods

Ethics Statement

This study adhered to guidelines of the Canadian Council on Animal Care, and all protocols were reviewed and approved by the University Committee on Animal Care and Supply - Animal Research Ethics Board of the University of Saskatchewan (Protocol Number 20100063 to CS), Environment Canada's Animal Care Committee (Protocol Numbers: EC-PN-07-008 (2007), EC-PN-08-026 to EC-PN-11-026 (2008 to 2011) to HGG) and by the Université du Québec à Rimouski (CPA-42-10-78 to JB).

Study species and field methods

Geese and eiders are partial capital breeders that molt all their flight feathers simultaneously after the breeding season. During incubation, female eiders and geese lose up to 40% of their body mass due to fasting (Gabrielsen et al., 1991). After hatching, female eiders must recover and regain body condition before initiating molt, another energetically costly event (Senechal et al., 2011). For this study we used data from long-term monitoring studies in which birds were fitted with permanent markers with unique combination to allow resightings in subsequent years (Descamps et al., 2010; Legagneux et al., 2012). Captures occurred prior to breeding in spring staging areas for geese and on the breeding colony for eiders. Geese were captured from 2006 to 2009 in the Saint Lawrence estuary near Québec city [47°00'N 70°33'W, see details in 36]. Eiders were captured on Mitivik Island (64°02'N, 81°47'W) in the East Bay Migratory Bird Sanctuary, Southampton Island, Nunavut, Canada from 2007-2011 (Descamps et al., 2011).

From late April to mid-May 2006-2009, we captured geese using baited canon-nets (see Morez et al., (2000) fro details. Adult females (> 1 year old) were weighed and measured shortly

after capture. Females were individually marked with neck collars (Gauthier et al., 2001). From mid-June to early July, eiders were captured using large flight nets as eiders flew over the island. Eiders were weighed and measured shortly after capture. Adult female eiders were banded with a metal band and two coloured alphanumeric Darvic bands (Pro- Touch, Saskatoon, Canada) (Buttler et al., 2011). All female common eiders were also marked with a unique color and shape combination of two temporary plastic nasal markers (Juno Inc., Minneapolis, MN) so that nasal tagged individuals could be identified on nests. We attached nasal markers with synthetic absorbable suture monofilament (Polydiaxanone suture, 2.0 or 3.0 metric; Ethicon, Markham, Canada). At capture, one flight feather (a central undercovert in greater snow geese and the second right rectrix in common eider) was plucked from each individual. Feathers were stored in an envelope in a dark and dry place until measurements in the laboratory.

We first selected feathers from all recaptured individuals (n = 16 geese and n = 65 eiders) to assess individual repeatability. For common eiders, the effect of previous breeding investment was assessed for 29 recaptured individuals for whom we had previous breeding information. We determined if these females attempted to breed or not in the previous year, based on whether they were resighted on nest and, for those that bred, we determined laying date (n = 16) and nesting success (a nest was considered successful when at least one egg hatched, n = 15). Precise information on reproduction (laying date of the first egg and subsequent nest success) was available through the daily monitoring (via a maximum of seven blinds within the colony) and nest visitation (15 to 78 nests each year) of marked females. Laying date of females was determined by monitoring > 358 nests annually at the colony.

We investigated possible links between relative pre-laying biomass for a given year and CORT levels sequestered in new feathers grown during the pre-basic molt of the same year

immediately following breeding (though feathers were plucked in the following year). We restricted these analyses to birds captured and weighed during the pre-laying period in order to avoid any change in body mass due to egg laying. To do so and for each year, we only included data from birds caught before the date at which >2.5% of the population had started laying. Individuals with known laying dates were subsequently added to the dataset if known laying date was later than capture date (with a buffer of 3 days to account for potential error on laying date estimation). This procedure was applied during all the years of the study, which yielded a sample of 16 eiders with known laying dates and 17 with known body mass.

Finally, we investigated the effect of environmental covariates on feather CORT variation with a larger sample size (n = 652) that included all captured individuals from 2007 to 2011 for which we had feather CORT data. For this last analysis, we included body size as an individual covariate because structural body size is a static variable that remains similar over time once eiders reach sexual maturity. Body size was assessed using a principal component analysis on morphometric measurements (culmen, bill, tarsus and head lengths). The four variables had loadings ranging from 0.34 to 0.57 on the first axis (PC1), which explained 56.2% of total variation in the body size data. We used individual PC1 scores as a measure of body size.

Environmental covariates

We addressed the influence of environmental conditions on CORT during molt only on eiders because not enough data was available in geese. Molting in females occurs outside the colony in our studied eider population, presumably in East Bay (Southampton Island) but precise information on molting period and molting sites are still lacking (but see Mosbech et al., 2006). Based on our knowledge of the study system, we considered a two months molting period (August-September; Goudie et al., 2000).

Climate

We used different climate indices (temperature, precipitation, and North Atlantic Oscillation). At the regional scale, large-scale indices are often better predictors of ecological processes than local climatic variables, likely because features of several weather components are combined (Stenseth and Mysterud, 2005). The North Atlantic Oscillation (NAO) is a major source of atmospheric mass balance measured as the mean deviation in average sea level pressure between the subarctic and subtropical Atlantic (Hurrell, 1995). By influencing the speed and direction of westerly surface winds across the North Atlantic, the NAO influences weather conditions over eastern Canada-USA and northern Europe (Hurrell, 1995; Stenseth et al., 2002). We obtained daily values of the NAO indices from the Climate Prediction Center of the National Weather Service (http://www.cpc.ncep.noaa.gov). We also used average values of temperature and precipitation from the meteorological station of Coral Harbour located 42 km from our study site and available at http://www.climat.meteo.gc.ca/climateData

Finally, we used two indices of ocean primary production of East Bay from remote-sensing data. Satellite-derived measurements of surface chlorophyll (Chla) and sea surface temperature (SST) are indication of ocean primary production (Field et al., 1998). Chla and SST were extracted using a resolution of 9 x 9 km from MODIS satellite images (SST 4 micron night and Chla). We selected one pixel centered on the bay and extracted mean values values of SST and Chla from August and September of each year. Using a greater area (108 x 27 km) covering more potential moulting sites did not affect the results (not shown). Data were available at http://gdata1.sci.gsfc.nasa.gov/

Trophic interactions

Ducklings are especially vulnerable to predation and females tending broods may increase their parental investment to defend their offspring when predation risk is high. Therefore, we expected that the level of duckling protecting behaviours displayed by females in response to predatory attacks could affect their ability to regain condition, and hence affect their feather CORT level during molt. The main predator of common eider eggs and ducklings on East Bay Island is the herring gull (*Larus argentatus*). As an index of gull abundance, we calculated the cumulative numbers of gulls counted daily around the island, including both breeders and transient birds. We considered the period 30 June (median hatching date) to 15 August (end of the colony monitoring), which was common to the five years of the present study. We assumed that gull abundance measured during that period was a good proxy for predation risk experienced by females during brood rearing.

CORT assays

Feather CORT assays were performed at the University of Saskatchewan, Canada, and at the Centre d'Études Biologiques de Chizé, France, for eiders and geese respectively. We followed previously established protocols (Bortolotti et al., 2008; Bortolotti et al., 2009). In eiders, inter and intra-assay coefficients of variation (%CV) were 13.7 and 5.4 % respectively (n = 18). In geese, such %CVs were 10.1 and 5.1% respectively (n = 16). The lowest detectable concentration was 10.3 or 11.4 pg CORT/assay tube (for University of Saskatchewan and CEBC respectively) while the lowest measurement was tenfold for both eiders and geese. The length of the feather was measured, the calamus was removed and discarded, and then the sample was cut into pieces less than 5 mm^2 with scissors. We then added 10 ml of methanol (HPLC grade, Fisher Scientific, Fairlawn, NJ, USA) and placed the samples in a sonicating water bath at room temperature for 30 min, followed by incubation at 50ºC overnight in a shaking water bath. The

methanol was then separated from feather material by vacuum filtration, using a plug of synthetic polyester fibre in the filtration funnel. The methanol extract was placed in a 50ºC water bath and subsequently evaporated in a fume hood. Extract residues were reconstituted in a small volume of phosphate-buffered saline (0.05 M, pH 7.6) and frozen at -20ºC until analysed by radioimmunoassay (RIA). We assessed the efficiency of the methanol extraction by including feather samples spiked with a small amount (approximately 5000 CPM) of ^{3}H-corticosterone in the extraction. RIA assays were performed on reconstituted methanol extracts, and samples were measured in duplicate. Serial dilutions of feather extracts produced displacement curves parallel to the standard curves. Data values are expressed as pg CORT per mm of feather, which gives a valid estimate of CORT per unit time of feather growth (Bortolotti et al., 2008; Bortolotti et al., 2009).

Statistical analyses

Intra-individual repeatability of feather CORT among years was calculated using the within and between-variance components in a linear mixed effects model, using the restricted maximum-likelihood method (REML) with bird identity as the grouping random factor (Lessells and Boag, 1987; Nakagawa and Schielzeth, 2010). Data were analysed using R studio v2.1 (R Development Core Team) and the package rptR to calculate repeatability (R) and associated standard errors based on bootstraps (1000 iterations) that generated the distributions of likelihood ratios.

To examine variations in feather CORT, we used generalized linear mixed models with climatic and predation indices as explanatory covariates. Year was considered as a random factor to account for multiple samples per year. Our dataset contains yearly covariates (temperature, SST etc.) that represent a case of potential pseudo-replication. To avoid any pseudo-replication

and over-parameterization (only 5 years studied), no more than one yearly parameter was entered in a competing model. Models were then ranked through a model selection procedure.
The model with the lowest AICc was chosen (Burnham and Anderson, 2002), unless the differences in AICc (ΔAICc) were smaller than 2, in which case the simplest model was selected (Lebreton et al., 1992). All data were log-transformed to follow assumptions of normality. Maximum and restricted maximum likelihood fitted models were used for model comparison and parameter estimation, respectively.

Results

Repeatability

The repeatability of feather CORT within individual eiders sampled more than once in different years was low and not significant (R = 0.1 ± 0.09 SE; P = 0.76 ; n = 65; Figure 2a). When examining only individuals recaptured in consecutive years, the repeatability was still very low and non-significant (R = 0.04 ± 0.13 SE; P = 0.58; n = 29). The same results were found in geese, with a repeatability of almost nil (R = 0.01 ± 0.15 SE; P = 0.50; n = 16 for all individuals and R = 0.01 ± 0.13 SE; P = 0.29 ; N = 8 for individuals sampled during consecutive years; Figure 2b).

We also considered repeatability analyses using relative feather CORT values (x-median of the yearly sample) for eiders, the species with the largest sample size. Similar results were found (R = 0.14; P = 0.45 and R = 0.31; P = 0.19 for all recaptures and for consecutive years respectively).

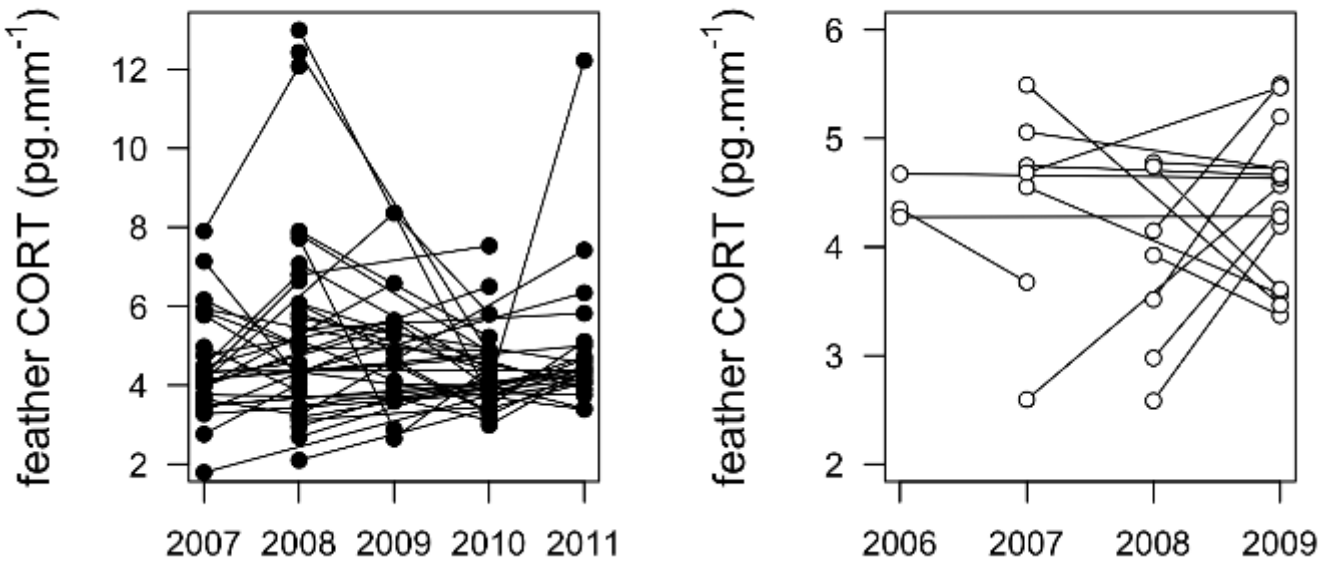

Figure 2. Repeatability of feather corticosterone. Repeatability is shown for the common eider (in black) and the greater snow geese (in white). Levels of feather CORT of recaptured individuals in different years are represented with lines connecting same individuals. (n = 65 and 16 respectively). Note that scale of the Y-axis differs between the two graphs.

Influence of breeding investment

CORT in eider feathers was not related to previous breeding investment (breeding propensity or nesting success; $F_{1,27} = 1.11$; $P = 0.30$ and $F_{1,13} = 0.18$; $P = 0.68$ respectively; Figure 3). Feather CORT in eiders was not related to relative pre-breeding body mass nor to relative laying date of the previous breeding season ($F_{1,15} = 0.05$; $P=0.82$ and $F_{1,14} = 0.50$; $P = 0.49$ respectively, Figure 4).

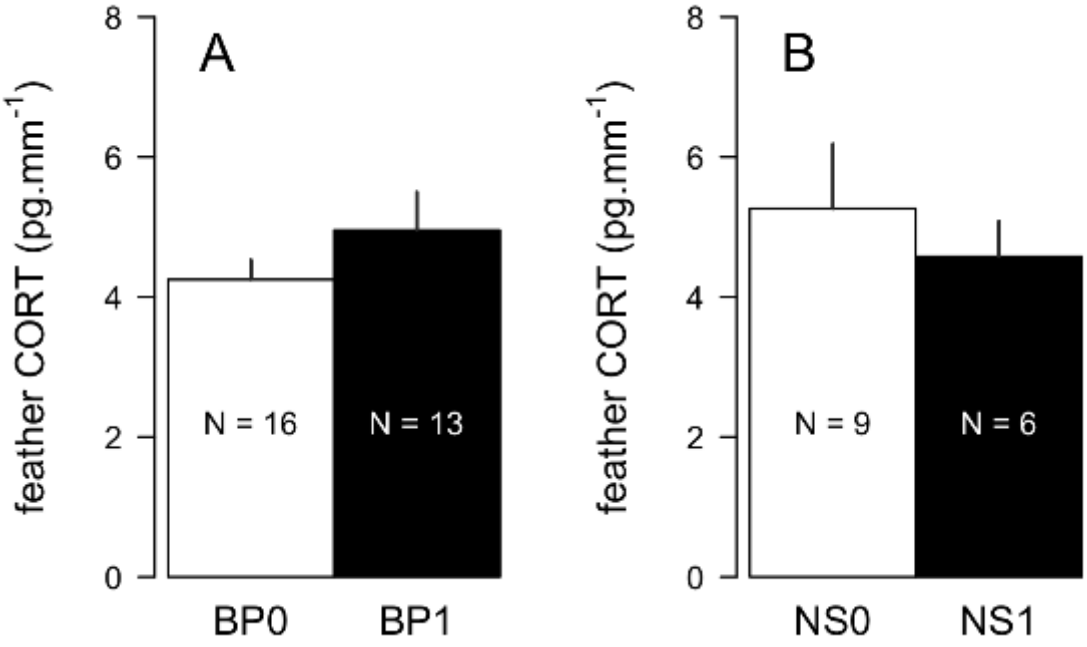

Figure 3. Feather corticosterone and previous breeding investment in common eiders. Average feather CORT (+ S.E.) with information on breeding propensity or nesting success during the previous year at East Bay. The effect of breeding propensity of nesting success on feather CORT was non significant.

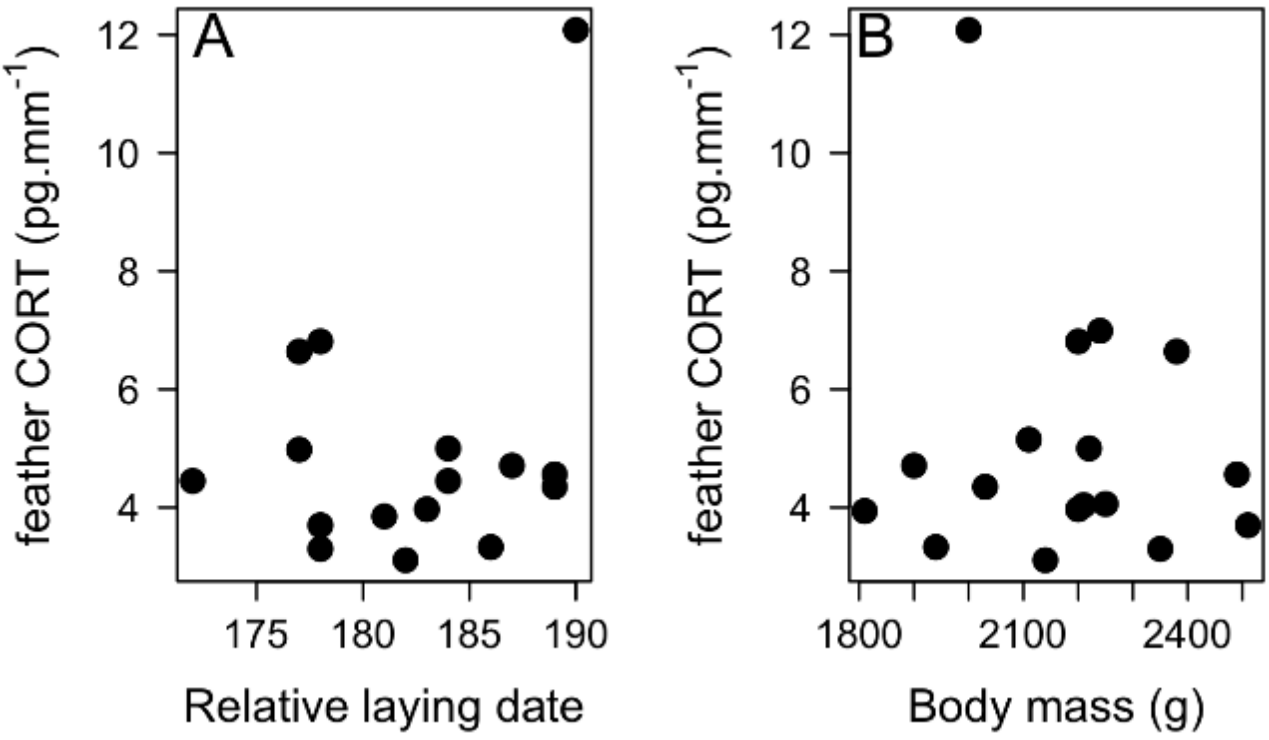

Figure 4. Feather corticosterone and previous laying date and body mass in common eiders. Relationship between feather CORT and previous laying date (relative to the median of all monitored individuals for a given year) and body mass measured in late June in female common eiders (residuals from a regression between capture date and mass for a given year). These graphs were performed from birds captured in consecutive years.

Environmental conditions

Our index of predation during the hatchling stage was not related to feather CORT in eiders (Table 1). Climatic variables such as Sea Surface Temperature or primary production (Chla) were poorly ranked (see model selection in Table 1). However, air temperature was the best variable explaining yearly variation in eider feather CORT (Table 1). Eider feather CORT levels were positively related to late summer temperatures ($\beta = 0.085 \pm 0.017$ SE, Figure 5) but not temperature variation (Table 1). A model with body size and temperature was also within 2 AICc (Table 1). The effect of body size was marginal here. Larger individuals tended to present lower CORT in their feathers ($\beta = 0.011 \pm 0.008$ SE).

Table 1. Model selection of the effects of local (average Aug-Sept temperature T and associated standard deviation TSD, precipitation PPT) and global (NAO) climate, and remote sensing covariates (SST and CHLa) on feather CORT variation on female common eiders. An index of predation pressure (number of gulls counted from June to mid August) was also considered. An index of eider body size (1st axis of a PCA based on head, culmen, wing and tarsus lengths) was also incorporated. Year was considered as a random factor. n observations = 652 females. k = number of parameters, ωi = model AICc cumulative weight, LL = log-likelihood.

Models	k	AICc	ΔAICc	ω_i	LL
T	4	353.47	0	0.39	−172.7
T+Size	5	353.75	0.29	0.34	−171.83
TSD	4	357.32	3.85	0.06	−174.63
TSD+Size	5	358.09	4.62	0.04	−174
NAO	4	358.65	5.18	0.03	−175.29
Null	3	359.51	6.04	0.02	−176.74
NAO+Size	5	359.62	6.15	0.02	−174.76
CHLa	4	359.77	6.3	0.02	−175.85
Size	4	360.26	6.79	0.01	−176.1
PPT	4	360.35	6.88	0.01	−176.14
STT	4	360.38	6.91	0.01	−176.16
CHLa+Size	5	360.48	7.01	0.01	−175.19
HEGU	4	360.55	7.08	0.01	−176.24
PPT+Size	5	361.27	7.8	0.01	−175.59
HEGU+Size	5	361.35	7.88	0.01	−175.63

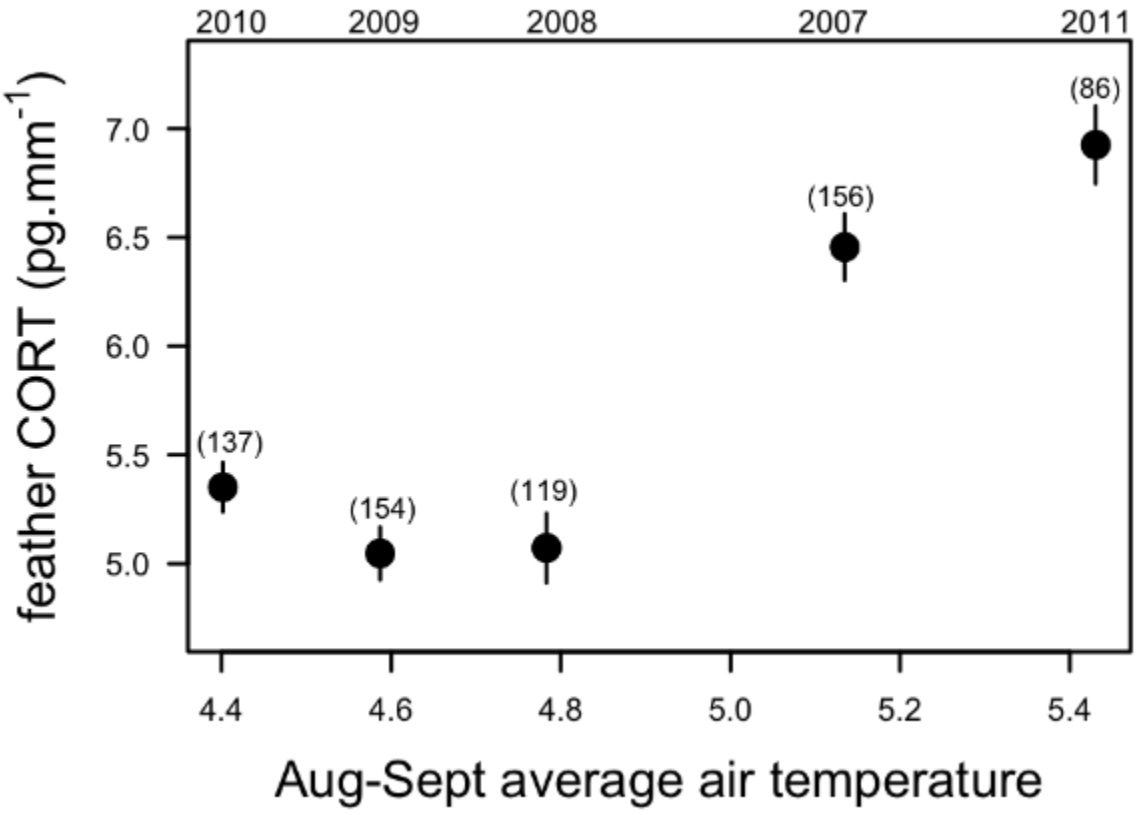

Figure 5. Feather corticosterone in common eiders and late summer air temperature. Average feather CORT (±SE) according to the average air temperature in August and September recorded in Coral Harbour (Southampton Island, Nunavut). Years of capture (above) and sample size (under brackets) are provided.

Discussion

To our knowledge, our study is the first to examine individual repeatability of feather CORT in wild birds. Feather CORT is an integrated measure of the stress responses and energetic demands experienced by an individual during the period of feather growth (Bortolotti et al., 2008; Lattin et al., 2011). In the literature, there are few examples of repeatability of CORT responses in birds. For instance, repeatability of stress-induced plasma CORT was observed in Adelie penguins (*Pygoscelis adeliae*) (Cockrem et al., 2009) and in female but not male Zebra finches (*Taeniopygia guttata*) (Wada et al., 2008). Repeatability of fecal CORT levels was observed in a semi-captive population of geese (*Anser anser*) across several seasons (Kralj-Fiser et al., 2007). Studies exploring intra-individual variability of baseline plasma CORT in wild animals have inconsistent results with repeatability being either context or sex-dependent (Ouyang et al., 2011; Wada et al., 2008; Romero and Reed, 2008). Angelier et al. (2010) reported high repeatability of basal CORT among individuals (especially in females) and an effect of reproductive investment on basal CORT measured at the beginning of reproduction in the wandering albatross (*Diomedea exulans*). In the present study, no repeatability in feather CORT level was found in either common eiders or snow geese, two species that share similar ecology and life history traits suggesting a prominent role of external conditions (such as previous breeding investment, climatic or foraging conditions). The timing of sampling also differs between our study (molting) and others (mostly pre-laying or breeding) focusing on CORT repeatability. Indeed, repeatability of baseline CORT levels can vary depending on stage of annual life cycle (Romero and Reed, 2008). In free ranging tree swallows *(Tachycineta bicolor*), repeatability of plasma CORT was found within the breeding season, but not across seasons or years (Ouyang et al., 2011). In captive house sparrow (*Passer domesticus*), basal

CORT repeatability was lower during molt than at other stages of the life cycle (Romero and Reed, 2008). Since, repeatability of basal CORT during other life stages was indeed found in waterfowl (Kralj-Fiser et al., 2007), it suggests an overall lower repeatability of CORT secreted during molt compared to other part of the life cycle.

External factors have been shown to have a strong influence on molt in birds, affecting timing of onset and rate of molt (Hahn et al., 1992). Our study suggests that feather CORT levels were more influenced by environmental conditions experienced by female eiders during the molting period than by individual quality.

The large inter-annual variability that we observed in eider feather CORT was not related to differences in breeding investment, timing of breeding or pre-breeding body condition prior to molting. This result contrasts with Bortolotti et al. (2008) which clearly demonstrates a positive relationship between breeding investment and CORT in feathers grown in late summer of the same year in captive red-legged partridges (*Alectoris rufa)*. It is surprising that this relationship was not observed in female common eiders, since they lose up to 40 % of their mass during incubation (Gabrielsen et al., 1991). We expected that such an important energetic demand would affect an individual's physiology up to and during the molting period. Based on different metrics of breeding investment, our results nonetheless indicate that the previous state of an individual (e.g., reproduction and associated fasting during incubation) did not lead to stress-associated carry-over effects during the molting period (e.g., through increased stress responses or energetic demands). Foraging conditions experienced during the summer and early fall could potentially affect eider condition and thus their CORT levels during molt. The lack of individual repeatability and the large inter-annual variability that we observed in eiders CORT feathers suggests an important role for environmental conditions affecting stress experienced during molt.

The positive association between August and September temperatures and feather CORT may reflect a causal link between weather and stress during or just prior to molting when females rebuild their energy fuels. A direct link may occur through increased metabolic activity associated with thermoregulation. However, Jensen et al. (1989) reported similar metabolic rates (although not measured during molt) in captive common eiders at 1.5 or 16-25˚C which gives little support for a direct link between temperature and CORT in feathers. Temperature can likely affect stress during molt indirectly, through food quality and quantity; for instance, the profitability (flesh-shell ratio) of bivalves, a key food resource for eiders (Senechal et al., 2011), increases when temperature is cold (Beukema et al., 2009). Petersen et al. (2003) have also shown that filtration rate of arctic bivalves starts to decrease when water temperature was > 6 degrees.

Our understanding of the importance of environmental effects on feather CORT in common eiders is based on data spanning five years. Establishing the mechanisms that affect energetic costs and stress responses during molting will require further studies either using longer-term data or experimental manipulation. Our study nonetheless provides evidence that CORT measured in feathers likely reflects environmental conditions (Fairhurst et al., 2012) and has the potential to be a valuable tool to study carry-over effects (Koren et al., 2011; Saino et al., 2012).

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APPENDIX B
PILOT STUDY TO EXPLORE MOUSE INOCULATION AS AN IMPROVED METHOD OF ISOLATING *PASTEURELLA MULTOCIDA* FROM AVIAN SWAB SAMPLES

Introduction and methods

A pilot study to examine to feasibility and sensitivity of a mouse bioassay for the recovery of *Pasteurella multocida* from PCR positive avian swab samples was conducted. As controls, CD-1 mice were injected intraperitoneally with 0.5 mL of 10^3 cfu/mL (2 mice), 10^2 cfu/mL (2 mice) and 10^1 cfu/mL (1 mouse) of pure culture *P. multocida* diluted in sterile physiological saline. Dilutions were confirmed by plate counts before and after mice were inoculated. The isolate used for positive controls was from a wild bird that died of avian cholera. An additional 4 mice were used to test avian swab samples spiked with *P. multocida*. Two mice were injected with 0.5 mL of sample spiked with 10^2 cfu/mL, and two mice were injected with 0.5 mL of sample spiked with 10^1 cfu/mL. Finally, three mice were injected with avian swab samples that tested positive for *P. multocida* using the PCR protocol described above. Swab samples in TSB glycerol were centrifuged and the pellet was resuspended in 0.5 mL of sterile physiological saline, which was injected intraperitoneally. Mice that did not die within 48 hours of injection were euthanized using overdose of isoflurane. The spleen and heart blood from euthanized or dead mice were aseptically removed, macerated under sterile conditions, streaked onto 5% blood agar plates, and incubated at 37ºC for 24 hours. Colonies that resembled *P. multocida* were confirmed using the PCR protocol as described in Chapter 2 for 2010-2011 swab samples.

Results

All mice injected with 10^3 or 10^2 cfu/mL *P. multocida* died or were euthanized due to clinical signs within 48 hours of inoculation. Culture of spleen and heart blood from these mice

resulted in pure growth of *P. multocida*. The single mouse injected with 10^1 cfu/mL was euthanized after 48 hours with no clinical signs. *P. multocida* was not cultured from tissues from this mouse. Of the mice injected with spiked samples, 3/4 died or were euthanized within 48 hours of inoculation, and pure growth of *P. multocida* was cultured from spleen and heart blood of all 3 mice. No growth was obtained from the culture of spleen and heart blood from a mouse inoculated with a sample spiked with 10^1 cfu/mL *P. multocida* which was euthanized 48 hours post inoculation, nor from the three mice injected with samples that were not spiked, which were also euthanized 48 hours post inoculation.

Discussion and conclusion

Inoculating mice with material from field samples to recover *P. multocida* has been used for decades as a method of bacterial isolation. There are some discrepancies among studies that have used this technique regarding its usefulness. For example, Muhairwa et al., (2000) found that mouse inoculation was significantly more sensitive than selective media for recovering *P. multocida* from poultry samples. Using mouse inoculation Muhairwa et al., (2001) found it to be an efficient method of isolating *P. multocida* from carrier poultry, and more sensitive method for detecting *P. multocida* spp. *multocida* compared to blood agar or selective media. In contrast, Lariviere et al., (1992) found that mouse inoculation was not superior to modified Knight's media with respect to isolating virulent strains of *P. multocida* from pigs. Although mouse inoculation has been used in studies of *P. multocida* since the 1950s (e.g., Sinha et al., 1957) and many strains of *P. multocida* are virulent in mice and can be successfully recovered (Kasten et al., 1997), there are a number of disadvantages to this method, including the use of a live animals, the expense, and the fact that only strains virulent to mice will be recovered (Kasten et al., 1997). In our study, *P. multocida* was successfully recovered from mice inoculated with at

least 10^2 cfu/mL of virulent *P. multocida* organisms. However, mouse inoculation was not successful in recovering isolates from three PCR positive field samples, and thus was not employed as an isolation method for this study, since it was determined that the disadvantages outweighed the benefits.

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APPENDIX C
PILOT STUDY TO EXPLORE SELECTIVE ENRICHMENT AS AN IMPROVED METHOD OF ISOLATING *PASTEURELLA MULTOCIDA* FROM WATER AND SEDIMENT SAMPLES

Introduction and methods

Culturing *Pasteurella multocida* from samples heavily contaminated with other microorganisms, or containing very low numbers of *P. multocida,* can be very challenging (Moore et al., 1994). In an attempt to improve recovery rates of *P. multocida* from field samples, various authors have developed and used selective media containing antibiotics and other inhibitors (e.g. Knight et al., 1983; Lariviere et al., 1993; Moore et al., 1994) with varying success. Lariviere et al., (1993) reported that using a modified Knight's media was as successful at isolating *P. multocida* from pig nasal cavities as mouse inoculation. A selective media using clindamycin, gentamicin, potassium tellurite and amphotericin B was used to successfully isolate *P. multocida* from oral swab samples from dogs and cats (Knight et al., 1983). Moore et al., (1994) developed two selective media, an agar and a broth, to improve isolation of *P. multocida* from pond water and heavily contaminated samples. The authors found that use of the selective media was more effective in isolating *P. multocida* from mixed cultures than using blood agar alone (Moore et al., 1994). Some of the procedures described by Moore et al., (1994) have been used in several studies to successfully isolate *P. multocida* from swab samples collected from wild apparently healthy birds (e.g., Samuel et al., 1997; 2005) and wetland water samples (e.g., Moore et al., 1998; Blanchong et al., 2006).

Our objective was to increase the likelihood of isolating *P. multocida* from pond water and sediment and nest soil samples collected from common eider colonies in the eastern Canadian Arctic. We conducted a pilot study to compare the effectiveness of using two selective

enrichment techniques (as described in Moore et al., 1994) to plating directly on 5% blood agar media for the recovery of *P. multocida* isolates from environmental samples. We also compared the effectiveness of culturing two different types of subsamples for each of the three techniques: a vortexed sample, or the pellet of a centrifuged sample.

Sample collection

Pond water and sediment samples were collected on East Bay Island, Nunavut, Cape Dorset, Nunavut, and Ungava Bay, Nunavik, in 2010 and 2011, as described in Chapter 2 (total n = 561). A subset of these samples were used for this study, and included water and sediment samples from East Bay Island collected after the avian cholera outbreak began (n = 127), and randomly selected pond water and sediment samples from ponds near Cape Dorset (n = 24) and Ungava Bay, Nunavik (n = 29). Collection, preservation, and storage of samples is described in Chapter 2.

Media preparation

Pasteurella multocida selective broth (PMSB) and *Pasteurella multocida* selective agar (PMSA) were prepared according to Moore et al., (1994). These media were used to culture 71 of the samples. For the remaining 109 samples, PMSB was used as described, but the PMSA preparation was modified and 0.5 µg/ml gentamicin was added to the media instead of 0.75µg/ml, since we suspected that the high gentamicin concentration in the PMSA may have inhibited growth of *P. multocida.*

Vortexed sample

Water and sediment samples were thawed for 30-60 minutes, then gently vortexed for 10 seconds. Immediately after vortexing, 100 µL of sample was spread on a blood agar plate. 100 µL of sample was also spread onto a PMSA plate. The blood agar plate was incubated at 37ºC

for 24 hours. The PMSA plate was incubated in the presence of 5-10% CO_2 at 37ºC for 24 hours. 500 µL of the vortexed sample was added to 5 ml of PMSB. The PMSB was incubated in the presence of 5-10% CO_2 at 37ºC for 14-16 hours. After incubation, PMSB was vortexed for 10 seconds, and a 10 µL aliquot was plated onto 5% blood agar and incubated at 37ºC for 24 hours. All the plates were examined for the presence of colonies resembling *P. multocida*. Any colonies that resembled *P. multocida* were streaked on blood agar and incubated at 37ºC for 18-24 hours to obtain a pure culture. Pure cultures were confirmed to be *P. multocida* using the real-time PCR assay as described in Corney et al., (2007) with modifications as described in Chapter 2 of this thesis for samples from 2010 and 2011.

Centrifuged sample

Immediately after the aliquots of the vortexed sample were plated or placed in PMSB, the remaining sample was transferred to a sterile 15 ml centrifuge tube. Samples were centrifuged at 2700 rpm for 10 minutes. After centrifuging, the supernatant was removed. Using a sterile 25 µL loop, approximately 1/3 of the pellet was streaked onto a blood agar plate. Approximately 1/3 of the pellet was also streaked onto a PMSA plate. The remaining third of the pellet was added to 5 ml of PMSB and gently vortexed for 5 seconds. Agar plates and broth were processed as described above.

For each batch of samples tested, pure cultures of *P. multocida* were used as positive controls and were cultured alongside the samples using the three methods (blood agar, PMSB, and PMSA) and incubated following the methods described above for the samples. Over the course of the project five different isolates were used as positive controls, with one or two isolates used per batch of samples. The positive control isolates included two isolates from common eiders that died during the avian cholera outbreak on East Bay Island in 2010, two

isolates from common eiders that died during the avian cholera outbreak on East Bay Island in 2011, and one isolate from a common eider that died during the avian cholera outbreak on East Bay Island 2009.

Results and Discussion

P. multocida was isolated from one pond water sample collected on East Bay Island in 2010 after the avian cholera outbreak was underway. The colonies were cultured from the vortexed and centrifuged samples plated on blood agar, and pre-cultured in PMSB and then plated on blood agar plates. *P. multocida* from this sample was not detected on any PMSA plates.

Five different *P. multocida* isolates were used throughout the study as positive controls. Only 1/5 positive control isolates grew on PMSA. All positive controls grew when using blood agar alone, and when using PMSB pre-culture followed by plating on blood agar.

Despite the results reported by Moore et al., (1994) we found that in our laboratory, very little bacteria from any samples grew on PMSA, no *P. multocida* colonies from any sample were detected on PMSA, and PMSB did not improve our ability to isolate *P. multocida* compared to blood agar alone. Based on our suspicion that the gentamicin concentration was too high in PMSA, we reduced the amount added to the agar from 0.75 µg/ml to 0.5 µg/ml, however, this did not improve our ability to recover *P. multocida* from samples. Furthermore, only one of the five *P. multocida* isolates used as a positive control in this study grew on PMSA. Since there did not appear to be any advantage to using the selective enrichment broth before plating the sample on blood agar, we used only direct plating on blood agar for the remaining 381 samples. For the first 180 samples analyzed in this pilot study, we found that using the pellets from centrifuged samples resulted in significant overgrowth of plates by contaminating bacteria from the sample,

making detection of any potential *P. multocida* colonies very difficult. Thus, we chose to use vortexed samples for culturing the remaining 381 water and sediment samples.

Lariviere et al., (1993) used several selective media in their study, including the selective Knight's media, which contains potassium tellurite and is similar to the media described in Moore et al., (1994). *P. multocida* from piglet nasal swabs was more likely to be isolated when the authors used the modified Knight's media, which did not contain potassium tellurite. In future, it may be beneficial to decrease or eliminate potassium tellurite from the Moore et al., (1994) media to determine if that improves recovery of *P. multocida*. Moore et al., (1994) reported that *P. multocida* was less likely to be isolated from tissues of birds suspected to have died of avian cholera when PMSA was used, as compared to blood agar. We suspect that other authors had poor success using PMSA as well, since both Samuel at al., (1997) and Samuel et al., (2005) report only using PMSB in their culture protocols for avian swabs, and wetland water samples collected by Moore et al., (1998) were processed using PMSB but not PMSA.

Based on the results of this pilot study, 381 of the 561 pond water and sediment samples and nest soil samples from East Bay Island collected in 2010 and 2011 and pond water and sediment samples from Ungava Bay collected in 2011 were cultured by plating 100 µL of vortexed sample on blood agar. Despite our efforts to improve our ability to culture *P. multocida* from pond water and sediment and soil samples, *P. multocida* was only cultured from 1/561 water, sediment and soil samples from the eastern Canadian Arctic (Chapter 2).

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