PACIFIC WALRUS USE OF HIGHER TROPHIC LEVEL PREY AND THE RELATION TO SEA ICE EXTENT, BODY CONDITION, AND TRICHINELLOSIS

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A

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Abstract

The changing Arctic ecosystem may prompt Pacific walruses (*Odobenus rosmarus divergens*) to change their usual diet of lower trophic level prey (e.g., benthic invertebrates) by increasing the consumption of higher trophic level prey (HTLP). Prey-switching may have consequences to walrus populations through increased energetic costs, increased stress response, declines in body condition, and exposure to diseases, including the zoonotic parasite *Trichinella* spp. *Trichinella* is possibly transmitted to walruses via predation or scavenging on seals. The goal of this study was to quantify reliance on HTLP using stable carbon and nitrogen isotope ratios, and assess potential correlations among consumption of HTLP and sea ice extent, sex, *Trichinella* infection, body lipid stores, and cortisol concentrations used as an index of the stress response. Walrus diet is comprised of ~1-22% HTLP and reliance on HTLP may be correlated with sea ice extent in a complex way. *Trichinella* was present in ringed seal (*Pusa hispida*, 1/57), Arctic fox (*Vulpes lagopus*, 3-7/32), and polar bear (*Ursus maritimus*, 1/1), but was not detected in walruses (0/137) regardless of %HTLP in the diet. Walrus blubber and attached skin contained 44.6 ± 12.4% lipid wet weight, which was lower than that found for other Arctic marine mammals; however, the inclusion of skin likely decreased our %lipid values. While the absolute value of %lipid from blubber and attached skin was not a suitable substitute for %lipid from blubber only, we were still able to detect the influence of biological factors, with sex-linked variability in walrus lipid stores observed. Cortisol analysis from full-thickness blubber resulted in a wide range of concentrations

(2.77 to 34.04 ng/g), but showed that this stress hormone can be extracted from blubber. While neither %lipid nor blubber cortisol was correlated with the proportion of HTLP in walrus diet, they may serve as minimally-invasive methods for health monitoring of walruses. Overall, dietary plasticity of walruses is robust and switching to HTLP is not likely to have immediate adverse effects on the Pacific walrus population.

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Chapter 1: General Introduction

The Arctic marine environment is experiencing unprecedented climatically-induced changes (Grebmeier 2012; Stroeve et al. 2012), with recent temperatures more extreme than those recorded in ice cores for the last 400,000 years (Petit et al. 1999). The increasing Arctic sea ice retreat resulting from these climatic changes has potential for severe consequences to ice-associated and ice-obligate marine mammal populations (Moore and Huntington 2008) intrinsically important to many Alaska Native communities. Compounding physical changes in the Arctic environment, retreating sea ice is expanding industry access to marine resources, thereby increasing potential impacts to marine mammal populations from anthropogenic activities (Huntington 2009). A solid understanding of how life history requirements of these species are affected by external variables is needed to accurately estimate the consequences of climate change and anthropogenic activities to Arctic marine mammals.

Pacific walruses (*Odobenus rosmarus divergens*) are long-lived, pagophilic pinnipeds that typically forage on benthic organisms (Fay 1982; Sheffield et al. 2001; Sheffield and Grebmeier 2009). The species is of particular importance to coastal communities along the Russian Chukotkan Peninsula and in Alaska, providing food and materials, in addition to being culturally significant (Fay 1982; Garlich-Miller et al. 2011; Robards and Garlich-Miller 2013). In the absence of successful hunting, communities reliant on walruses can experience economic impacts (Parnell 2013). Walruses winter on the sea ice throughout the Bering Sea with females, calves, and some males migrating northward into the Chukchi Sea each summer (May through July) with the retreating sea

ice (Fay 1982; Garlich-Miller et al. 2011). The remaining males migrate southwest to the Bristol Bay region, and west to the Russian coastline (Fay 1982). However, a greater proportion of males may be migrating northward in recent years (Garlich-Miller et al. 2011). As seasonal sea ice extent increases each fall, walruses return to their wintering grounds (Fay 1982). Mating occurs during December-March; however, delayed embryonic implantation results in a gestation period of over a year, with calves born in April through June (Fay 1982; Garlich-Miller et al. 2011).

Walruses use sea ice both as a diving platform over shallow waters, which reduces the energetic costs of migration (Fay 1982). The species is physiologically ill-adapted for deep diving (Fay and Burns 1988), which limits their ability to access benthic prey in deep waters. Walruses can dive to depths greater than 250 m, but typically forage at depths of 80 m or less (Fay and Burns 1988; Born et al. 2003; 2005). When sea ice retreats to areas with bathymetry too deep for benthic foraging, walruses may seek alternate habitat (Jay and Fischbach 2008; Garlich-Miller et al. 2011; Robards and Garlich-Miller 2013). Terrestrial haulouts are common on the Russian Chukotkan coastline and were historically present to a moderate extent along the Alaska coast, although no large haulout existed on the northern Alaska coastline (i.e., in the Chukchi and Beaufort seas) prior to 2006 (Robards and Garlich-Miller 2013; Figure 1.1). The recent drastic retreat of summer sea ice may be causing walruses to aggregate at terrestrial haulouts along both sides of the Bering Strait in much higher densities than historically documented (Jay and Fischbach 2008; Garlich-Miller et al. 2011; Jay et al. 2012; Robards and Garlich-Miller 2013). In addition to increased presence at Alaska

terrestrial haulouts in the Chukchi Sea, and decreased use of terrestrial haulouts in the Bristol Bay region (Fay 1982; Weiss and Morrill 2014), researchers have documented higher numbers of calf abandonment than previously observed (Cooper et al. 2006), and calves make up a disproportionate majority of fatalities resulting from trampling events (Udevitz et al. 2013). Increased rates of calf abandonment and fatality may be of concern given the naturally low fecundity and growth rates of the species; females typically give birth to one calf every other year and calves remain with their mother for two years (Fay 1982). Other population-level impacts resulting from increased reliance on terrestrial haulouts may include intensified competition for food sources and increased contact with density-dependent infectious agents (Kutz et al. 2005; Fischbach et al. 2007; Burek et al. 2008; Garlich-Miller et al. 2011; MacCracken 2012). For example, in 2011, a yet-to-be-identified pathogen caused illness and death among multiple species of Arctic pinnipeds, including walruses (NMFS 2012). However, walruses have traditionally used large terrestrial haulouts during summer and fall months, particularly on the Russian coastline (Fay 1982; Robards and Garlich-Miller 2013) without apparent detrimental health effects. This historical haulout use suggests that at least some locations can support seasonal aggregations of walruses on a long-term basis.

Walrus-specific variables that may be affected by changing climate, such as diet, growth rate, and disease prevalence, are difficult to detect and to compare. Due to the life history characteristics and habitat of the Pacific walrus population, historical data on such basic management information as population counts are difficult to accomplish (Fay et al. 1997; Speckman et al. 2010). In 2011, concern for population impacts resulting from

forecasted declines in sea ice, led to the Pacific walrus being listed as a candidate species for protection under the U.S. Endangered Species Act (76 FR 7634 7679 2011).

Walruses are benthic invertebrate specialists feeding primarily on bivalves and gastropods (Fay 1982; Sheffield and Grebmeier 2009). However, walruses will forage on a diverse array of prey, including seals and seabirds (Fay 1960; Fay et al. 1977; 1990; Lowry and Fay 1984; Sheffield et al. 2001; Sheffield and Grebmeier 2009). While predation on seals and seabirds by walruses is not novel (Fay 1960; Krylov 1971; Fay et al. 1977; Sease 1984; Burns et al. 1985; Merrick and Hills 1988; Muir et al. 1995; Mallory et al. 2004; Fox et al. 2010), some studies suggest that this foraging strategy is increasing in both Pacific and Atlantic walrus (*Odobenus rosmarus rosmarus*) populations (Lowry and Fay 1984; Fay et al. 1990; Wolkers et al. 2006). Fay (1960), Rausch et al. (2007), and Jay and Fischbach (2008) hypothesized that reliance on higher trophic level prey (HTLP) would increase during unfavorable sea ice conditions or other nutritionally stressful situations when benthic invertebrates are inaccessible or unavailable.

Prey-switching by marine mammals has often been associated with increased stress response and adverse impacts to health due to insufficient daily energy gains and/or increased food handling costs (Fryxell and Lundberg 1994; Rosen and Trites 2000; Rosen et al. 2007; Rosen 2009). These general impacts may hold true for walruses relying more heavily on seals and seabirds or, alternately, the dense caloric properties of seal and bird tissues, blubber in particular, may offset the aforementioned costs to individual fitness (Kuhnlein and Soueida 1992; Hondolero et al. 2012). Regardless, consumption of HTLP

can impact population health through alteration of host-parasite dynamics, increases in disease exposure and increases in contaminant loads (Muir et al. 1995; Votier et al. 2004; Burek et al. 2008; McKinney et al. 2009). Reliance on HTLP may affect walrus health and could have acute and long-term consequences for Russian and Alaskan coastal communities that rely on walruses as a food source by exposing consumers to potential zoonotic diseases. This could possibly decrease the availability of walruses for subsistence harvest. Understanding how walrus foraging strategies impact disease transmission and walrus health/body condition would be useful to better estimate trends in the Pacific walrus population. Additionally, possible correlations between HTLP reliance and sea ice extent could provide wildlife managers with a better understanding of the species. One impact to both walruses and humans from increased reliance by walruses on HTLP may be infection with the zoonotic disease trichinellosis or trichinosis, an incurable, potentially fatal disease (causative agent: the parasitic nematode *Trichinella* spp., Despommier et al. 2005). Walrus meat is a source of periodic outbreaks of trichinellosis in Alaska Native subsistence communities (Margolis et al. 1979; Proulx et al. 2002; Pozio and La Rosa 2003; Schellenberg et al. 2003; Moller et al. 2005). Contraction of *Trichinella* occurs primarily through ingestion of infected muscle; although the transmission pathway in the marine environment is poorly understood. Fay (1960) suggested that walruses may contract the parasite through consumption of infected seals. Thus, exposure to *Trichinella* may rise if walruses change their diet in response to changes in sea ice.

Potential impacts of *Trichinella* infection on walruses is difficult to assess. Few studies have examined potential impacts of *Trichinella* infection on non-human mammalian hosts (reviewed in Forbes 2000). Experimental studies of terrestrial wildlife indicate that *Trichinella* infection may lead to declines in fecundity and offspring survival as well as behavioral changes (Worley et al. 1983; Rau 1985; Meagher and Dudek 2002). Decreased fecundity could negatively impact the walrus population (MacCracken 2012), which is of particular concern, given that evidence from biological samples recently analyzed from subsistence-harvested walruses suggests that a decline in fecundity may already be occurring (Garlich-Miller et al. 2006).

Trichinella has been documented in Alaska marine mammals since 1950 (Forbes 2000), but historical prevalence of the parasite in Alaska marine and marine-associated mammals may have been underestimated due to the method of detection (i.e., trichinoscopy, Brandly and Rausch 1950; Forbes et al. 2003). Estimates of prevalence are needed to evaluate the risk of *Trichinella* to human communities and the walrus population. Increased reliance on HTLP by walruses may be associated with declines in overall body condition and increased exposure to stressors. Assessment of Pacific walrus body condition and any stress response has historically been limited due to the logistical difficulties of studying free-ranging sea mammals in remote areas. In recent decades, tissue samples have been obtained from natural mortality events or have been provided cooperatively by Alaska Native subsistence harvests and provided to wildlife managers and other researchers. However, such sampling methods may be biased towards weak

animals (in the case of natural mortalities) or those selected by humans for subsistence use.

Non-lethal techniques to assess body condition and stress response of walruses would be useful to better understand trends and correlations between body condition, exposure to stressors, reliance on HTLP, and influence of other environmental and biological parameters. Traditional methods to evaluate proxies of body condition and stress response in marine mammals (e.g., Thomson and Geraci 1986; Fossi and Marsili 1997) include measurement of blubber thickness as an indicator of energy stores, foraging success and prey accessibility, and blood cortisol levels as an indicator of exposure to environmental or physiological stressors. Research shows blubber %lipid to be a more reliable proxy for body condition than blubber thickness (Beck et al. 1993; Koopman et al. 2002; Gulland et al. 2005). Blubber samples can be obtained via biopsy dart, and this minimally-invasive method of tissue collection is valuable for multiple types of analyses (e.g., contaminant levels and stable isotope values used in dietary and migration studies). Furthermore, recent success in measuring blubber steroid concentrations suggests that cortisol, a corticosteroid produced in response to stressors, may also be quantified from this tissue (Kellar et al. 2006; 2009; 2013; Perez et al. 2011). Walrus blubber may provide a record of long-term stress responses because it serves as a sequestration site for lipophilic hormones, which have a longer biochemical residence time in this tissue as compared to serum (Liggins et al. 1993; Jarman et al. 1996; Zapol 2011). Combined with dietary analyses and disease surveys, walrus blubber %lipid and cortisol concentration may offer information on walrus health in light of changes in

reliance on HTLP. This information can be used to better manage the walrus population under changing conditions.

This dissertation is part of a larger study assessing and monitoring the Pacific walrus population (including work from the U.S. Fish and Wildlife Service's Walrus Harvest Monitoring Program and the U.S. Geological Survey's Biological Research Division). These larger efforts involve federal and state agencies, Alaska Native coastal communities and organizations, academic institutions, and co-management groups in a broad-scale evaluation of walrus population health. This study seeks to achieve a better understanding of walrus diet and dietary impact on disease prevalence, stress response, and body condition. Chapter 2, "Occurrence and Genotypic Analysis of *Trichinella nativa* in Alaska Marine-Associated Mammals of the Bering and Chukchi Seas", provides current prevalence and genotype(s) of *Trichinella* spp. present in walruses and select Alaska marine and marine-associated mammals (Seymour et al. 2014a). This information provides Alaska Native coastal communities and management agencies with data to better assess the risk of *Trichinella* transmission to local communities and wildlife populations. This chapter has been published in Veterinary Parasitology. Chapter 3, "Proportion of Higher Trophic Level Prey in the Diet of Pacific Walruses", examines the potential use of multiple biological tissues (including those obtained by non-lethal collection methods) to be used in stable carbon and nitrogen isotope analysis, providing researchers with less invasive methods for examining walrus diet (Seymour et al. 2014b). Additionally, Chapter 3 investigates the use of a Bayesian mixing model to estimate the proportional contribution of HTLP to walrus diet, and biological variables influencing

their reliance on HTLP. This chapter is in press for publication in Polar Biology. Chapter 4, “Inter-Annual Variability in the Proportional Contribution of Higher Trophic Levels to the Diet of Pacific Walruses”, correlates the Bayesian mixing model estimates of HTLP across years with historical sea ice extent to evaluate the hypothesized influence of sea ice retreat on walrus foraging and dietary preference (Seymour et al. 2014c). This information provides a better understanding of how future sea ice trends may affect walrus foraging, with potential consequences on species distribution and habitat use. This chapter has been published in Polar Biology. Chapter 5, “Influence of Diet on Lipid Content and Cortisol Concentration in the Blubber of Adult Pacific Walruses”, investigates variability of %lipid in walrus blubber, as a sex- and reproductive status-dependent indicator of body condition. This index of %lipid provides insight into biological and dietary influences on walrus body condition. Additionally, Chapter 5 assesses cortisol concentrations in walrus blubber (in combination with blubber %lipid values), and correlates diet and other indicators of health to determine whether prey-switching by walruses may result in an increased stress response. This chapter has been submitted to Marine Mammal Science. Combined, this research provides a suite of quantitative and often minimally-invasive methods and baseline data for monitoring walrus diet and health. These results many also be useful in estimating future impacts of climate change on the Pacific walrus population.

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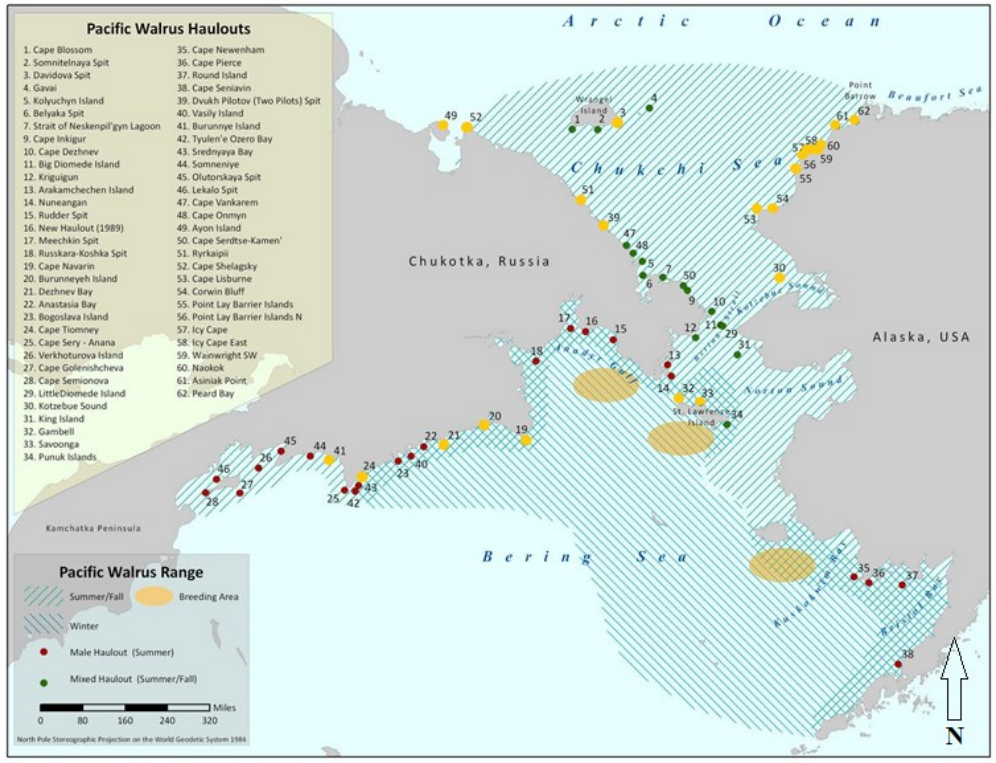

Fig. 1.1 Pacific walrus haulout locations

Orange dots denote haulouts that are new or have increased in size since 2006 (adapted from U.S. Fish and Wildlife Service and Robards and Garlich-Miller 2013, available at: http://www.fws.gov/alaska/fisheries/mmm/walrus/pdf/fwsreg7_pacific_walrus_range_map4.pdf.

Chapter 2: Occurrence and Genotypic Analysis of *Trichinella* in Alaska Marine-Associated Mammals of the Bering and Chukchi Seas[1]

2.1 Abstract

The zoonotic parasite *Trichinella* is the causative agent of trichinellosis outbreaks in the circumpolar Arctic. Subsistence communities are particularly vulnerable to trichinellosis due to traditional meat preparation methods and regional presence of a freeze-tolerant *Trichinella* species (*Trichinella nativa*). This study is the first application of a validated artificial digestion method in determining incidence of *Trichinella* spp. in Alaska mammals. Infection incidence in pinniped species (*Erignathus barbatus, Eumetopias jubatus, Odobenus rosmarus divergens,* and *Pusa hispida*) was low, with only 1/57 ringed seals infected. Polymerase Chain Reaction assays indicated *T. nativa* as the only species present in northern Alaska. Analysis of an archived polar bear (*Ursus maritimus*) muscle sample shows freeze-tolerance and longevity for *T. nativa* to -20 ˚C for 10 years and short-term freeze resistance to -80 ˚C when morphology was used to determine presence of live larvae. However, larval motility suggests 0% survival. An approach that combines artificial digestion with PCR based species identification has excellent potential for *Trichinella* spp. detection and identification of archived tissues. Overall, *Trichinella* in Alaska mammals, particularly marine mammals of subsistence importance to Alaska Native communities, appears to be a minor problem. These modern diagnostic techniques

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[1] Seymour J, Horstmann-Dehn L, Rosa C, Lopez JA (2014) Occurrence and genotypic analysis of *Trichinella* in Alaska marine-associated mammals of the Bering and Chukchi seas. Vet Parasitol 200:153-164

provide accurate insight into the presence of *Trichinella* in the Alaska marine environment.

Keywords: *Trichinella*, walrus, seal, Arctic fox, polar bear, digestion assay, PCR

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2.2 Introduction

The genus *Trichinella* groups parasitic nematodes infecting a broad range of mammalian, avian, and reptilian hosts, and includes some of the world's most widely-distributed nematodes (Despommier et al., 2005). Infection with *Trichinella* spp. causes the disease commonly referred to as trichinellosis (also termed trichinosis). This disease originally became well known from *T. spiralis* infection in the species' reservoir host, the domestic pig, resulting in slaughterhouse investigations and industrial testing programs in North America and Europe (Despommier et al., 2005). Currently, there is a low incidence of human trichinellosis in the United States, n=66 cases between 2002 and 2007, with most cases occurring as scattered outbreaks, mainly from the consumption of game meat (Roy et al., 2003; Kennedy et al., 2009). *Trichinella* is contracted through the consumption of raw or undercooked tissue harboring a nurse-cell-larvae complex. Larvae, released by the action of gastric digestive juices, from consumed tissue migrate to the small intestine, where adult development and sexual reproduction occur. Second generation larvae then move into the bloodstream and to body musculature where they form nurse-cell complexes through modification of the host's cell. These nurse-cell complexes nourish the larvae, which thrive for the duration of the host's lifespan or longer (Despommier et al., 2005).

While symptoms of trichinellosis in the wild animal host are generally unknown, in humans, clinical features of mild, moderate, and severe infections have been reviewed (Capo and Despommier, 1996). The presentation of the disease varies over time, resulting

in a wide variety of clinical conditions and is therefore often misdiagnosed. The severity of the disease is dependent on the number of larvae ingested, further complicating accurate diagnosis. Early onset of trichinellosis presents as gastroenteritis associated with diarrhea, abdominal pain, and vomiting. In severe cases, acute muscle pain, breathing difficulties, and heart failure can develop (Proulx et al., 2002). Laboratory studies on mice (*Mus musculus*) and field studies of grizzly bears (*Ursus arctos*) suggest correlations between *Trichinella* infection and increased aggression as well as adverse reproductive and physiological effects. These effects include possible discontinued lactation, increased energy expenditure, and decreased mass of offspring, with second generational impacts suggesting potential long-term consequences for infected populations (Worley et al., 1983; Rau, 1985; Meagher and Dudek, 2002).

Trichinellosis outbreaks have been documented in native communities in the circumpolar Arctic for several decades, with the primary source of transmission to the human host being consumption of marine mammal tissue (Proulx et al., 2002; Moller et al., 2005). In September 2008, two fatalities (with *Trichinella* as the causative agent) were reported in a Chukotkan community after the consumption of raw Pacific walrus (*Odobenus rosmarus divergens*) meat (Independent Newspaper, 2008). In September 2010, multiple people in Nunavut, Canada sought medical treatment after ingesting meat from *Trichinella* infected walruses (Canadian Broadcasting Corporation, 2010). In 2012 one individual in Alaska was diagnosed with trichinellosis after consuming infected bear meat (Anchorage Daily News, 2012). Archeological evidence suggests that the presence of *Trichinella* in Alaska predates Western-European contact (Zimmerman and

Aufderheide, 1984). Current incidence within the State of Alaska is 1.8 per 100 000 individuals, compared to 0.05–0.06 incidence rate per 100 000 people in the overall U.S. (Hotez, 2010).

The last 60 years have shown that *Trichinella* has a wide variety of reservoir hosts in the Arctic, including Pacific walruses, Arctic foxes (*Vulpes lagopus*), polar bears (*Ursus maritimus*), and domestic dogs (*Canis lupus*, Kapel et al., 1999, Table 2.1). The transmission mechanism in the Arctic, particularly in the marine environment, is poorly understood. Current theories suggest that walruses contract the parasite through consumption of infected ice seals (i.e., ringed seals (*Pusa hispida*), bearded seals (*Erignathus barbatus*), spotted seals (*Phoca largha*); Fay, 1960), which are known to be consumed by Pacific walruses at an unknown frequency (Lowry and Fay, 1984; Fay et al., 1990). Experimental infections also suggest the possibility of marine paratenic hosts and confirm cross-placental vertical transmission in several carnivores (Fay, 1968; Webster and Kapel, 2005).

Historical analysis of several Alaska marine mammal species (Table 2.1) has shown the presence of *Trichinella* at sporadic and low levels (<2%, reviewed by Forbes, 2000) with the exception of polar bears, which have an infection incidence of 55-60% in Alaska populations (Fay, 1960; Weyermann et al., 1993). However, previous incidences in Alaska were determined using trichinoscopy, rather than the more accurate digestion assays (Forbes et al., 2003). Forbes et al. (2003) found digestion assays three times more likely than trichinoscopy to identify positive samples, which suggests a potential for trichinoscopy-based monitoring to underestimate low-level infections (Forbes and

Gajadhar, 1999; Forbes et al., 2003; Leclair et al., 2003). While enzyme-linked immunosorbent assay (ELISA) testing of serum or muscle fluid is another possible detection method (Beck et al., 2005; Gajadhar et al., 2009), the ELISA method can produce false-negatives if the animals tested were in early stages of infection (Gajadhar et al., 2009). Recently, real-time PCR was shown to be a promising option for *in situ* detection (Cuttell et al., 2012); however this method was not in practice at the time of our study. Studies of *Trichinella* spp. isolated from Arctic foxes in Greenland and Svalbard (confirmed or assumed to be cold-tolerant *T. nativa*) determined larval cysts were still viable and capable of infection after freezing at -18 ˚C for 4 years (Kapel et al., 1999). Many studies have used morphology and/or motility (*in vitro* assessments) as an indication of live, potentially infective larvae (e.g., Kapel et al., 1999); however, Davidson et al. (2008) suggests this technique is unreliable and experimental inoculation (*in vivo* assessments) is the only confident method for detecting infectivity. Most recent studies conduct both *in vitro* and *in vivo* assessments (Bolas-Fernandez and del Corral Bezara, 2006; Wu et al., 2007). Historical assessments of *Trichinella* in Alaska assume the species present to be either *T. spiralis* or *T. nativa*, but genotypic analyses are needed to confirm the identity of infecting species as possible differences in virulence exist. Generally, *T. spiralis* produces greater infection loads in both humans and animal hosts, particularly in carnivorous mammals (Smith, 1983). However, *T. spiralis* has not been recorded in Arctic regions since differentiation of the *Trichinella* genus into multiple species (Pozio et al., 2009).

At present there are 12 recognized species of *Trichinella* (Pozio et al., 2009). Five species occur in North America (*T. spiralis, T. nativa, T. pseudospiralis, T. murrelli,* T_6, Pozio et al., 2009). With climate change impacting habitats, temperature regimes, and seasonal cycles, there is a pressing need to characterize the current prevalence and incidence of this parasite within the Arctic. Warming air and water temperatures and northward range expansion of marine species are expected to alter disease exposure rates as well as create new transmission routes which, in turn, may ultimately impact Alaska Native coastal communities (Hinzman et al., 2005; Fischbach et al., 2007; Rausch et al., 2007; Burek et al., 2008). The ability of wildlife management and public health agencies to determine *Trichinella* incidence will be dependent on monitoring of this pathogen. For example, commercially raised pork products are subject to country-mandated inspection protocols which have largely eradicated *Trichinella* from mass-sale products (Gajadhar and Gamble, 2000).

The goals of this study were to 1) assess current incidence of *Trichinella* among select Alaska marine and marine-associated mammals using the double-separatory funnel digestion assay diagnostic method, and 2) identify the species of *Trichinella* present in these mammals using multiplex PCR analysis. We hypothesized that modern, more accurate diagnostic techniques would uncover a higher incidence in these species than historically documented and that these infections would be dominated by the freeze-tolerant *T. nativa* species.

2.3 Materials and Methods

2.3.1 Sample Collection

For each specimen from which tissue was collected, the species, sampling date and sex (when available) were recorded, and each individual was given a unique identifier (Table 2.2). Muscle (100 g or entirety of tongue muscle if the organ weighed <100 g) was sampled from the tongue (including tongue apex) of each animal. Tongue muscle was selected as it has been shown to be the preferred site for *Trichinella* in walruses and most other mammals (Kapel et al., 1995). Samples were stored in Whirlpak™ or Ziploc™ bags, and frozen at -20 °C until analysis. All samples, except several Arctic fox and walrus samples, were assessed as carcass code 1 (fresh, Geraci and Lounsbury, 1993).

2.3.1.1 Pacific Walruses

Pacific walrus tongue muscle samples (n=137) were opportunistically collected during the Spring/Summer Alaska Native subsistence harvest during 2009 and 2010 near Little Diomede and St. Lawrence islands, Barrow, and Wainwright, Alaska. Samples were obtained in collaboration with Alaska Native subsistence hunters in these communities, as well as the Eskimo Walrus Commission, U.S. Fish and Wildlife Service (USFWS), North Slope Borough Department of Wildlife Management (NSB-DWM), and the Alaska Department of Fish and Game (ADF&G, Table 2.2). Additionally walrus tongue samples were collected from a 2009 mortality event (Liedberg et al., 2009) at Cape Peirce, Alaska in collaboration with the USFWS Togiak National Wildlife Refuge

(Table 2.2). Due to logistical difficulties that delayed sampling at the Cape Peirce, tissue samples from the mortality event were assessed as carcass code 2 (Geraci and Lounsbury, 1993). In summer 2011, a tongue sample from a walrus harvested near Barrow whose stomach contained parts of two ringed seals was provided by the NSB-DWM. Tissues of subsistence-harvested walruses in Barrow and Wainwright were collected under the authority of permit number MA134907-0 issued to T. Hepa, Director, NSB-DWM. All other walrus samples used in this study were obtained under a letter of authorization to Dr. Horstmann-Dehn through the USFWS.

2.3.1.2 Ice Seals

Ringed seal (*n*=57), bearded seal (*n*=65), and spotted seal (*n*=49) tongue samples were opportunistically collected during the spring/summer 2008, 2009, and 2010 subsistence harvests near Barrow, Hooper Bay, Little Diomede, Point Hope, Savoonga, Gambell and Shishmaref, Alaska. Samples were obtained in collaboration with Alaska Native subsistence hunters in these communities, as well as the NSB-DWM and ADF&G (Table 2.2). Additionally, whole tongues from 10 Steller sea lions (*Eumetopias jubatus*) were provided by the National Oceanographic and Atmospheric Administration (NOAA) from a natural mortality event in 2010 in Southeast Alaska. Steller sea lion samples were collected under permit number 932-1905-00/MA-009526 issued to NOAA Fisheries, Alaska Region (Table 2.2).

2.3.1.3 Arctic Foxes

Arctic fox tongue samples (*n*=32) were opportunistically collected in Barrow and Prudhoe Bay, Alaska and were provided to this study by researchers at the University of Alaska Fairbanks (UAF) from on-going studies of Arctic fox home ranges, diet, and disease (Table 2.2). Some fox samples were moderately decomposed (carcass condition codes of 3, Geraci and Lounsbury, 1993); however, this did not appear to impact the ability to isolate larvae from samples (see Section 2.4.1.2).

2.3.1.4 Polar Bear

An archived sample of polar bear skeletal muscle was obtained through the USFWS under a letter of authorization to the NSB-DWM. The sample was taken from a polar bear harvested for subsistence purposes by Alaska Natives on 10 April 2000. This sample had been archived, frozen at -20 ˚C for over 10 years (Table 2.2).

2.3.2 Digestion Assays

All samples were analyzed at the UAF Marine Mammal Laboratory following the digestion assay protocol for detection of *Trichinella* spp. outlined by Forbes and Gajadhar (1999) and by Leclair et al. (2003). This technique simulates the natural digestive process of the mammalian gastric environment under which the parasite is released into the host's system by using concentrated hydrochloric acid and granulated pepsin to digest muscle and the protective nurse-cell coating, while leaving the larvae intact. Samples were allowed to thaw long enough to subsample, and any remaining

tissue was immediately refrozen at -20 ˚C. Pooled tissue samples (by species) totaling between 25 to 50 g were used (5 animals per pooled test, 10 g tissue samples from each animal when possible, when not possible, due to the size of the tissue sample, a minimum of 5 g was used). Tongues were laid ventral-side up on a clean stainless steel cutting board and bisected laterally. When total tongue muscle sample exceeded 10 g per animal (i.e., Pacific walrus, Steller sea lion, polar bear), parallel incisions, approximately one cm apart and running from the anterior to posterior of the tongue, were made. Mirrored incisions were prepared along both halves of the tongue to prevent missing a localized infection in one half of the tongue. These strips of muscle were then cut free of the tongue and subsectioned into 1-2 cm cubes and placed into a tared clean weigh boat to allow for weight of individual tissue samples or total pooled sample weight to be determined. Tongues from smaller species (where the total tongue muscle sample did not exceed 10 g per animal) were bisected in the manner described above and then trimmed of all fascia, adipose, and epidermal tissue so that only muscle remained prior to subsectioning the sample into 1-2 cm cubes. This allowed for maximum extraction of muscle tissue from the sample. To reduce possible contamination, subsampled tissue was not allowed to come in contact with the cutting surface. Fascia and adipose tissue were removed from subsamples to prevent lipids from clouding the final digestion fluid and impairing microscopic analysis. Each digestion assay was performed as soon as the tissue reached room temperature (to prevent excessive cooling of the digestive fluid during the blending process). Pooled samples not immediately processed were re-frozen at -20 ˚C in individual Ziploc™ bags to retain the integrity of the samples. All sets of samples were

analyzed within 1 week of the initial subsampling process and no tissue samples were allowed to thaw and re-freeze at any other time than during subsampling.

Following the protocol outlined by Forbes and Gajadhar (1999) and by Leclair et al. (2003) each 50 g pooled sample set was pureed in a blender with approximately 350 mL of diluted hydrochloric acid (1% v/v HCl) to which 30 g of granulated pepsin (American Laboratories Incorporated, pepsin 1:10,000 granular) was dissolved (quantities of diluted HCl and pepsin were reduced by half for 25 g samples). The blended mixture was then transferred to a clean 4 L beaker and incubated for 90 minutes at 45 ± 1 °C, while continuously stirred. After incubation, the digestion fluid was strained through a 180 µm metal sieve into a 4 L separatory funnel and diluted with 100 mL of water. The diluted fluid was allowed to settle for 30 minutes, after which approximately 125 mL was drained into a 500 mL separatory funnel and diluted with 375 mL of water. After fluid settled for 10 minutes, approximately 25 mL of sediment was drained onto a gridded (1 cm x 1 cm) petri dish. Particulate matter, including potential larvae, were permitted to settle to the bottom for at least 1 minute to allow for accurate count. After complete screening of the first petri dish, a second petri dish of 25 mL of sediment was poured and screened to reduce the possibility of false negative results due to larvae settling at slower than expected rates. Modification to the artificial digestion technique we used has since been proposed (Gajadhar et al., 2009). These modifications increase the amount digestive fluid per g tissue and alter blender speed, seeking to optimize sample digestion. We encourage implementation of the recommendations detailed in Gajadhar et al. (2009) in future digestion studies, however note that the Leclair et al.

(2003) and Forbes and Gajadhar (1999) method resulted in full digestion of the tissues tested. The double separatory funnel technique used in this study has also been found to be as effective as the method recommended by the European Union (Gajadhar and Forbes 2002).

A Zeiss Stemi 2000 stereomicroscope with 16x magnification was used to examine each grid for the presence of *Trichinella* larvae. An additional ~25 mL of sediment was drained into a second petri dish to ensure a representative count of larval contents in the muscle sample. When *Trichinella* larvae were present, separate digestion assays of 25 g or the whole remaining tissue sample (in the case where the remaining tissue sample was >25 g) were performed on each individual sample to identify the positive sample(s) from each sample pool. To ensure the sensitivity and reliability of the digestion assay technique, known positive samples of polar bear or pooled samples of Arctic fox were utilized for quality control. For every ten assays performed, one known positive sample was assayed. We note that the accuracy of the larval densities we measured could not be determined. Accuracy in the artificial digestion method is quantified by using proficiency samples containing a known number of larvae (Gajadhar et al., 2009). Proficiency samples are provided by a regulatory lab or by having captive, inoculated animals on site to provide the diagnostic lab with constant access to live larvae. We were not able to measure accuracy because experimental infection is beyond our laboratory's capabilities and authorizations, and because any shipment of larvae would have to cross international boundaries (there are no Alaska-based sources for

Trichinella proficiency samples). Shipment of infectious agents into the U.S. requires special permits authorized by the U.S. Center for Disease Control (CDC, 2013).

Larvae were categorized morphologically as "alive/viable" or "dead/non-viable" based on coiling shape and presence or lack of movement. Live *Trichinella* larvae usually maintain a typically tightly coiled position, with uncoiling occurring temporarily during movement (Leclair et al., 2004). This contraction of the body ceases upon larval death, with larvae forming a characteristic "C" shape (Leclair et al., 2004; Davidson et al., 2008, Fig. 2.1).

2.3.3 Multiplex Polymerase Chain Reaction Analysis

Four *Trichinella* larvae were isolated via pipette from the digestion fluid from each positive sample and frozen in phosphate buffered saline (PBS) in Cyrovials™ at -20 ˚C until DNA extraction for genotype analysis using the multiplex PCR method outlined in Pozio and La Rosa (2003) and Zarlenga et al. (1999).

For genotypic analysis individual larvae were placed in 0.5 mL Cyrovials™ with 5 µL of PBS and 2 µL of 1 mM Tris-HCl (pH 7.6). One drop of sterile mineral oil was then added to the sample before placement in a heating block at 90 ˚C for 10 minutes. After cooling on ice, 3 µL of proteinase K solution (QIAGEN) was added, and the sample was centrifuged for 30 seconds at 6000 rpm. The sample was then incubated for 3 hours at 48 ˚C followed by heating to 90 ˚C for 10 minutes and subsequently cooled on ice. Larval DNA extracts were stored at -20 ˚C until PCR amplification.

Each larval DNA extract was slowly thawed atop a tray of ice prior to their addition to the PCR mixture (to prevent excessive warming, and thus degradation of the sample). Final PCR reagent concentrations were: 1X GoTaq® PCR buffer (Promega), 0.8 µM dNTPs, 2 mM $MgCl_2$, 0.4 uM of each primer (10µM, see below), 0.01 µ/L of *Taq* DNA polymerase (GoTaq® DNA Polymerase, Promega). Reaction templates were 4 µL of crude larval DNA extract, Reactions were performed in 50 µL volumes in 0.2 mL thin-walled reaction vials. Vials were kept on ice during reaction assembly. Samples were next incubated at 94 ˚C for five minutes, followed by 35 cycles at 94 ˚C for 20 seconds, 58 ˚C for 30 seconds, and 72 ˚C for one minute, followed by an extension cycle at 72 ˚C for four minutes as described by Pozio and La Rosa (2003). Primers (Integrated DNA Technologies, Inc.) used in the above multiplex PCR analysis were cp –IF, cp –IR – II.F cp, cp- II.R, cp- III.F, cp- III.R, cp- IV.F, cp- IV.R, and cp cp –VF –VR. Following thermal cycling, the size of PCR products was determined by polyacrylamide gel electrophoresis on 10 % polyacrylamide gel (9.5 × 10 cm, 1-mm thick*)* in TBE buffer. PCR product sizes were determined by comparison with a standard 100-base pair (bp) DNA ladder (50 µg/mL, "Quick-Load 100 bp DNA Ladder", New England BioLabs). Electrophoresis conditions were constant 110 volts for 30 minutes. Gels were stained by immersion in ethidium bromide (0.5 µg/mL) and photographed under UV light. PCR product lengths were compared with those reported by Pozio and La Rosa (2003) to determine larval genotype.

2.4 Results

2.4.1 Digestion Assays

Out of a total of 380 individuals tested for *Trichinella* spp. representing seven different species, two marine mammal species were positive: ringed seal (*1/57*) and polar bear (*1/1*), both originating from Barrow, Alaska. All samples from Pacific walruses (*n*=137), bearded seals (*n*=65), spotted seals (*n*=49), unidentified ice seals (*n*=7), and Steller sea lions (*n*=10) tested negative for presence of the parasite (0% incidence, Table 2.3). Multiple Arctic fox specimens from Barrow and Prudhoe Bay were positive (for which resolution to individual level was not possible to the small size limitations of available tissue samples, Table 2.3). It is possible that these data may underestimate infectivity, as dead (i.e., uncoiled) larvae likely have longer sedimentation times than live (coiled) larvae due to higher drag coefficients and slower sinking speeds (Okubo, 1987). A decrease in the abundance of larvae in the second petri dish compared to petri dishes containing the initial sediment, and lack of test results in which only the second petri dish contained larvae, suggests that the possibility of false negatives is low.

2.4.1.1 Ringed Seals

One ringed seal (1/57) tested positive for *Trichinella* (0.2 larvae per gram (lpg), Table 2.3). This larval load is likely an underestimation of the infection in this individual, due to the small quantity of tissue available (25 g), which required digestion of the entire remaining sample, including dermal layers, to ensure all possible muscle was digested. *Trichinella* incidence in all analyzed ringed seal samples was 1.8% (Table 2.3). All larvae

within the ringed seal sample were determined to be dead and non-infective based on comma-shaped morphology and lack of larval movement.

2.4.1.2 Arctic Foxes

Three of eight pooled Arctic fox samples (five animals per pool) tested positive for *Trichinella*. Due to the small quantity of tissue available for processing, individual assays were only possible on four of 15 potentially positive tongues from these pools. All samples large enough for individual testing were negative, while seven samples were too small for assaying independently and had to be pooled. A minimum of three foxes infected and a maximum of seven individuals infected out of 32 total fox samples was evident, resulting in an incidence of 9.4-21.9% (Table 2.3). Due to the high parasite load in the positive pool of Arctic fox samples (>50 lpg), quantification of live and dead larvae was not possible. The majority of larvae were motile and tightly coiled despite the mild to moderate decomposition level of most fox tissue samples.

2.4.1.3 Polar Bear

Larval density in the polar bear sample was 3.6 lpg, a moderate to low infection based on previous accounts (0.4-33 lpg, Table 2.1), (Rausch et al., 1956; Born and Henriksen, 1990). Based on morphology, survival of larvae in the polar bear tissue sample stored for ten years at -20 ˚C was 98% (87 of 89 total larvae isolated). A subsequent digestion assay of the subsampled tissue from the same polar bear muscle frozen at -80˚C further indicated that *Trichinella* may be capable of surviving -80 ˚C conditions for a minimum of 7 days, with larval survival of 32% (17 of 53 larvae, Table

2.3, Fig. 2.1). However, while the majority of larvae maintained the characteristic coiled shape, none were observed to move or alter conformation, even after incubation at 45 °C for 10, 15, 30 and 45 minutes. Lack of movement suggests that the larvae were non-viable, despite holding a coiled shape. In addition, larvae were observed to be transparent, an indicator of mortality (Leclair et al., 2004).

2.4.2 Multiplex PCR-based Species Identification

Larval DNA extracts from individual mammal specimens (*n*=4 from each infected species), i.e., polar bear, Arctic fox, and ringed seal, were identified as *Trichinella nativa* based on a comparison with previously reported PCR results (Pozio and La Rosa, 2003). Species determinations were made by comparing the size of PCR fragments produced in this study with those reported from multiple species of *Trichinella.* All larval DNA isolated in this study yielded a PCR fragment at approximately 127 bp from expansion segment V of the ribosomal RNA gene, a pattern of amplification from these primers that is unique to *T. nativa* (Fig. 2.2a, b).

2.5 Discussion

2.5.1 Digestion Assays

In this study, the double separatory funnel method was accurate at detecting *T. nativa* infections as low as 0.2 lpg. The previously proven sensitivity (≥ 0.3 lpg) of this technique and successful isolation of larvae from quality control samples in our laboratory suggests that the likelihood of not detecting an infection is minimal under

ideal conditions (i.e., 150 g or more of each sample and tissue available for analysis and frozen at -20 °C immediately after death, Leclair et al., 2003). For some individuals (i.e., Arctic fox, ringed seal, bearded seal, spotted seal), size of the sample was limited by the size of the individual and/or the nature of opportunistic sample collection, resulting in less than 10 g available for pooled and/or individual testing. In the case of these samples, the probability of an infection remaining undetected increases with decreasing mass of the sample. However, our positive results from both Arctic fox and ringed seal analyses indicate that detection of low level infections is still possible using the digestion assay technique under non-ideal conditions (i.e., tissue samples <10 g), and provides reliable results at least to the level of identifying infection, although parasite load may not be quantifiable.

All Pacific walruses tested negative for *Trichinella*, including the known seal-eating individual. All samples were of ideal volume for analysis, thus it is unlikely that the quantity of tissue sampled resulted in false negatives. All samples from animals harvested for subsistence were frozen shortly after harvest. Samples from the Cape Peirce mortality event were sampled in the field two weeks after the death of the animals due to difficult logistics in accessing the remote area where the event occurred. Sub-zero (Celcius) temperatures at Cape Peirce during this time period may have helped preserve tissue samples via freezing of the carcasses. While the amount of tissue decomposition (carcass code 2, Geraci and Lounsbury, 1993) that occurred may have resulted in a decreased effectiveness in the digestive assay's ability to isolate larvae, *Trichinella* larvae

are known to survive in naturally decaying muscle tissue for up to 6 weeks during winter months (Riva et al., 2012).

The mechanism of parasite transmission in the marine environment remains elusive. We observed a similar or lower incidence of *Trichinella* in Pacific walruses than previously documented (Table 2.1), despite the use of more accurate detection techniques. The highest recorded infection incidence for walruses (40%) was reported from the Atlantic walrus population (*Odobenus rosmarus rosmarus*, Gajadhar and Forbes, 2010). Predation incidence on seals by Atlantic walruses is thought to be higher than in Pacific walruses (Lowry and Fay, 1984; Muir et al., 1995; Wolkers et al., 2006). Comparatively lower infection incidence in Pacific walruses versus Atlantic walruses may be expected if walruses contract the parasite through the consumption of infected seal tissue. However, our results and previously published accounts do not indicate that infection incidence of *T. nativa* in seal populations is high enough to support the historical incidence found in Pacific and Atlantic walruses or the high incidence in polar bears (~78%, Table 2.1, Åsbakk et al., 2010).

Our study suggests a higher *Trichinella* incidence in ringed seals (1.8%) than the 0.7% previously reported by Dau and Barret (1981; Table 2.1). It is important to note that these incidence data are the result of two positive animals from Dau and Barret's study and one positive animal in this study. The very small number of infected individuals may have skewed incidence calculations to create the impression of increase in *Trichinella* incidence over time, when in fact rates of infection in the species may not have changed.

Ringed seals are a major prey species for polar bears and occasional prey of walruses (Lowry and Fay, 1984; Fay et al., 1990). *Trichinella* infection does occur in ringed seals (Table 2.1; Forbes, 2000), and the consumption of ringed seals may lead to infection in walruses. Higher prevalence of *Trichinella* in Atlantic walruses (up to 40% in *O. rosmarus rosmarus*, compared to 2.0 % in *O. rosmarus divergens*) and greater frequency of predation on seals as well as cannibalism in the Atlantic stock does suggest a correlation between seal-eating and infection with *Trichinella* in walruses (Fay, 1960; Kozlov, 1968; Pozio, 2001; Gajadhar and Forbes, 2010; also Table 2.1). Further dietary studies quantifying the contribution of ringed seals to the diet of Pacific walruses are needed to confirm a correlation between seal-eating and infection with *Trichinella* in walruses.

The source of *Trichinella* in Holarctic ringed seal populations remains unknown and is beyond the scope of this study. However, ringed seals prey on a variety of pelagic fishes as well as pelagic and benthic crustaceans (Lowry et al., 1980a; Dehn et al., 2007), which could serve as transmission vectors. There are conflicting results as to whether Arctic marine amphipods may serve as transmission routes (Fay 1968, Forbes 2000). The low incidence of *Trichinella* infection in bearded seals (0.7-3.0% from previous accounts, 0% in this study, Table 2.1; Forbes, 2000) suggests that benthic amphipods are not a good vector for *Trichinella* transmission, if they are a vector at all. Bearded seals are primarily benthic feeders, foraging on crustaceans, mollusks, and benthic fishes (Lowry et al., 1980b; Dehn et al., 2007). Due to the nature of *Trichinella* transmission (primarily via

consumption of infected tissue) and overlap in ringed and bearded seal habitat, it is likely that both phocids could contract the parasite from a similar (unknown) vector or vectors.

Trichinella transmission route through ingestion of fecal matter from an infected animal has been suggested to be a viable pathway based on laboratory experimentation in which extremely large dosages of *Trichinella* larvae were administered, however such levels of infection would be unlikely to occur naturally (Zimmerman et al., 1959; Robinson and Olson, 1960). Additionally, larvae remained alive within the feces for only short periods of time, with the majority of parasites shed in fecal matter during the first 24 hours post-infection (Zimmerman et al., 1959). It is possible, however, that transmission could occur in high density areas, such as the terrestrial haulouts used by Pacific walruses, where there is a higher likelihood of physical contact with fresh infected feces due to relatively high densities of animals in prolonged contact with each other compared to sea ice haulouts (Jay et al., 2012).

Spotted seals did not harbor *Trichinella* in this study and no prior research has identified this species as a host. Spotted seals prey mainly on mid-water column fishes such as herring (*Clupea pallasii*), but may consume cephalopods and occasionally crustaceans (Dehn et al., 2007; Carroll 2012). As with other pagophilic phocids, spotted seals are an uncommon prey item of Pacific walruses (Lowry and Fay, 1984; Sheffield et al., 2001; Sheffield and Grebmeier, 2009). All ice seal samples not identified to species tested negative for *Trichinella*, providing further evidence of low incidence of *Trichinella* in ice-associated pinnipeds within the Alaska Arctic marine ecosystem.

Samples from Steller sea lions tested negative for infection with *Trichinella*. Infection status in this species has not been previously documented. The general southerly distribution of Steller sea lions in comparison to the more northerly distribution of known Arctic *Trichinella* hosts suggests that risk of infection with *T. nativa* is currently low via direct exposure to the parasite. However, this 45richino species is occasionally observed in the Chukchi Sea in the summer months (Burns, 1970; Allen and Angliss, 2013) and overlaps with bearded and ringed seals as well as walruses in the northern Bering Sea/Bering Strait region into late fall (December) (Burns, 1970; Allen and Angliss, 2013), and thus cross-species transmission should be considered. Interestingly, *Trichinella* has not been reported in marine mammals such as California sea lions (*Zalophus californianus*) and harbor seals (*Phoca vitulina*) inhabiting temperate regions of the Pacific Ocean. The causative agent for *Trichinella* infection in temperate-region marine mammal species would most likely be *T. spiralis* and could transpire from utilization of coastal or nearshore habitat in close proximity to agricultural areas where run-off from livestock operations occurs, as *T. spiralis* is a commonly associated with pork. However, infection in U.S. commercially produced pork is low (Kennedy et al., 2009).

It has been hypothesized that the transmission cycle of *Trichinella* to Arctic foxes and polar bears is linked and separate from the strictly marine environment (Forbes, 2000). Periods of decline in *Trichinella* infection incidence in polar bear populations coincide with declines of the parasite in Arctic fox populations occupying the same region (Forbes, 2000). Results of our analyses indicate a *Trichinella* incidence of 9.4-

21.9% in Alaska Arctic fox populations (Table 2.3). The lower end of this range of estimates is consistent with those previously reported (Table 2.1). Arctic foxes are opportunistic predators and may scavenge on marine mammal carcasses or actively prey on ringed seal pups when available (Roth, 2003; Smith, 1976), suggesting a possible link between infection in Arctic foxes and sources/hosts within the marine food web. However, Arctic foxes also prey upon rodents. Previous research has also shown that rodents are capable of harboring *Trichinella* (Robinson and Olsen, 1960); however, little research has addressed *Trichinella* incidence in Alaska rodents. Rausch et al. (1956) found the parasite present in eight rodent species during a comprehensive survey of *Trichinella* in 2,433 species of Alaska mammals: ground squirrel (*Citellus undulates*), red squirrel (*Tamiascuirus hudsonicus*), brown lemming (*Lemmus sibiricus trimucronatus*), red-backed vole (*Clethrionomys rutilus dawsoni*), narrow-skulled vole (*Microtus iniurus muriei*), muskrat (*Ondatra zibethica*), and beaver (*Castor canadensis*). It is also known that larvae may be transmitted via intermediate insect hosts feeding on the remains or feces of infected individuals (Holliman and Meade, 1980). Whether Arctic insects can perform a similar role in this transmission cycle has not been established. In addition, as discussed previously, fresh feces may play a role in non-marine transmission cycles affecting both foxes and polar bears (Zimmerman et al., 1959; Robinson and Olsen, 1960). Survival time of the cold resistant *T. nativa* in fecal matter is unknown and longer survival times outside a host may be possible. Arctic foxes and polar bears are known to opportunistically consume rodent species such as lemmings (*Dicrostonyx* spp.) and may contract the parasite through this route or by scavenging discarded meat near human

settlements (Stempniewicz, 1993). Further research is underway to assess potential correlations between *Trichinella* infection in Arctic foxes and proportional contribution of marine-based prey items via bulk stable isotope analysis (Lehner et al., unpublished). Cyclical population growth of lemmings may explain the similar fluctuations in *Trichinella* incidence among Arctic fox and polar bear populations, if infection in rodents is positively correlated with high lemming population numbers. Alternately, it is thought that transmission mechanisms in polar bears may involve a cycle separate from pinnipeds and foxes: bear-to-bear transmission (Born and Hendriksen, 1990). Consumption of polar bear carcasses is hypothesized to be a source of the parasite in Arctic foxes, and positive correlations among infected bears and infection in Arctic fox populations have been recorded (Larsen and Kjos-Hanssen, 1983). *T. nativa* has also been isolated from red foxes in a variety of countries, including Norway (Davidson et al., 2006). As a species with wide-spread distribution, red fox may serve as a transition vector between Arctic and more temperate climates.

Our one positive polar bear sample is supported by the high prevalence of *Trichinella* in polar bears of up to 78% (Åsbakk et al., 2010, Table 2.1). Our study has demonstrated that, under certain environmental conditions, *Trichinella* can be detected in tissues long after the death of the host, providing another example of *Trichinella* detection from an aged tissue (Tryde 1952). Analysis may provide insight into the incidence of this parasite in Arctic wildlife over long time periods and potential correlations with external environmental factors such as temperature, sea ice extent and quality, and other short-term climatic patterns, potentially allowing for cautious

predictions of future incidence. Recurring outbreaks of various diseases (e.g., rabies, lungworm, *Echinococcus multilocularis*) associated with cyclic prey abundance and population densities are well known in the Arctic (Rausch, 1972; Kutz et al., 2005). Understanding past and future occurrence of *Trichinella*, particularly when considering rapid climate change and changing food web assemblages in the Arctic and potentially new routes of exposure, is especially useful for species of subsistence and cultural importance.

2.5.2 Assessment of Viability

The ability of *T. nativa* to remain viable for years under freezing conditions increases potential threat to human health due to the ingestion of frozen meats (particularly among Arctic Native cultures, where traditional foods are sometimes served uncooked, Kapel et al., 1999). Our study indicates the need for a consistent protocol for ascertaining viability and infectivity of *Trichinella*. The larvae isolated in this project were assessed as potentially viable based on the traditional method of morphology (coiled or "C"-shaped, Fig. 2.1), although not confirmed by motility. Further indications of viability and infectivity would require experimental inoculation and were beyond the scope of this study. The inconclusive nature of viability tests on larvae isolated from the polar bear skeletal muscle (coiled, but non-motile) is a reminder that the use of morphology alone to identify live larvae may results in erroneous estimations, as suggested by Davidson et al. (2008). The ability to extract larvae from samples stored frozen for an extended period, as well as successful extraction of DNA and identification

of these larvae, allows for future research and temporal trend analyses of parasite incidence in the Arctic to consider samples that may have otherwise been excluded from examination.

2.5.3 Multiplex PCR-based Species Identification

Confirmation of *T. nativa* in Alaska marine and marine-associated mammals is consistent with previous findings by Pozio and La Rosa (2003), indicating this to be the dominant species of *Trichinella* within Arctic ecosystems. We acknowledge that the lack of detection of other *Trichinella* species in Alaska does not confirm their absence. As previously discussed, it is possible that the sedimentation time was not sufficient to allow dead larvae to settle into the digestion sediment, potentially resulting in false negatives, particularly of non-freeze-tolerant species killed by the freezing of samples prior to analysis. As our analysis did examine multiple, but not all, individual larvae from each tissue sample, it is possible that mixed infections or other species exist in Alaska and were missed in this study. However, the only other *Trichinella* species (*Trichinella* T6, the only other highly freeze-tolerant *Trichinella* spp*.,* Pozio et al., 2009) documented in Alaska since the differentiation of the genus into multiple species, occurs as far west as the Rocky Mountain range, with distribution along this region ranging from Alaska to Idaho (Pozio et al., 2009) (prior to the differentiation, all *Trichinella* larvae were identified as *T. spiralis,* Rausch et al., 1956). This makes mixed infections with both *T. nativa* and T6 unlikely in the animals included in our analysis (Pozio and La Rosa, 2003). To completely rule out the occurrence of mixed infections or the presence of other

Trichinella species in Alaska, an extensive genotypic study assessing all larvae within each positive sample would be recommended. Studies using frozen tissues should also consider extending the sedimentation time to allow larvae of non-freeze tolerant species to settle into the sediment.

While *T. nativa* is known to be extremely freeze-tolerant, the impacts of a warming Arctic climate on the distribution and success of this parasite are unknown (Davidson et al., 2008; Polley et al., 2010). Incidence of *Trichinella* in both marine and marine-associated species will be dependent on both the parasite and host species' abilities to acclimatize to environmental changes. Anticipated and documented shifts associated with climate change include use of novel habitat, altered host population densities, introduction of novel diseases, and alterations in feeding ecology (Kutz et al., 2005; Fischbach et al., 2007; Burek et al., 2008; Garlich-Miller et al., 2011). Impacts resulting in poor host body condition from declines in food quantity or quality may make animals more susceptible to *Trichinella* or other parasitic or secondary infections (Beldomenico et al., 2008).

2.6 Conclusion

The ability to assess the public health threat of *Trichinella* will depend on monitoring incidence of infection in host species to determine trends associated with environmental change. To monitor *Trichinella* in marine-associated systems, a better understanding of the organism and its transmission cycle is required. The development and optimization of reliable and highly sensitive diagnostic and analytical tools provides

the opportunity to assess the incidence of *Trichinella* in Alaska mammals and ultimately determine the appropriate degree of concern to public health. While previous studies have provided some documentation, it has been approximately 50 years since a multi-species survey has been undertaken examining incidence in Alaska marine mammals and no survey to date has utilized the digestion assay technique developed by Forbes and Gajadhar (1999) and Leclair et al. (2003) for use in wildlife.

Our study reconfirms that larvae can be isolated and genotyped from decade-old tissues. Thus, the analysis of samples archived for up to 10 years (maybe longer) is encouraged to provide temporal estimates of *Trichinella* incidence. This opportunity underlines the importance of archival tissue banks, such as the now-defunct Alaska Marine Mammal Tissue Archival Project and the few currently maintained by the National Institute of Standards.

The transmission mechanism of *Trichinella* in the marine environment remains elusive and further exploration into this topic is needed. It is highly recommended that on-going collaborative efforts assessing Alaska marine mammal population health include diagnostic evaluation for *Trichinella* via digestion assay of muscle samples. This method may be more easily executed in rural Alaskan communities than ELISAs as the technique is robust, simple to execute, and requires less specialized equipment. This in turn reduces costs of laboratory set up and testing, and increases the likelihood that community members will be able to take leading roles in sampling local foods with little formalized training. Additionally, continued monitoring through collaborative sample

collection between subsistence communities and researchers is encouraged to provide current information on infection incidence.

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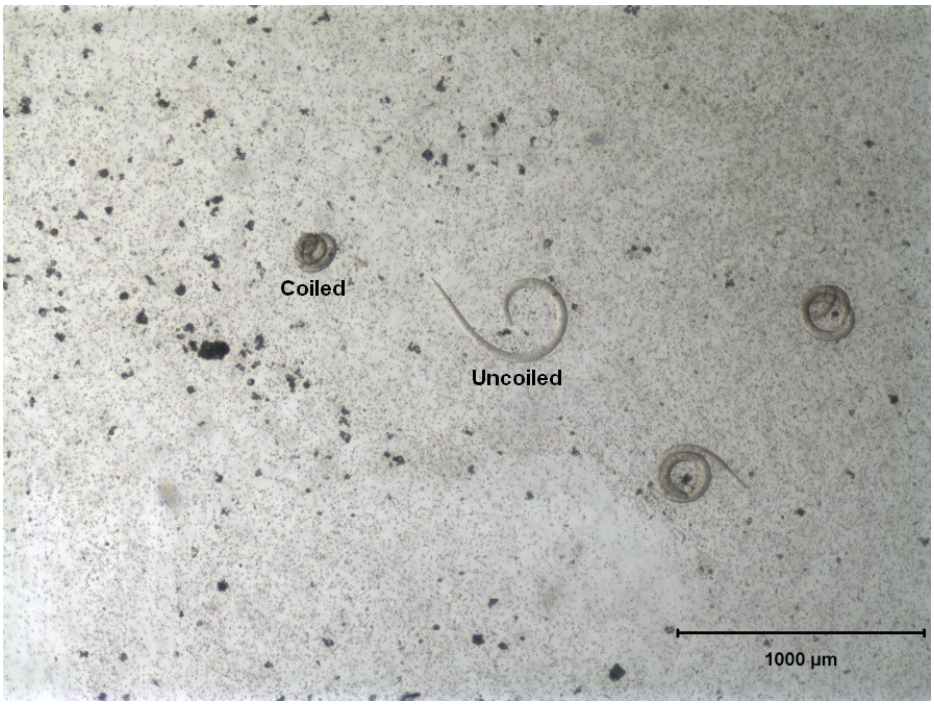
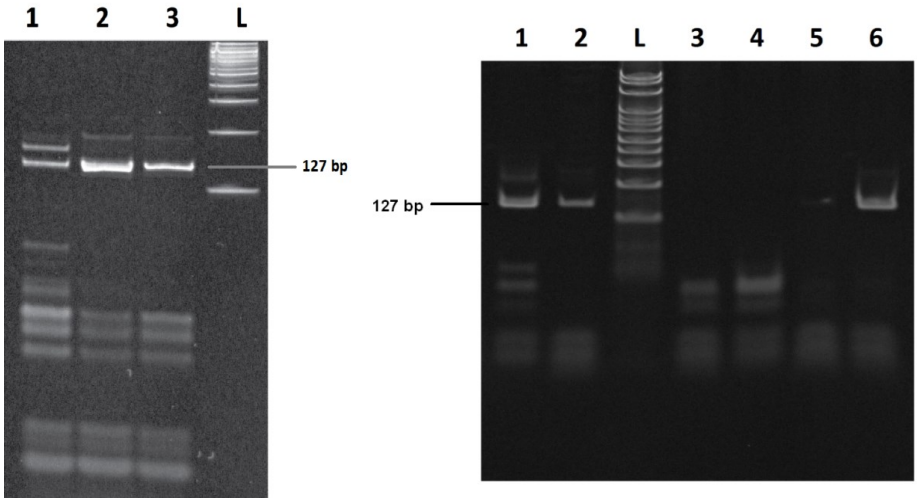


Fig. 2.1 *T. nativa* larvae isolated from decade-old polar bear muscle

T. nativa larvae isolated from decade-old polar bear muscle tissue stored at -20 °C demonstrating both "coiled" and "C-shaped" morphological categories historically hypothesized to be indicative of live and dead larvae, respectively.



A) B)

Fig. 2.2 Ethidium bromide stained polyacrylamide gels under ultraviolet light

Ethidium bromide stained polyacrylamide gels under ultraviolet (UV) light. A) Lanes 1, 2, and 3 represent DNA extracts from individual *Trichinella* larvae isolated from Alaska Arctic fox muscle. B) Lanes 1 and 2 represent DNA extracts from individual *Trichinella* larvae isolated from ringed seal muscle. Lane 3 is a standard blank (primers only). Lane 4, 5, and 6 show extracts from individual larvae isolated from a 10-year-old sample of polar bear muscle.

Note - PCR amplification was unsuccessful in lanes 4 and 5. Additional PCR analyses of larvae extracted from the polar bear tissue in which all DNA amplifications were successful (n=10) indicate that lack of amplification was most likely due to the degraded condition of the larvae tested in those lanes. DNA ladder 100 (L) shows sizes of base pairs (bp). All isolates are *T. nativa* as indicated by the presence of PCR fragments at approximately 127.

Table 2.1 Historic incidence of *Trichinella* spp. in circumpolar Arctic wildlife species addressed in this study*

Host Species		Year*	Location	Prevalence† (*n*)	Source
Common Name	Scientific Name				
Bearded seal	*Erignathus barbatus*	1949	Greenland	3% (*28*)	Roth, 1949
		1949-1953	Arctic coast, AK	0.8% (*148*)	Rausch et al., 1956, Rausch unpublished *in* Fay, 1960
		1953	Greenland	0.8% (*243*)	Roth and Madsen, 1953
		1961	Greenland	0.8% (*242*)	Madsen, 1961
		1981	Arctic coast, AK	0.7% (*148*)	Dau and Barrett, 1981
		2008-2010	Various, AK	0% (*65*)	This study
Ringed seal	*Pusa hispida*	1950	Greenland	1.9% (*52*)	Roth, 1949
		1953	Greenland	0.06% (*1561*)	Roth and Madsen, 1953
		1961	Greenland	0.1% (*1775*)	Madsen, 1961
		1981	Arctic coast, AK	0.7% (*300*)	Dau and Barrett, 1981
		2008-2010	Various, AK	1.8% (*57*)	This study
Unidentified seals		1949-1953	Arctic coast, AK	0.6% (*310*)	Rausch et al., 1956
		1961	Greenland	0.1% (*1657*)	Madsen, 1961
		1962	Arctic coast, Russia	2.4% (*210*)	Britov, 1962
		2008-2010	Various, AK	0% (*7*)	This study
Atlantic Walrus	*Odobenus rosmarus rosmarus*	1950	Greenland	0.9% (*207*)	Roth, 1949
		1950	Greenland	0.2% (*481*)	Roth and Madsen, 1953
		1954	Eastern Arctic	4.3% (*394*)	Kuitunen, 1954
		1954	Eastern Arctic, Canada	1.0% (401)	Brown et al., 1949, Kuitunen, 1954 *in* Fay, 1960‡
		1956	Barents Sea, Greenland Sea	9.5% (*74*)	Thorshaug and Rosted, 1956
		1961	Greenland	1.0% (*245*)	Madsen, 1961
		1982	Greenland	1.6% (*126*)	Born et al., 1982
		1998	Eastern Arctic	2.6% (*156*)	Makivik Magazine, 1998
		2000-2010	Various, Canada	40% (*32*)	Gajadhar and Forbes, 2010

Table 2.1, Continued

Host Species		Year*	Location	Prevalence†	Source
Common Name	Common Name			(*n*)	
Pacific Walrus	*Odobenus rosmarus divergens*	1960	Arctic coast, AK	0.9% (*53*)	Fay, 1960
		1965	Chukotka Peninsula, Russia	2.0% (*50*)	Kozlov, 1968
		2006	Chukotka Peninsula, Russia	1.5% (*138*)	Bukina and Kolevatova, 2007
		2009-2011	Various, AK	0% (*137*)	This study
Arctic fox	*Vulpes lagopus*	1949	Greenland	2.8% (*101*)	Roth, 1949
		1949	Alaska	100% (*1*)	Rausch *in* Connell, 1949
		1949-1953	Various, AK	7.2% (*222*)	Rausch et al., 1956
		1954-1980	Svalbard	3-67% (*77*)	Larsen and Kjos-Hanssen, 1983
		1963	Arctic coast, Russia	2.9% (*270*)	Lukashenko and Brzheskij, 1963 *in* Lukashenko et al., 1971
		1980-1986	Canadian Arctic	2.7% (*1567*)	Smith and Snowdon, 1988
		1983-1989	Svalbard	8.5% (*697*)	Prestrud et al., 1993
		1992-1993	Greenland/ Svalbard	7.5% (*319*)	Kapel et al., 1999
		2000-2010	Various, Canada	10.7% (*28*)	Gajadhar and Forbes, 2010
		2009-2010	Barrow and Prudhoe Bay, AK	9.4-21.9 % (*32*)	This study
Polar bear	*Ursus maritimus*	1949	Greenland	31.6% (*19*)	Roth, 1949
		1949	Spitsbergen	87.5% (*8*)	Aaser *in* Connell, 1949
		1949	Various, AK	66.7% (*3*)	Rausch *in* Connell, 1949
		1949	Northwest Territories, Canada	67% (*3*)	Brown et al., 1949, Brown, 1949
		1950-1970	Svalbard	23-58% (*376*)	Larsen and Kjos-Hanssen, 1983
		1953	Greenland	23% (*247*)	Roth and Madsen, 1953
		1956	Various, AK	55% (*104*)	Rausch et al., 1956, Rausch unpublished *in* Fay, 1960
		2000	Barrow, AK	100% (*1)*	This study

Table 2.1, Continued

Host Species		Year*	Location	Prevalence† (*n*)	Source
Common Name	Common Name				
Polar bear	*Ursus maritimus*	1991-2008	Svalbard	51-78% (*500*)	Åsbakk et al., 2010
		2000-2010	Various, Canada	65.9% (*85*)	Gajadhar and Forbes, 2010

Note - Until the late 20th century incidence was determined via trichinoscopy, a technique generally less accurate than the method used in this study. Therefore, incidence may have been underestimated in older reports. Only studies with positive results are included.

* Only positive findings are presented (excepting this study). An exhaustive review of *Trichinella* surveys of marine mammals is presented by Forbes (2000). Since publication of Forbes (2000) no surveys of marine mammals have produced negative results. Likewise, all studies of *Trichinella* in Arctic foxes have resulted in positive findings

† Year is reported as the year(s) samples were collected unless sample collection year is unknown. Otherwise, the year of publication is noted in lieu of collection year to provide an approximate time frame for historical incidence

‡Incidence is reported to the greatest significant digit provided by each published report

!Report includes animals from Kuitunen, 1954.

Table 2.2 Species, sampling year, location, and sample sizes for tissue specimens

Species	Sampling Year	Location	*n*
Bearded Seal	2009	Barrow	*24*
		Hooper Bay	*1*
		Little Diomede	*6*
		Point Hope	*1*
	2010	Barrow	*33*
Ringed Seal	2008	Hooper Bay	*1*
		Savoonga	*1*
		Shishmaref	*13*
	2009	Gambell	*3*
		Hooper Bay	*20*
		Little Diomede	*2*
		Savoonga	*4*
	2010	Barrow	*13*
Spotted Seal	2008	Gambell	*1*
		Shishmaref	*20*
	2009	Barrow	*1*
		Gambell	*10*
		Shishmaref	*16*
		Unknown	*1*
	2010	Barrow	*1*
Unknown Ice Seal	2008	Gambell	*1*
		Nome	*1*
		Savoonga	*1*
		Shishmaref	*3*
	2009	Little Diomede	*1*
Steller Sea Lion	2010	SE Alaska	*10*
Pacific Walrus	2005	Little Diomede	*1*
	2009	Barrow	*3*
		Cape Peirce	*5*
		Gambell	*16*
		Little Diomede	*1*
		Point Hope	*1*
		Savoonga	*32*
		Wainwright	*2*

Table 2.2, continued

Species	Sampling Year	Location	*n*
Pacific Walrus	2010	Barrow	*2*
		Gambell	*40*
		Savoonga	*33*
Pacific Walrus	2011	Barrow	*1*
Arctic Fox	2010	Barrow	*22*
		Prudhoe Bay	*10*
Polar Bear	2000	Barrow	*1*

Table 2.3 *Trichinella* prevalence and infection severity

Species	Prevalence	Infection Severity (lpg)
Bearded Seal	0/65	-
Ringed Seal	1/57	0.2
Spotted Seal	0/49	-
Unknown Ice Seal	0/7	-
Steller Sea Lion	0/10	-
Pacific Walrus	0/137	-
Arctic Fox	3 to 7/32	>50
Polar Bear	1/1	3.56

Trichinella prevalence and infection severity (in larvae per gram muscle tissue, lpg) for each species analyzed in this study.

Note - Arctic fox data are given as a range due to insufficient tissue sample size to perform individual confirmation assays. Arctic fox infection was too great for quantification, but was higher than 50 lpg.

Chapter 3: Proportions of Higher Trophic Level Prey in the Diet of Pacific Walruses (*Odobenus rosmarus divergens*)[2]

3.1 Abstract

During nutritionally stressful situations, Pacific walruses (*Odobenus rosmarus divergens*) may switch from preying on benthic invertebrates to higher trophic level prey (HTLP) (e.g., pinnipeds and/or seabirds). We applied a Bayesian mixing model to stable isotope (C and N) data from analyses of various tissues (tongue and lumbar muscle, skin, and liver) to quantify the proportional contribution of HTLP to walruses (n=293 individuals). The mode contribution of HTLP to walrus diet was ~22% (± 10%) based on muscle mixing models, which is consistent with results from contaminant studies of Atlantic walruses (*Odobenus rosmarus rosmarus*), but higher than estimates based on historical stomach content analyses of Pacific walruses. A broader range in the proportion of HTLP (0-60%) shown by mixing models using stable isotope data from liver and skin of walruses indicated they pursue an opportunistic foraging strategy. Data from the HTLP-consuming walruses were comparable with our stable isotope data of a known "seal-eating" walrus. No significant difference was evident between the estimated contributions of HTLP to the diet of male *vs*. female walruses (P>0.01). This finding suggests that changes in diet base for walruses are not influenced by the sex of the predator.

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Keywords: walrus, isotopes, pinnipeds, eiders, mixing model

3.2 Introduction

Environmental changes can alter the abundance, distribution, size, digestibility, and energetic content of prey (Barboza et al. 2009). Prey-switching is a common phenomenon of organisms in response to external factors, including declines in prey abundance and increases in predator populations (Bowen et al. 2006; Beaulieu et al. 2009). When preferred prey decline in density or abundance, the energetic cost of foraging and prey handling may increase, and these costs may eventually outweigh the predator's energy gains (Rosen et al. 2007; Barboza et al. 2009). Increased reliance on alternate prey species can mitigate the loss of caloric energy from declines in availability of traditional prey, allowing predators to persist and traditional prey populations to recover.

The changing environment in the Arctic may be prompting Pacific walruses (*Odobenus rosmarus divergens*) toward adopting alternative foraging strategies (Rausch et al. 2007; Jay and Fischbach 2008). Such alterations may take the form of direct changes (e.g., altered diet), or indirect changes in the form of differences in food web dynamics, decreased biomass input to the benthos, and decreases in prey quality (Grebmeier et al. 2006; Grebmeier 2012; Wang et al. 2013). Sea ice, the diving platform that provides walruses with energetically efficient access to benthic invertebrate prey (Fay 1982), has recently displayed unprecedented annual declines (National Snow and Ice Data Center, 2012; Stroeve et al. 2012). As the Arctic sea ice retreats to deeper waters, walruses are left without ready access to benthic foraging grounds and may need

to adopt alternate strategies to obtain prey, as they are not physiologically adapted to deep diving (Fay 1982). Dietary studies suggest that walruses have increased reliance on high trophic level prey in recent years, possibly as a result of environmental changes (Seymour et al. 2014).

Walruses are considered benthic specialist predators using highly adapted facial musculature to obtain invertebrate prey from sediments and extract bivalves from their shells, with numerous other taxa commonly utilized (Fay 1982; Sheffield et al. 2001; Sheffield and Grebmeier 2009). However, walruses may feed on other available resources, and seal predation or carcass scavenging by walruses are not novel foraging strategies (Fay 1960; Fay et al. 1977; Lowry and Fay 1984; Muir et al. 1995; Sheffield et al. 2001; Mallory et al. 2004; Wolkers et al. 2006; Fox et al. 2010). Fay (1960) suggested that in nutritionally stressful situations or during unfavorable sea ice conditions walruses may prey on seals or birds. Further, increased use of centralized terrestrial haulouts, such as the thousands of walruses hauling out near Point Lay, Alaska in past years (Jay et al. 2012), may lead to localized invertebrate prey depletion and/or create energetically costly increases in travel distances to foraging grounds. This may then result in opportunistic foraging on higher trophic level prey (HTLP), such as pinnipeds and seabirds. Additionally, recent *in situ* observations suggest active predation by Pacific walruses on spectacled eiders (*Somateria fischeri*, Lovvorn et al. 2010). Marine mammal trophic level prey shifts may have unforeseen energetic consequences, potentially resulting in declining body condition and fecundity, increased disease susceptibility, decreased offspring survival, and changes in contaminant exposure; all of which can lead to

population declines (Kutz et al. 2005; Fischbach et al. 2007; Burek et al. 2008; Garlich-Miller et al. 2011). The degree to which walruses rely on HTLP is not well understood as walrus dietary studies have historically relied on stomach content analysis, a method which is biased towards hard-bodied organisms (Pierce et al. 2004). Walruses preferentially ingest the soft tissues of seals and birds (Lowry and Fay 1984), and stomach content analysis may therefore underestimate the contribution of HTLP to diet of walruses (although the keratin-rich composition of portions of epidermal tissue result in longer digestion times for seal hide, Sheffield et al. 2001).

Analyses of stable nitrogen isotope ratios ($^{15}N/^{14}N$) can be used to assess trophic position of an organism relative to its prey (Kelly 2000). A stepwise enrichment of 3-5‰ per trophic level is generally assumed for marine mammals (Hobson et al. 1996). Stable carbon isotope ratios ($^{13}C/^{12}C$) can be used as an indicator of geographic origin (sourced by primary production as food web base), and little enrichment (0-1‰ per trophic level) occurs between prey and consumers in the marine environment (Kelly 2000; Kurle and Worthy 2002). When more than one prey species or prey of different trophic levels are consumed by a predator, isotopic mixing models can be applied to determine the proportional contribution of each source to the predator's diet (Phillips et al. 2005). Depending on fractionation and cellular turnover of different tissues, and the metabolic rate of the predator, stable isotope (SI) signatures can reflect the integrated diet of a consumer from days to years (Hobson et al. 1996; Newsome et al. 2010; Seymour et al. 2014).

We used a Bayesian stable isotope mixing model (SIAR) to examine the proportional contribution of HTLP to the diet of Pacific walruses. While SI analysis provides low taxonomic resolution compared to other methods of dietary analysis, such as stomach content and fecal examinations, it provides a tool to assess the importance of prey from different trophic levels to a consumer's diet without biases towards hard-bodied organisms. Assessment of the frequency and consequences of prey-switching by walruses in the Arctic ecosystem would be informative for management of this species, particularly when considering climate change effects on biological diversity and health of the Arctic Ocean.

We hypothesize that walruses rely on HTLP to a larger extent than observed from stomach content analyses. Furthermore, under the suggestion that predation on seals and seabirds is more common among male walruses (Fay 1982; Lowry and Fay 1984), we would expect a greater proportion of HTLP in samples from males. The objectives of this study were to: 1) quantify the proportional contributions of HTLP to the diet of Pacific walruses, 2) determine if multiple tissue types (i.e., lumbar and tongue muscle, skin, and liver) provide homologous SI and Bayesian mixing model results, and 3) investigate whether male and female walruses have different proportional contributions of HTLP in their diet.

3.3 Materials and Methods

3.3.1 Walrus Sample Collection

Sources and types of walrus tissues collected and analyzed for this study are presented in Table 3.1. In 2011, we obtained muscle and liver from a known "seal-eating" walrus (the stomach contained identifiable parts of 2 ringed seals, *Pusa hispida*; Seymour et al. 2014) in collaboration with the North Slope Borough Department of Wildlife Management (NSB-DWM) and hunters in Barrow, Alaska.

Tongue and muscle samples were stored in Ziploc™ bags and frozen at -20 °C. Full-thickness blubber samples with attached skin were wrapped and stored in aluminum foil in individual Ziploc™ bags at -80 °C. Archived tissue samples (muscle, skin, and liver) were stored at -80 °C in Cyrovials® (with the exception of Russian-sourced samples, which were stored in trace clean I-CHEM jars with Teflon lining). Further details of walrus tissue sample collection are presented in Chapter 4 (Seymour et al. 2014).

3.3.2 Walrus Stable Isotope Analysis

Approximately 3 g of tissue were subsampled from each sample using sterile knives and dissection scissors on a clean, stainless steel tray. Subsamples were transferred to scintillation vials, refrozen at -20 °C, and then freeze-dried for 24 to 48 hours. Following lyophilization, samples were ground into a fine powder using a mortar and pestle. For each sample, 0.2–0.4 mg of tissue was weighed into tin capsules. Walrus

samples were analyzed for both stable carbon and nitrogen isotope ratios at the UAF Alaska Stable Isotope Facility using a Costech Elemental Analyzer (ESC 4010) coupled to a Finnigan MAT DeltaPlusXL stable isotope ratio mass spectrometer. Isotope values were expressed relative to atmospheric N_2 (for nitrogen) and Vienna PeeDee Belemnite (VPDB) (for carbon) using the following equation:

(1) $$\delta X = [(R_{sample}/R_{standard})\text{-}1] \times 10^3$$

where differential notation (δX) equals the relative difference between sample and standard stable isotope ratios (R_{sample} and $R_{standard}$, also notated as $^{15}N/^{14}N$ for nitrogen or $^{13}C/^{12}C$ for carbon). Peptone was analyzed as an internal laboratory working standard every 10 samples. Instrument precision, expressed as one standard deviation calculated from multiple (n=46) analyses of the peptone standard, was ±0.2‰ for $\delta^{13}C$ values and ±0.1‰ for $\delta^{15}N$ values.

The presence of large amounts of lipid in some tissue types (i.e., skin and liver) can skew the $\delta^{13}C$ value obtained from SI analysis unless the sample is lipid-extracted or values are mathematically-corrected after the SI analysis (Sweeting et al. 2006). The lean quality of marine mammal muscle, however, allows for analysis of muscle without lipid extraction (Hoekstra et al. 2002). SI analysis of lipid-extracted duplicate skin samples from five walruses were used to develop a lipid normalization equation (below). This equation was then applied to the $\delta^{13}C$ values from the 22 walruses represented solely by skin to compare walrus lipid-corrected skin to muscle/tongue.

(2) $$\delta^{13}C' = -14.58 - 0.07\delta^{13}C$$

The equation is the linear regression (r=0.60) between the δ^{13}C values of non-lipid-extract and lipid-extracted skin samples (n=5), where $\delta^{13}C$ is the SI value of the non-lipid-extracted sample and $\delta^{13}C'$ is the lipid-corrected value. Lipid extraction of skin was performed using the method outlined by Bligh and Dyer (1959). Briefly, samples were freeze-dried, then vortexed in 4:1 chloroform:methanol. The supernatant was then removed via pipette and discarded. The extraction process was repeated at least four times or until chloroform:methanol added to the sample remained clear. Samples were then air-dried overnight, freeze-dried for 48 h, and analyzed for stable carbon isotopes.

δ^{13}C values were corrected for the Suess effect to adjust for the depletion in δ^{13}C values as a result of increased input of anthropogenically sourced CO_2 into the atmosphere. The corrective equation (below), modified by Misarti et al. (2009), incorporates the maximum annual rate of δ^{13}C value decrease in the North Pacific (-0.014, from Quay et al. 1992; calculations for Arctic regions are not currently available):

(3) $$\textit{Suess Effect Correction Factor} = -0.014^{(b*0.027)}$$

where b is the year of the animal's death subtracted from 1850 (the start of the Industrial Revolution); the constant 0.027 describes the curve for change in the δ^{13}C values of the world's oceans from 1945 through 1997 as calculated by Gruber et al. (1999).

3.3.3 Prey Stable Isotope Analysis

The HTLP mixing model utilized C and N isotope estimates from 3 potential prey items. The $\delta^{13}C$ and $\delta^{15}N$ values of *Serripes* spp. soft tissue (a common prey item of walruses, Ray et al. 2006; *n*=12) were used to represent lower trophic level prey. The $\delta^{13}C$ and $\delta^{15}N$ values of ringed and bearded seal (*Erignathus barbatus*) muscle, and spectacled eider muscle were used to represent the contribution of HTLP in the diet of walruses. Prey sample sizes, sources, and SI values are presented in Table 3.2. While consumption of lipid-rich tissues, such as seal blubber and eider fat, can affect $\delta^{13}C$ values of the predator and are consumed by walruses (Lowry and Fay 1984; Seymour et al. 2014) these tissues were excluded from analysis to maintain the simplicity of the model. To examine the possibility that lipid-rich tissue consumption might influence model output, an isotope biplot was generated (Figure 3.1).

Clam soft tissues were extracted from their shells and analyzed for stable carbon and nitrogen isotope ratios as described above. Mean and standard deviation of non-lipid-extracted bulk stable isotope values were used in the SIAR mixing model (lipid-extracted bulk isotope values are not currently available for *Serripes* spp.).

Ice seal tissue samples were processed using the same steps described above for SI analysis of walrus tissue. As with walrus samples, ice seal and spectacled eider $\delta^{13}C$ values incorporated into the mixing model were not lipid-extracted due to the lean quality of the muscle (Hoekstra et al. 2002; Dehn et al. 2007). Ribbon seals (*Histriophoca fasciata*) were omitted for the purposes of this study as this species has not been documented in walrus stomach contents nor are there any accounts of walruses foraging

on this species (Fay 1960; Burns 1970; Sheffield et al. 2001). While spotted seal (*Phoca largha*) remains have occasionally been retrieved from walrus stomachs (Lowry and Fay 1984), this species was excluded from modeling input as SI data were only available for young-of-the-year animals whose SI signatures are not necessarily representative of the entire population due to maternal influences (Jenkins et al. 2001). SIAR models were run both with (hereafter EI model) and without (hereafter NE model) SI values for spectacled eiders to examine the influence of inclusion of eiders in walrus diet on resulting HTLP estimates.

3.3.4 Mixing Model

Stable Isotope Analysis in R (SIAR), a Bayesian mixing model within R (version 2.12.2, R Development Core Team 2011), was used to estimate the proportional contribution of prey items (i.e., clam, ice seal, eider) to the diet of walruses. The SIAR program incorporates the $\delta^{13}C$ and $\delta^{15}N$ values of predators and representative prey species as well as tissue-specific turnover rates to produce high, low, mean, and mode estimates of the proportion of prey taxa in the diet of an individual predator (Parnell et al. 2010). Following Parnell et al.'s recommendations, mode estimates were used in this study. The model's use of Bayesian statistics allows for incorporation of a greater number of prey sources into the mixing model as well as uncertainty and variation of prey stable isotopes (Parnell et al. 2010). It is ideal to use tissue-specific turnover rates for the predator species; however, for many species, including walruses, these rates are not known (Bond and Diamond 2011). In the absence of this information, we used the

turnover rate for ringed seal muscle (2.4‰ $\delta^{15}N$, 1.3‰ $\delta^{13}C$, Hobson et al. 1996), the closest evolutionary relative to walruses with a known turnover rate.

3.3.5 Statistical Analyses

Statistical analyses and visual representations of data were performed in SigmaPlot (version 10.0, TE Sub Systems Inc and Sax Software 2006). Data failed normality and homogeneity assumptions ($P<0.05$), and so one-way ANOVAs on ranked data were performed (Iman and Conover 1979), followed by Dunn's multiple comparison tests to assess differences in $\delta^{13}C$ and $\delta^{15}N$ values among different walrus tissues (i.e., muscle, tongue, skin, and liver) and to examine differences in the mixed model HTLP estimates among walrus tissue types. A Mann-Whitney's test was used to assess differences in tissue-specific mixing model outputs between NE and EI models. One-way ANOVA on ranks followed by Dunn's multiple comparison test were also applied to each data set (muscle NE model, muscle EI model, liver NE model, liver EI model) to examine possible differences in $\delta^{15}N$ and $\delta^{13}C$ values between sexes (skin models were excluded as all skin samples were from female walruses). All statistical analyses were run with $\alpha = 0.05$.

3.4 Results

SI signatures of walrus muscle, liver, and skin, and mean SI values and standard deviation of the 3 representative prey items are presented as a biplot (Figure 3.1). Walrus tissues with depleted carbon isotope signatures were assumed to have ingested lipid-rich

tissue, which themselves have lower carbon SI signatures (DeNiro and Epstein 1977). Mean, standard deviation, data range, and sample size of $\delta^{13}C$ and $\delta^{15}N$ values for all walrus tissues are provided in Table 3.3. These values were compared among walrus muscle, tongue, and lipid-corrected skin from a subset of 28 individuals to determine if multiple tissues can provide statistically similar isotopic information (Table 3.4). Liver was not available in combination with the other walrus tissue types, thus no comparison among liver and other tissues from the same individual could be made. Statistical analysis showed no significant difference between $\delta^{15}N$ values from walrus muscle, tongue, and lipid-corrected skin ($P>0.05$, Table 3.3). A significant difference was found when $\delta^{13}C$ values from different walrus tissues were compared ($P<0.001$) with lipid-corrected skin being more enriched in carbon-13 compared with muscle.

Based on statistical analysis, muscle (lumbar and tongue) data were pooled for use in mixing model analyses as pooling allowed for a greater sample size with respect to model output. If more than one type of walrus tissue was available from the same individual, muscle was used preferentially in the SIAR model because muscle was the most abundant tissue sampled. In the absence of lumbar muscle, the order of preference for other tissue types was tongue muscle, skin, and liver. This order was selected based on turnover rate, with tongue muscle likely having the turnover rate most comparable to lumbar muscle (and statistically similar bulk stable isotope signatures), followed by skin, and then liver. Separate mixing models were generated for walrus liver and skin due to significant differences in their $\delta^{13}C$ values compared with muscle.

3.4.1 Higher Trophic Level Feeding

Mode and standard deviations of the proportional contributions of HTLP to walrus diet for both the NE and EI models are presented by tissue type in Table 3.3. Percent HTLP estimates and bulk stable isotope signatures of individual tissue samples are detailed in Appendix A. The mode contribution of HTLP when walrus tongue and muscle tissues were combined was 22% ± 10% for the NE model and 23% ± 10% when eiders were considered in the model (EI model). When muscle/tongue and liver mixing model outputs were compared, there was a statistically significant difference (P<0.01) between HTLP estimations, with contribution of HTLP estimated from walrus liver being significantly lower (10% ± 10%, Table 3.3). There was a significant difference between lipid-corrected skin HTLP estimates when all possible walrus tissue data were compared (muscle and tongue versus skin) (P<0.01, both models). However, comparisons between the muscle and lipid-extracted skin of the five walruses from which the lipid correction equation was developed, showed no significant difference in HTLP proportions between muscle and skin (P=0.20).

3.4.1.1 Contribution of HTLP and Sex

The sex of the sampled walrus did not significantly impact the proportional contribution of HTLP in either model utilizing walrus muscle (P>0.05, Table 3.5). Likewise, $\delta^{15}N$ values did not vary significantly with sex regardless of tissue type (P>0.05, Table 3.3). Sex influenced $\delta^{13}C$ values in walrus muscle, with males (n=46) having relatively higher values compared with females (n=111, P<0.05, Table 3.5). No

significant difference in $\delta^{13}C$ values between males and females was evident from the walrus liver analyses ($P>0.05$, Table 3.5).

3.5 Discussion

Diet assessments of marine mammals provide a basis for understanding the impacts of environmental change and alterations in prey base on both an individual and population-level. Dietary analyses, of marine mammals in particular, can be logistically difficult and are often invasive (Burns et al. 1998). The ability to assess diet of walruses using bulk stable isotopes in a variety of tissues (especially tissues that can be obtained during minimally invasive biopsy sampling, i.e., skin) is thus extremely valuable. However, caution must be exercised in the choice of tissue type(s). The isotopic information contained in a specific tissue is the result of fractionation factors and cellular turnover (i.e., tissue turnover rates) and the metabolic rate of the predator (Hobson et al. 1996; Newsome et al. 2010). For walruses, different skeletal muscles (tongue and lumbar muscle) from the same individual produce $\delta^{13}C$ and $\delta^{15}N$ values more similar to each other than non-muscle tissue. This is reasonable as metabolic/isotopic turnover rates in different muscles are likely to be closer than turnover rates between skin and muscle or liver and muscle (Hobson et al. 1996; Newsome et al. 2010; Todd et al. 2010). While we initially concluded from comparison of all 293 individuals (with one tissue type representing each animal) that $\delta^{13}C$ values were not homogeneous between muscle and lipid-corrected skin, comparison between the tissue sets of the five individuals (i.e., both skin and muscle samples from each animal) from which our lipid correction equation was

developed proved similar (P=0.09). These findings suggest that the $\delta^{13}C$ and $\delta^{15}N$ values of different tissues from the same walrus may provide statistically similar results and that the non-homogeneity observed when our entire sample pool was compared by tissue type could have been the result of comparing different animals with each other (i.e., variation may not be due to comparing skin with muscle, but rather comparing individuals with each other). Such variation among individuals can arise from differences in metabolic rate, which often result from changes in energetic demand associated with migration, reproduction, lactation, growth, and body condition (Barboza et al. 2009; Newsome et al. 2010). Alternately or in addition, variation can be the result of individual dietary preferences and history. Further, it is possible that the sample size of our pilot study was too small to capture significant variation. Additional research comparing SI values of different tissues from a larger sample set and use of captive dietary studies could confirm whether the SI values of different tissues can be directly compared.

3.5.1 Higher Trophic Level Feeding

The overall proportional contribution of HTLP to walrus diet, as indicated by both the muscle NE and EI Bayesian mixing models, is ~22.0 and 23.0%, respectively. These proportions are higher than anticipated based on walrus stomach content analyses from the 1980s (~10%, Lowry and Fay 1984). However, our results support the hypothesis by Rausch et al. (2007) and by Lowry and Fay (1984) that predation on seals by walruses has been increasing over the last 40 years. We note, however, that the above historical data come from stomach content analysis and is thus not directly comparable to the

results of our mixing models. Furthermore, the proportion of HTLP estimated by our models closely matches the magnitude of HTLP consumption suggested from bio-accumulating contaminant analysis of Atlantic walruses (25.0%; 13 of 53 animals, Muir et al. 1995), where predation on seals and birds has been well documented (Muir et al. 1995; Mallory et al. 2004; Fox et al. 2010). Our findings are supported by stomach content and SI analysis of the known seal-eater we examined in 2011, which contained the remains of two ringed seals as well as quantities of *Mya* spp. siphons (a common benthic bivalve prey of walruses) and displayed a muscle HTLP value of 42% (Seymour et al. 2014). Further support for opportunistic prey selection comes from the wide range in HTLP values indicated by the liver mixing models (>1-22%, Table 3.3), if we assume that walrus liver turnover rate is higher than that of muscle (Tieszen et al. 1983).

While energetic requirements for Pacific walruses are unavailable, Born et al. (2003) estimated that Atlantic walruses require a gross 896 kJ/kg wet body weight daily. Applied to Pacific walruses (using weights from Fay 1982), an adult male walrus (~1,200 kg) would need 256,800 kJ per day (and a female weighing ~830 kg needs 191,744 kJ per day). We estimated the quantity of lower versus higher trophic level prey needed to meet these requirements as follows, using bivalves as a representative lower trophic level prey and ringed and bearded seals as representative higher trophic level prey. Average *Serripes* spp. (values are not available for *Mya* spp.) contain 21 kJ/g dry weight (dw) (Hondolero et al. 2012) and dw represents 61% of the shell-free wet weight of the animal (Ricciardi and Bourget 1998). At 3.05 g dw/clam (Hondolero et al. 2012), male walruses must consume approximately 12 kg dw of the soft parts of bivalves per day (~4,000

clams) and a female ~9 kg dw per day (~3,000 clams, assuming similar metabolic rates). Ice seal blubber is much more energy dense at 34.5 kJ/dw (Kuhnlein and Soueida 1992). Based on an estimated maximum water content of 17% for pinniped blubber (measured for harbor seals (*Phoca vitulina*) by Bowen et al. 1992), an adult male walrus would only need to consume ~7.5 kg blubber per day (and females ~6 kg per day). An average adult ringed seal weighs 50-70 kg (Usher and Church 1969), about 40% is blubber (Ryg et al. 1990), thus an adult walrus would only require ~1/3 of an adult ringed seal per day to meet caloric requirements. Fewer animals would be needed if bearded seals were consumed, which weigh 200-250 kg, with similar % blubber mass (Reeves et al. 1992). Ultimately, relatively fewer and/or shorter successful foraging trips would be required daily if blubber constitutes a regular or semi-regular portion of the walrus diet compared to solely relying on benthic invertebrates. However, consumption of uncommon or novel prey items is often associated with increased energy expenditures related to prey capture, and mechanical and chemical digestion (Barboza et al. 2009). Whether predation on HTLP offers a more efficient net energy gain remains unknown, as the energy expenditure associated with walruses capturing and processing seals or seabirds has not been determined. Furthermore, benthic-pelagic uncoupling in the Arctic and sub-Arctic marine environments (Grebmeier et al. 2006) could influence reliance on HTLP by walruses and perceived potential benefits of "seal-eating". Documented alterations in Arctic and sub-Arctic prey populations in response to environmental variables are not yet fully understood (Grebmeier 2012), but decreases in prey quantity, quality, and distribution all can affect the energetic costs of foraging.

We note that while walruses occasionally forage on spectacled eiders and other seabirds, the values generated by the muscle EI mixing model (6-8% of the diet, Table 3.3) in this study should be considered objectively, given historical analyses of walrus stomach contents rarely found evidence of seabird consumption (Fay 1982; Fay et al. 1990). Recent studies do suggest seabirds may now comprise a larger portion of the Atlantic walrus diet (Mallory et al. 2004; Fox et al. 2010). It is also plausible that SIAR's ability to delegate proportions of prey is reduced in the EI models as the $\delta^{15}N$ values of eiders (14.0 ± 0.2‰) falls approximately midway between that of ice seals (17.0 ± 0.8‰) and *Serripes* spp. (9.0 ± 1.5‰) and $\delta^{13}C$ values of all three representative prey species are within 1 SD of each other (-19.0 ± 0.1‰, -19.0 ± 1.4‰, and -18.0 ± 0.6‰, respectively), making both HTLP isotopically similar to each other and difficult to apportion for the model. The distribution of walrus tissue SI values was negatively correlated between the ice seal and spectacled eider SI signatures (Figure 3.1), sometimes an indication that the SIAR model will be unable to differentiate between two prey sources (Inger et al. 2010).

As an alternative or in combination with consumption of ice seals and seabirds, it is conceivable that prey with similarly high $\delta^{15}N$ values (such as scavenging benthic crustaceans or predatory gastropods) may contribute to HTLP estimates found in this study. Scavenging crustaceans from the Chukchi Sea region display relatively high $\delta^{15}N$ values (16.0‰ for *Sclerocrangon boreas*, Dehn et al. 2007, 16.0‰ for *Buccinum* spp., 14.0‰ for *Neptunea heros,* Feder et al. 2011), and have been identified in walrus

stomach contents (Sheffield and Grebmeier 2009), and the presence of these invertebrates likely reflects variations in regional habitat use.

Contrary to our hypothesis that males constitute the portion of the population preying on HTLP, our models indicate that prevalence of HTLP in both males and females is apparently similar. This finding is divergent from Alaskan local ecological knowledge (LEK) asserting that rogue male walruses are the only individuals to prey on seals and are obligatory seal-eaters (Fay 1960). However, the lack of a significant difference in HTLP consumption between males and females is consistent with previous stomach content analyses (Fay et al. 1977; Fay 1982; Lowry and Fay 1984; Sheffield and Grebmeier 2009). Based on female and calf stomach content analyses, Fay et al. (1977) further hypothesized that females might preferentially seek out calorically-dense seal blubber to offset the high energetic costs of reproduction. It is likely that obligate seal-eaters exist within the Pacific walrus stock, as Alaskan hunters report seal-eating walruses as morphologically distinct (i.e., yellow tusks and greasy appearance, Fay 1960). The one walrus harvested near Barrow that had recently eaten seals (Seymour et al. 2014) did not exhibit the morphologically distinct characteristics of an obligate seal predator (per. comm. G. Krafsur, NSB-DWM). SI analysis of a whisker from this animal showed fluctuations in its reliance on HTLP over time (Seymour et al. 2014) and indicated that this walrus had consumed seals.

The significant difference in $\delta^{13}C$ values of males versus females shown by the muscle SI analysis implies that sexes utilize different habitat for foraging. While a preliminary explanation might be differences in ingested % lipid (lipids typically have

relatively lower $\delta^{13}C$ values compared to proteins, DeNiro and Epstein 1977), this difference is unlikely the result of variation in tissue lipids as C:N ratios were comparable (P=0.62). C:N ratios would be higher for individuals with lower $\delta^{13}C$ values if the difference in $\delta^{13}C$ values was the result of consuming comparatively lipid-rich prey. It is known that $\delta^{13}C$ values vary geographically (Crawford et al. 2008). Seasonal changes in $^{13}C/^{12}C$ are driven by fluctuations in primary productivity (Hobson et al. 1996). However, as extrapolated turnover estimates show walrus muscle $\delta^{13}C$ values to be integrated over roughly two years (Seymour et al. 2014), it is unlikely that differences in the $\delta^{13}C$ values are seasonally driven. Walruses occupy geographically distinct regions throughout the year based on semi-sexually segregated migrations (Fay 1982; Garlich-Miller et al. 2011). Additionally, elemental tooth composition suggests that the Pacific walrus population may be comprised of several stocks (Jay et al. 2008). The opportunistic nature of the sample collection in our study precludes us from analyzing regional influence on diet as our samples are not regionally representative of the walrus range.

Sexual partitioning of foraging areas (proximity to shore or geographical region) would result in varying intensities of impact on walrus population segments from environmentally-induced changes in prey base. Female walruses with dependent calves may be most adversely affected by declines in prey quantity or quality due to their higher energetic demands (Barboza et al. 2009). On-going monitoring efforts have revealed declines in fecundity and lipid stores among female walruses (Garlich-Miller et al. 2006) and increased calf-abandonment related to poor sea ice conditions (Metcalf and Robards 2008). The magnitude of these impacts will be dependent on whether the severity and

extent of alterations in the prey base exceeds walrus dietary plasticity (Barboza et al. 2009). Alternately, sexually-segregated migrations may be declining as more mixed-sex walrus herds have been observed in recent summer months in the Chukchi Sea, an area historically used by females and calves (Garlich-Miller et al. 2011). This trend may explain the lack of differences in reliance on HTLP and absence of a difference in $\delta^{13}C$ values between sexes when liver models are analyzed, assuming that liver turnover rates are higher than muscle and represent a time period of approximately 3 months as described above (Table 3.5). A continued increase in mixed-sex herds could disperse localized climate change impacts more evenly among the population. The ability to understand the impacts of climate change on walrus population will be dependent on unraveling correlations among climate, consumer, and prey.

The wide range in mode proportional dietary contribution of HTLP (average of ~22% ± 10%, range of 2-38% based on muscle) suggests that most walruses feed opportunistically on seals or HTLP with similar $\delta^{15}N$ signatures when and where these prey are available. Seasonal reliance on HTLP would be reflected by more consistent %HTLP across most samples, as the majority of samples were collected during the spring subsistence harvest, April-May. This finding is supported by the fluctuating $\delta^{15}N$ signature found along the length of a whisker from a known seal-eating walrus (Seymour et al. 2014). The proportional contribution of HTLP found in our analyses is higher than the historical 10%, suggesting climate impacts on walrus foraging brought on by changes in sea ice quality and extent or more complex environmental and ecological variables.

However, historical analyses based on gut content may underestimate the past significance of ice seals and sea birds as walrus prey.

Walruses' ability to forage on a diverse assemblage of prey types is supported by this study. The dietary breadth and plasticity of the species will serve to mitigate effects from abrupt and/or short-term changes in the prey base. To accurately establish the proportional contribution associated with specific prey species, a combination of methods, including bulk and compound-specific SI analysis, fatty acid signature analysis, and stomach content analysis combined with developing techniques in fecal DNA analysis, will prove most useful. Continued monitoring of diet through a combination of these analytical methods will provide the best understanding of trends in walrus foraging, and a better foundation for assessing population level dietary consequences of climate change.

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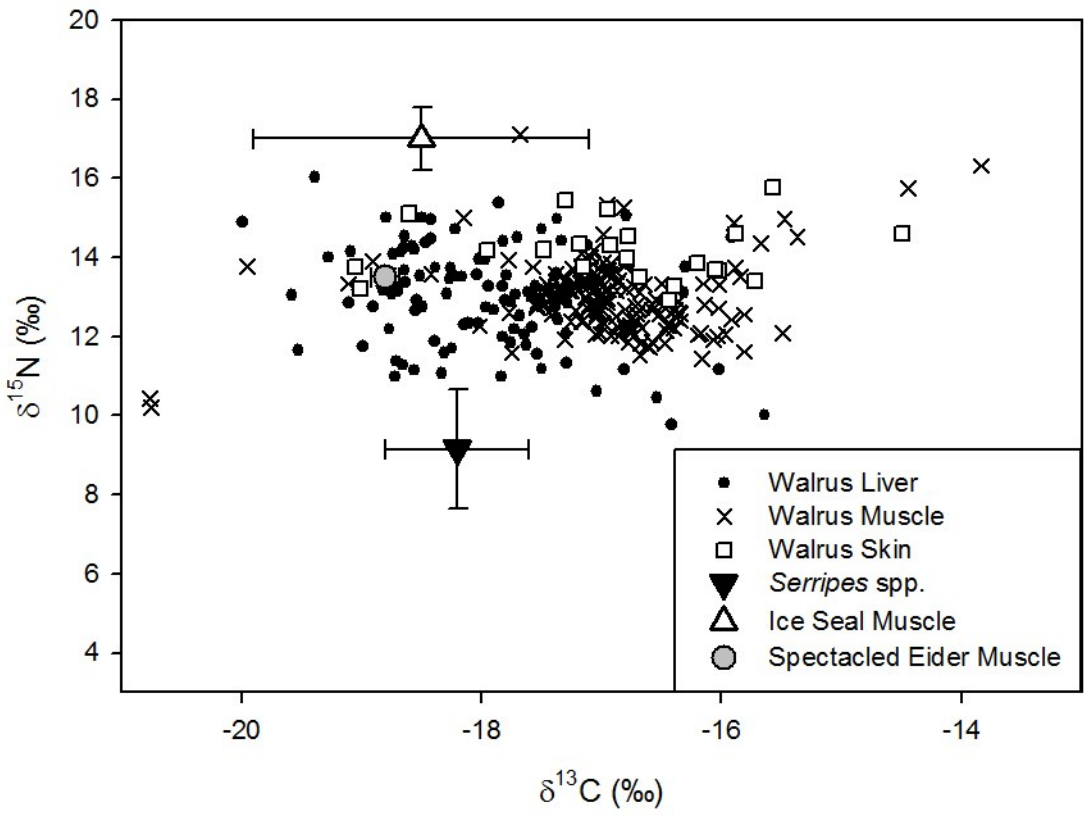

Fig. 3.1 Walrus muscle stable isotope ratios and stable isotope ratio of representative walrus prey

Scatter plot of Pacific walrus stable nitrogen versus stable carbon isotopes by tissue type, and stable isotope means and standard deviations for representative higher and lower trophic level walrus prey.

Table 3.1 Sampling year, location, and sample sizes for walrus tissues used in this study

				Available Sample (*n*)			
Location	**Source**	**Type**	**Date**	**Tongue**	**Muscle**	**Liver**	**Skin**
St. Lawrence Island	USFWS	Subsistence	2010	73	74	–	–
			2009	49	48	–	5
			1994	–	–	24	–
			1993	–	–	15	–
			1992	–	–	10	–
Chukotka Peninsula	USFWS	Free-Ranging USA/USSR Cruise	1991	–	–	10	–
Barrow, Wainwright	NSB-DWM	Subsistence	2009	–	6	–	6
Little Diomede Island	USFWS/	Subsistence	2005	1	–	–	–
			2004	–	2	–	–
			2003	5	7	–	–
			1994	–	–	11	–
			1993	–	–	7	–
			1992	–	–	37	–
Cape Peirce	National Park Service/ Togiak National Wildlife Refuge	Mortality Event	2009	5	9	–	–
Bering Sea	USGS	Free-Ranging	2009	–	–	–	22
Various, Alaska	ADF&G	Subsistence	2009	3	–	–	–
Various, Alaska	UA Museum of the North	Subsistence	1981-2006	–	32	15	1

Table 3.2 Mean and range of prey stable isotope values incorporated into SIAR

Species	Sample Size (*n*)	Mean $\delta^{15}N \pm SD$ [‰]	Mean $\delta^{13}C \pm SD$ [‰]
Serripes spp. soft	12	9.15 ± 1.5	-18.2 ± 0.6
Mean ice seal	137	17.0 ± 0.8	-18.5 ± 1.4
Ringed seal muscle[b]	82	16.0 ± 0.6	-18.5 ± 0.8
Bearded seal	55	16.8 ± 0.9	-17.1 ± 0.5
Spectacled eider	42	13.5 ± 0.2	-18.8 ± 0.1

Mean ± standard deviation (SD) and range (in parentheses) of bulk stable isotope values of prey items incorporated into the SIAR mixing model.

[a] Stable isotope values for *Serripes* spp. were provided by Dr. K. Iken, UAF. *Serripes* spp. were collected during the 2004 Bering Sea Integrated Ecosystem Research Project (BEST-BIESP) research cruise

[b] Stable isotope values for ringed and bearded seals were provided by Dr. L. Horstmann-Dehn, UAF (Dehn et al. 2007). Seal samples were collected from subsistence harvests between 1996 and 2003 in the Bering and Chukchi seas. Both seal species consume benthic and pelagic fishes, crustaceans, and benthic invertebrates, and both occupy a similar trophic level distinct from typical invertebrate walrus prey. We therefore used mean and SD SI values from both species combined in the mixing model

[c] Stable isotope values for lipid-extracted spectacled eider muscle were provided by Dr. J. Lovvorn, University of Wyoming, Department of Zoology and Physiology. Non-lipid-extracted SI values were unavailable. Eider muscle samples were collected in April 2009 in the wintering area of the species in the Northern Bering Sea

Table 3.3 Walrus stable isotope values and contribution of higher trophic level prey to walrus diet

Tissue type	Sample size (*n*)	$\delta^{15}N$ diet-tissue isotope fractionation factor [‰]	$\delta^{13}C$ diet-tissue isotope fractionation factor [‰]	Protein turnover half-life (days)	Mean $\delta^{15}N \pm$ SD [‰] (*range*)	Mean $\delta^{13}C \pm$ SD [‰] (*range*)	HTLP (NE model) (*range*)	HTLP (EI model)	
								Ice Seal (*overall range*)	Eider
Muscle	154	2.4	1.3	~160[a]	13.0 ± 0.9 (*10.0 to 17.0*)	-17.0 ± 0.8 (*-14.0 to -20.0*)	22.0% ± 10% (*9.0 to 38.0*)	15.0% ± 10% (*2.0 to 38.0*)	9.0% ± 10%
Tongue	10	n/a	n/a	n/a	13.0 ± 0.5 (*12.0 to 14.0*)	-17.0 ± 0.5 (*-15.0 to -19.0*)	23.0% ± 10% (*13.0 to 36.0*)	16.0% ± 9% (*17.0 to 37.0*)	9.0% ± 10%
Skin-lipid-corrected	22	2.3	2.8	~20[a]	14.0 ± 0.7 (*13.0 to 16.0*)	-17.0 ± 1.1 (*-13.0 to -16.0*)	44.0% ± 8% (*31.0 to 49.0*)	61.0% ± 4% (*57.0 to 64.0*)	1.0% ± 3%
Liver	107	3.1	0.6	~1.9-6.7[a]	13.0 ± 1.2 (*10.0 to 16.0*)	-18.0 ± 0.8 (*-16.0 to -21.0*)	10.0% ± 10% (*1.0 to 22.0*)	5.6% ± 4% (*3.4 to 20.0*)	6.0% ± 10%

Mean ± standard deviation (SD) and range (in parentheses) of walrus bulk stable isotope values and proportional contribution of higher trophic level prey (HTLP) to walrus diet by tissue, including enrichment factors (mean diet-to-tissue isotope fractionation values) (Hobson et al. 1996) and protein half-life turnover rates (muscle, Seymour et al. 2014; skin, Welle 1999; liver, Kurle and Worthy 2002) using both inclusion (EI) and exclusion (NE) of spectacled eiders in the mixing model (SIAR).

[a] Tissue turnover rates for walrus tissues are currently unknown. Seymour et al. (2014) presents an extrapolated turnover rate for walrus muscle tissue. To date, Kurle and Worthy (2002) present the only measured turnover rates for pinniped liver (from northern fur seals, *Callorhinus ursinus*). Pinniped skin turnover rates have not been yet been measured. Welle (1999) reported skin turnover rates for humans, which are presented here to provide a rough estimate of pinniped skin turnover rates

Table 3.4 Tissue comparison of estimated contribution of higher trophic level prey to walrus diet

Tissue comparison[a]	Sample size (*n*)	Significant difference in HTLP (*P*<0.05)
Muscle and tongue	28 (muscle), 11 (tongue)	No (*P*=0.67)
Muscle and lipid-corrected skin	28 (muscle), 16 (skin)	Yes (*P*<0.001)
Tongue and lipid-corrected skin	11(tongue), 16 (skin)	Yes (*P*<0.001)

Tissue comparison (one way ANOVA on ranks) of SIAR mixing model estimates for proportional contribution of higher trophic level prey (HTLP) to the diet of walruses.

[a] Liver was not available in combination with other tissue types, thus, no comparison among liver and other tissues from the same individual could be made

Table 3.5 Walrus stable isotope values and contribution of higher trophic level prey to diet by tissue and sex

Sex	Tissue type[a]	Sample size (*n*)	Mean $\delta^{15}N \pm SD$ [‰]	H_0[b]	Mean $\delta^{13}C \pm SD$ [‰]	H_0[b]	HTLP (NE model)	HTLP (EI model)		H_0[c]
								Seal	Eider	
Female	Muscle/Tongue	111	13.0 ± 0.6	*P*=0.95 (NE)	-17.0 ± 0.6	*P*=0.04 (NE)	21.0% ±7%	14.0% ±6%	11.0% ±5%	*P*=0.57 (NE)
Male	Muscle/Tongue	46	13.0 ± 1.4	*P*=0.18 (EI)	-17.0 ± 1.2	*P*=0.01 (EI)	23.0% ±12%	19.0% ± 12%	5.0% ± 12%	*P*=0.18 (EI)
Female	Liver	65	13.0 ± 1.1	*P*=0.75 (NE)	-18.0 ± 0.7	*P*=0.15 (NE)	10.0% ±9%	5.0% ± 4%	6.0% ± 7%	*P*=0.87 (NE)
Male	Liver	25	13.0 ± 1.5	*P*=0.75 (EI)	-18.0 ± 1.0	*P*=0.14 (EI)	9.0% ± 10%	5.0% ± 8%	6.0% ± 7%	*P*=0.14 (EI)

Mean ± standard deviation (SD) of walrus bulk stable isotope values and proportional contribution of higher trophic level prey (HTLP) to diet by tissue and sex using both inclusion (EI) and exclusion (NE) of spectacled eiders in the mixing model (SIAR)

Note - Tongue and lumbar muscle bulk stable isotope data were pooled as no significant differences were found in $\delta^{13}C$ values ($P \geq 0.05$), $\delta^{15}N$ values ($P=0.61$), and HTLP estimates ($P \geq 0.05$) .

[a] Skin samples were excluded from sex-categorical analysis as skin was only available from females

[b] H_0 testing lack of difference in stable isotope signatures between sexes

[c] H_0 testing lack of difference in %HTLP between sexes

Chapter 4: Inter-Annual Variability in the Proportional Contribution of Higher Trophic Levels to the Diet of Pacific Walruses[3]

4.1 Abstract

Pacific walruses (*Odobenus rosmarus divergens*) depend on Arctic sea ice as a resting and foraging platform; however, recent years have seen unprecedented seasonal reductions in ice extent. Previous researchers proposed that during unfavorable ice conditions walruses might prey on other pinnipeds. To examine this hypothesis, we analyzed carbon and nitrogen stable isotope ratios of muscle from walruses (n=155) sampled from the Bering and Chukchi seas during 2001-2010. We used a Bayesian stable isotope mixing model to examine the proportional contribution of higher trophic level prey (HTLP) (e.g., seals, seabirds) to walrus diets and extrapolated a tissue-specific turnover rate to compare diet of individuals over time. Mode HTLP across years was 19%± 8%. Results indicate a significant decrease (P<0.05) in the reliance on HTLP during 2008-2009 (mode HTLP 13%), one of two sampling periods that experienced great seasonal loss of pan-arctic sea ice (the other being 2007-2008 with mode HTLP of 23%). We also reveal intra-annual fluctuations in the contribution of HTLP to the diet of a walrus sampled in 2011 with seal remains in its stomach through high-resolution sectioning along a whisker length. Our findings suggest that walruses forage

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[3] Seymour J, Horstmann-Dehn L, Wooller MJ (2014) Inter-annual variability in the proportional contribution of higher trophic levels to the diet of Pacific walruses. Polar Biol 37:597-609

opportunistically as a result of multiple environmental factors and that sea ice extent alone does not drive consumption of HTLP.

Keywords: walrus, seal, sea ice, Arctic, whisker, stable isotopes, SIAR mixing model

4.2 Introduction

Arctic sea ice minima have been reported during 2007-2013 (National Snow and Ice Data Center (NSIDC) 2012a; Stroeve et al. 2012), and the forecasted trend for decreasing sea ice extent and thickness has prompted listing of several Arctic pinniped species as threatened, endangered, or candidate species under the U.S. Endangered Species Act (USFWS 2011; NMFS 2012a, 2012b). Pacific walruses (*Odobenus rosmarus divergens*) rely on sea ice for a large portion of their life history, including the use of sea ice as a resting platform between foraging trips and a diving platform providing energetically efficient access to regionally diverse benthic invertebrate prey (Fay 1982; Sheffield and Grebmeier 2009). The decrease in Arctic sea ice extent may prompt walruses to implement alternative foraging strategies (Rausch et al. 2007; Jay and Fischbach 2008). These adjustments may take the form of direct changes, such as altered feeding ecology.

Although walruses are considered specialized benthic feeders who use highly adapted facial musculature to extract bivalves from their shells (Fay 1982), numerous other invertebrate taxa (e.g., amphipods, polychaetes, gastropods) have been identified from the stomach contents of walruses (Sheffield and Grebmeier 2009). During unfavorable ice conditions or other energetically stressful situations, walruses opportunistically forage on other available prey, including seals and seabirds (Fay 1960). For example, increased use of centralized terrestrial haulouts by walruses, such as those documented at Point Lay and Cape Peirce, Alaska, during the 2009, 2010, 2011, and

2013 summers, may lead to localized prey depletion and/or create energetically costly increases in travel distances to foraging grounds (Garlich-Miller et al. 2011; Jay et al. 2012; Monson et al. 2013). Increased energy demands on walruses may prompt them to opportunistically forage on higher trophic level prey (HTLP), such as pinnipeds and seabirds. Although, seal-eating and carcass scavenging by walruses is not a novel activity for them, the incidence of this foraging strategy appears to be increasing in both the Atlantic and Pacific subspecies (Fay 1960; Lowry and Fay 1984; Wolkers et al. 2006).

Bulk stable isotope (SI) analysis is a common tool for examining feeding ecology. Stable nitrogen isotope ratios ($^{15}N/^{14}N$) are used to assess trophic position of an organism relative to its prey (Kelly 2000). A stepwise enrichment of 3-5‰ per trophic level is generally assumed for marine mammals (Hobson et al. 1996). Stable carbon isotope ratios ($^{13}C/^{12}C$) are used as an indicator of geographic origin (sourced by primary production as food web basis), and little enrichment (0-1‰ per trophic level) occurs between prey and consumer in the marine environment (Kelly 2000; Kurle and Worthy 2002). When more than one prey species or prey of different trophic levels are consumed by a predator, isotopic mixing models can be applied to determine the proportional contribution of each source to the predator's diet (Phillips et al. 2005). Depending on fractionation factors and cellular turnover of different tissues (i.e., tissue turnover rates) and the metabolic rate of the predator, stable isotope (SI) values can reflect the integrated diet of a consumer from days to years (Hobson et al. 1996; Newsome et al. 2010). While SI analysis provides low taxonomic resolution compared to other methods, such as

stomach content and fecal analyses, it provides a tool to assess the importance of prey from different trophic levels to a consumer's diet without biases towards hard-bodied organisms (Pierce et al. 2004). Whiskers and other keratinous tissues are laid down over time and once deposited, no cellular turnover occurs within any given layer (Schell et al. 1989; Hobson et al. 1996; Cherel et al. 2009). Variation in the importance of different trophic level prey can therefore be assessed from SI analysis along the length of the keratin structure, providing an extended dietary timeline for an individual.

Assessing temporal variability in the proportional contribution of HTLP by Pacific walruses in the Arctic ecosystem is important for management of this species as well as associated prey, particularly when considering climate change influences on biological diversity and health of the Arctic marine ecosystem. We hypothesize that recent years show a higher incidence of walruses relying on HTLP due to changes in benthic biomass (e.g., abundance, distribution), potentially in relation to Arctic sea ice extent. To this end, we examined whether reliance on HTLP is increasing over time (as suggested by Rausch et al. 2007), potentially in response to changes in sea ice extent (Fay 1960). We analyzed tissue samples from walruses (n=155) taken from the Bering and Chukchi seas during the spring and summer of 2001 - 2003, and 2009 - 2010 for their carbon and nitrogen stable isotope ratios. We applied these data to a Bayesian SI mixing model (SIAR) to examine the proportional contribution of higher trophic level prey (e.g., seals and seabirds) to walruses' diets. We also compared these data with SI data from a whisker removed from a known seal-eating walrus.

4.3 Materials and Methods

4.3.1 Sample Collection

Walrus lumbar muscle (hereafter "muscle") and tongue muscle (hereafter "tongue") (*n*=155) were opportunistically collected during the Spring/Summer 2009 and 2010 Alaska Native subsistence harvests near St. Lawrence Island, in the northern Bering Sea (*n*=118), and from Barrow and Wainwright, Alaska, in the Chukchi Sea (*n*=6, Table 4.1). Samples were obtained in collaboration with these communities, as well as the Eskimo Walrus Commission, the U.S. Fish and Wildlife Service (USFWS), and the North Slope Borough Department of Wildlife Management (NSB-DWM). Walrus muscle and tongue samples (*n*=11) were also collected from a Fall 2009 natural mortality event (not disease related) at Cape Peirce, Alaska in collaboration with the USFWS and the Togiak National Wildlife Refuge (Table 4.1, Liedberg et al. 2009). Tissues (including a 9.55 cm-long whisker) were also collected from a known-seal eating walrus subsistence-harvested near Barrow in July 2011. Predation on seals by this walrus was confirmed by the presence of the remains of two ringed seals (*Pusa hispida*) in the stomach.

Tissues of Alaska Native subsistence-harvested walruses in Barrow and Wainwright were collected under the authority of permit number MA134907-0 issued to T. Hepa, NSB-DWM. Archived walrus muscle samples from 2001-2003 from the Chukchi and Bering Sea regions were provided for analysis by the University of Alaska Fairbanks (UAF) Museum of the North (*n*=19, Loan # 2010.006.Mamm). All other

walrus samples used in this study were obtained under a letter of authorization to L. Horstmann-Dehn through the USFWS.

Tissue samples from all animals were collected immediately after death, with the exception of samples from Cape Peirce. Due to the isolated location of the Cape Peirce mortality event and logistical difficulties of access during fall months, samples were collected one to two weeks post-mortem. However, as temperatures were well below freezing, carcasses were frozen and therefore well-preserved upon sample collection. Sex and sampling date were recorded whenever possible, and each animal was given a unique identifier. Samples from tongue and muscle were stored in Ziploc™ bags and frozen at -20 °C. Archived tissue samples were stored at -80 °C in Cyrovials®.

In development of the time series, years for which less than 5 samples were available were excluded to maximize robustness of results and minimize bias. Samples from the excluded years (2004- 2006) were also generally all of the same sex and age class, and taken from the same geographic location over the course of several days. Because of this duplicity they could not be assumed to be representative of the entire population.

4.3.2 Stable Isotope Analysis

Approximately 3 g of walrus muscle tissue was sub-sampled from each sample using sterile knives and dissection scissors on a clean, stainless steel tray. Any fascia and fatty tissues were trimmed from muscle so as not to bias SI analyses due to the presence of lipids. Samples were transferred to clean scintillation vials, refrozen at -20 °C, and

then freeze-dried for 24 to 48 hours. Following freeze-drying, samples were ground into a fine powder using mortar and pestle. For each sample, 0.2–0.4 mg of tissue was weighed into tin capsules. Samples were analyzed for both stable carbon and nitrogen isotope ratios at the UAF Alaska Stable Isotope Facility using a Costech Elemental Analyzer (ESC 4010) coupled to a Finnigan MAT DeltaPlusXL Isotope Ratio Mass Spectrometer (IRMS). SI values are expressed in delta notation relative to atmospheric N_2 and Vienna PeeDee Belemnite using the following equation:

(1) $$\delta X = [(R_{sample}/R_{standard})\text{-}1] \times 10^3$$

where differential notation (δX) equals the relative difference between sample and standard stable isotope ratios (R_{sample} and $R_{standard}$, also notated as $^{15}N/^{14}N$ for nitrogen or $^{13}C/^{12}C$ for carbon). Peptone, as a laboratory-working standard, was analyzed every 10 samples. Instrument precision, expressed as one standard deviation, was calculated from multiple (n=46) analyses of peptone (±0.2‰ for $\delta^{13}C$ values and ±0.1‰ for $\delta^{15}N$ values).

The 9.55 cm-long whisker from the walrus with seal remains in its stomach was cleaned with de-ionized water followed by chloroform/methanol (2:1). Under a dissecting scope, sections (equating to <0.1 mm thickness) were incrementally removed along the length of the whisker using a razor blade. Incremental re-measurements of the remaining intact whisker after removal of every 20 samples allowed us to estimate the average thickness of each individual slice of whisker and track the position of the 568 samples along the length of the whisker. Each section was freeze-dried and weighed into a tin capsule for stable isotope analyses, as described above. While the growth rate of walrus

whiskers is unknown, a growth rate was estimated using known mean rates for Steller sea lion (*Eumetopias jubatus*) whiskers of 0.1-0.2 mm d^{-1} (Hirons et al. 2001)

4.3.3 Mixing Model

Stable Isotope Analysis in R (SIAR), a Bayesian mixing model program within R (version 2.12.2, R Development Core Team 2011), was used to estimate the mode proportional contribution of prey items (i.e., clam (low trophic), ice seal (high trophic)) to the diet of walruses. The SIAR program incorporates the stable carbon and nitrogen isotope signatures of predators and representative prey species, as well as tissue-specific turnover rates to produce high, low, mean, and mode estimates of the proportion of prey types in the diet of an individual predator (Parnell et al. 2010). Mode estimates were used in this study, following guidelines by Parnell et al. (2010). While it is still necessary to pool prey types that occupy similar isotopic space, the SIAR program is not limited by the number of prey sources; a problem encountered with earlier SI mixing model programs, such as IsoError (Phillips and Gregg 2001). The model's use of Bayesian statistics allows for incorporation of a greater number of prey sources into the mixing model as well as uncertainty and variation of prey stable isotopes (Parnell et al. 2010). It is ideal to use tissue-specific turnover rates for the predator species; however, for many species, including walruses, these rates are not known (Bond and Diamond 2011). In the absence of this information, we used the turnover rate for ringed seal muscle (2.4‰ $\delta^{15}N$ value, 1.3‰ $\delta^{13}C$ value, Hobson et al. 1996), the closest evolutionary relative to walruses with a known turnover rate.

4.3.3.1 Suess Effect

$\delta^{13}C$ values were corrected for the Suess effect to adjust for the depletion in $\delta^{13}C$ values as a result of increased input of anthropogenically sourced CO_2 into the atmosphere. The corrective equation (below), modified by Misarti et al. (2009), incorporates the maximum annual rate of $\delta^{13}C$ value decrease in the North Pacific (-0.014, from Quay et al. 1992; calculations for Arctic regions are not currently available):

(2) $$\textit{Suess Effect Correction Factor} = -0.014^{(b*0.027)}$$

where *b* is the year of the animal's death subtracted from 1850 (the start of the Industrial Revolution); the constant 0.027 describes the curve for change in the $\delta^{13}C$ values of the world's oceans from 1945 through 1997 as calculated by Gruber et al. (1999).

4.3.3.2 Prey Species

For the mixing model, bivalve $\delta^{13}C$ and $\delta^{15}N$ values were provided by K. Iken, UAF. Bivalve samples (*Serripes* spp., a common walrus prey; n=12) were collected during the 2004 Bering Sea Integrated Ecosystem Research Project (BEST-BSIERP) research cruise (Ray et al. 2006). Clam soft tissues were extracted from their shells and analyzed for $\delta^{13}C$ and $\delta^{15}N$ values. Mean and standard deviation of non-lipid-extracted bulk isotope values were used in the mixing model (Table 4.2, Fig. 4.1). To address potential HTLP items contributing to walrus diets, $\delta^{13}C$ and $\delta^{15}N$ values for ringed and bearded (*Erignathus barbatus*) seal muscle were provided by L. Horstmann-Dehn, UAF (Dehn et al. 2007, Table 4.2, Fig. 4.1). Seals were collected from Alaska Native

subsistence harvests between 1996 and 2003 in the Bering and Chukchi seas. Both species consume benthic and pelagic fishes, crustaceans, and benthic invertebrates and both occupy a similar trophic level distinct from typical bivalve walrus prey (Dehn et al. 2007). We therefore used mean and standard deviation of $\delta^{13}C$ and $\delta^{15}N$ values for both ice seal species combined in the mixing model. As with walrus samples, ringed and bearded seal tissues were analyzed without lipid-extraction due to the lean quality of the muscle (Hoekstra et al. 2002; Dehn et al. 2007). Ribbon seals (*Histriophoca fasciata*) were omitted for the purposes of this study as they have not been documented in walrus stomachs (Burns 1970; Sheffield et al. 2001). While spotted seal (*Phoca largha*) remains have occasionally been retrieved from walrus stomach content (Lowry and Fay 1984), this species was excluded from modeling input as the only isotopic information available was for young-of-the-year (YOY) animals. The isotope signatures of tissues from YOY may not be representative of the entire population due to maternal influences (Jenkins et al. 2001). We acknowledge that maternal influence on SI values of YOY varies by species (Jenkins et al. 2001) and previous research has shown no age-related difference for SI values for spotted seals (Dehn et al. 2007). However, spotted seals were also omitted from the model because habitat overlap of spotted seals with walruses is not as extensive as the overlap among walruses and ringed and bearded seals (Lowry and Fay 1984). This suggests that spotted seals are less likely to be subject to predation by walruses than other pinniped species. Demersal fishes were excluded from the model because of the rarity of their occurrence in walrus stomachs regardless of consideration for differing rates at which different species' bodies are digested, due to variations in

tissue composition between species (Fay 1982; Sheffield and Grebmeier 2009). Research by Fay et al. (1983) also indicated that when seals are consumed, they constitute by weight the greatest proportion of the stomach contents, followed by benthic invertebrates. Large quantities of prey, even if ingested only occasionally, have a greater impact on the isotopic signature of consumer tissues than smaller quantities of occasionally consumed prey (Peterson and Fry 1987).

Lumbar muscle was the preferred tissue for SI analysis when multiple tissues were available from a single walrus due to ease of obtainment, future use in comparison studies, and popularity of this tissue in other marine mammal stable isotope investigations (Atwell et al. 1998; Newsome et al. 2010; Hückstädt et al. 2011). In the absence of lumbar muscle, tongue was analyzed as previous work by our lab has indicated that these tissues provide statistically comparable stable isotope data (Tukey, $P<0.05$, tongue 23.0% ± 10, $\delta^{15}N$ 13.0 ± 0.05‰, $\delta^{13}C$ -17.0 ± 0.05; muscle 22.0 ± 10%, $\delta^{15}N$ 13.0 ± 0.9‰, $\delta^{13}C$ -17.0 ± 0.8‰). Table 4.2 and Fig. 4.1 present mean carbon-13 and nitrogen-15 isotope signatures of Pacific walrus muscle (lumbar and tongue combined) and representative prey.

4.3.3.3 Tissue Turnover Rate

To date, the turnover rate of walrus tissues has not been investigated. To provide a temporal context to our results, we extrapolated muscle turnover for male and female walruses using previously published muscle turnover rates for mice (*Mus musculus*), gerbils (*Meriones unguiculatus*), alpacas (*Lamas pacos*), and steers (*Bos primigenius*) to

create a curvelinear regression from which the half-life of isotopic incorporation rate of the mammal muscle tissue with known body mass can be predicted, following the extrapolation equation (below) developed by Carroll (2012):

(3) $Half\text{-}life\ of\ Isotopic\ Incorporation\ into\ Muscle\ (d) = 180.15 - 369.35 * (x^{-0.25})$

where x is the lean body mass of the animal (in g) (Tieszen et al. 1983; MacAvoy et al. 2005; Sponheimer et al. 2006; Bahar et al. 2009; Kolokotrones et al. 2010). Tissue turnover rate was calculated as four times the half-life (half-life equals 50%, 2x half-life equals 75%, 3x half-life equals 87.5%, 4x half-life equals 93.75%).

While pinniped muscle is lean, the mass of the blubber layer is substantial and dictates the need to account for it when estimating tissue turnover rates (Harington 2008). Mean percent blubber has only been documented for Atlantic walruses (*Odobenus rosmarus rosmarus*) measured during the summer months (April-August), with blubber constituting 15% of total body mass in adult males and 19% of body mass in adult females (Knutsen and Born 1994). Pacific walrus lean mass was calculated by subtracting percent blubber from mean body mass of adult males (1210 kg) and females (832 kg) (documented by Fay 1982). We note that these estimates utilize available % blubber values during summer months and that blubber thickness may be higher during the winter season.

Estimates from the SIAR model of the proportional contribution of HTLP to the diet of each walrus was also compared with the normalized weighted mean annual sea ice extent (mil km^2) for the Bering Sea (encompassing the greater part of Pacific walrus

range) obtained from the National Snow and Ice Data Center (NSIDC) for years reflected by the isotopic signature of each muscle sample. To assess possible patterns between walrus diet and sea ice extent over a larger geographical and ecological scale, comparisons were made between HTLP and the normalized weighted mean Arctic sea ice minima (i.e., September) for the entire Arctic Ocean (provided by NSIDC 2012a, b).

4.3.4 Statistical Analyses

Statistical analyses and visual representation of data were performed in SigmaPlot (version 10.0, TE Sub Systems Inc and Sax Software 2006). Data failed normality and homogeneity assumptions, thus distribution-free statistics (i.e., Spearman rank correlations) were run among proportional contribution of HTLP, year, and sea ice extent. As overall sample size was large (n=155) and normality likely failed due to the opportunistic nature of sample collection, multilinear regressions were performed with standardized sea ice data. Among-year comparisons of HTLP (to identify differences in HTLP among years/ice extents) failed normality assumptions and were performed using one-way analysis of variance (ANOVA) on ranks (Iman and Conover 1979) followed by Tukey's pairwise multiple comparison test. An alpha of ≤0.05 was considered significant for all statistical analyses.

4.4 Results

Walrus muscle turnover rate was estimated at ~2 years for adults, regardless of sex (Table 4.3, Fig. 4.2). In addition, the use of either total or lean body mass did not affect the turnover rate (Table 4.3). Mode proportional contribution of HTLP varied

among sample years, from 13.0% ±8% for samples collected in 2010 to 23.0% ±6% for 2009 samples; Table 4.4). Based on these results normalized, the weighted mean sea ice extent and sea ice minima were calculated as follows:

(4) *Weighted mean ice extent= ([Year A ice extent*80%] + [Year B ice extent*20%])*

where "Year A" is the year immediately prior to sample collection and "Year B" is the year prior to "Year A". The percentages are normalized turnover rates for year A (75% normalized to 100%) and Year B (18.75 normalized to 100%). Weighted mean ice extents are presented in Table 4.4.

Results of the multiple linear regression indicated that there is a correlation between time period and diet (P=0.009). Our one way ANOVA showed that the integrated diets of walruses sampled in 2010 had significantly lower contributions of HTLP compared to 2002, 2003, and 2009 (P<0.01; Fig. 4.3). Comparisons of HTLP estimates among years indicate a significant difference between diets of walruses sampled in 2002, 2003, and 2009 and diets of animals sampled in 2010 (Tukey, P<0.05; Table 4.5, Fig. 4.3). Though %HTLP increased from 2001-2009, no significant difference was found between the diets of walruses sampled during that period (P>0.05, Table 5, Fig. 4.3), nor was the estimate of HTLP for walruses sampled in 2001 different from individuals sampled in 2010 (P>0.05, Table 4.5, Fig. 4.3). For walruses sampled in 2001-2003, and 2009, reliance on HTLP remained relatively steady (between 19.0% and 22.0%, Table 4.4, Fig. 4.3). However, walruses sampled in 2010 displayed a decrease in reliance on HTLP of about 10% (Table 4.4, Fig. 4.3).

SI analysis of whisker subsections showed that the $\delta^{15}N$ and $\delta^{13}C$ values changed over the length of the whisker, ranging from 16.7‰ to 13.1‰ and -15.5‰ to -17.4‰, respectively (Fig. 4.4). The fluctuations in $\delta^{13}C$ values were negatively correlated to the $\delta^{15}N$ values ($r = 0.89$). Based on *in situ* studies of Steller sea lion whisker growth rates (Hirons et al. 2001), the whisker of the seal-eating walrus used in this study is estimated to reflect the past 2.5 years of the animal's diet. It should be acknowledged, however, that the growth rate of walrus vibrissae and seasonal variations thereof are unknown, thus this estimate, though accurate to the best of our ability may deviate from the actual growth rate.

4.5 Discussion

Factors influencing walrus reliance on HTLP are complex. Our mixing model analysis of walrus stable isotope data indicates that between 1999 and 2009, over the seven years reflected by our samples, contribution of HTLP varied between 13.0 and 23.0%, producing a mode of 19.0% ±8% for the entire data set examined. While this proportion is higher than historical stomach content analyses of Pacific walruses, which put reliance on HTLP at approximately 10% (Lowry and Fay 1984; Sheffield and Grebmeier 2009), it more closely matches the level of HTLP consumption suggested from bio-accumulating contaminant analysis of Atlantic walruses (24.5%; 13 of 53 animals, Muir et al. 1995). Stomach content analysis is a method biased towards hard-bodied prey items, and thus prior research not taking the effects of digestion into account may have underestimated the contribution of HTLP as walruses preferentially consume

muscle and blubber of seals (Lowry and Fay 1984; Fay et al. 1990).The overall higher reliance on HTLP by walruses revealed by our research when compared to stomach contents data (Fay 1960; Fay et al. 1977; Lowry and Fay 1984; Sheffield and Grebmeier 2009) could also partially be the result of benthic-pelagic uncoupling in the Arctic and sub-Arctic marine environments (Grebmeier et al. 2006). Alterations in Arctic and sub-Arctic prey populations in response to environmental variables have been documented and are not yet fully understood (Grebmeier 2012). Quakenbush et al. (2011a) documented that between 1970 and 2010 ringed seals transitioned to pelagic prey from benthic crustaceans and demersal fishes. Over the same time period, the frequency of mollusks in bearded seal stomachs declined (Quakenbush et al. 2011b). These findings indicated that observed dietary changes correspond to changes in the Arctic food web as a result of the 1976/77 regime shift (Quakenbush et al. 2011a, b). The regime shift triggered a cascading benthic-pelagic uncoupling (Grebmeier et al. 2006), with decreased energy exported to the benthos, thus producing a decrease in benthic productivity, and favoring a pelagic ecosystem. Recent stable isotope diet studies of ringed and bearded seals also indicate declines in reliance of these ice seal species on benthic prey, possibly corresponding to the recent Arctic sea ice minima (Carroll et al. 2013). Conversely, Coyle et al. (2007) reported a 50% decrease in the Arctic amphipod prey of gray whales (*Eschrichtius robustus*) during 2002-2003 compared to the 1980s, likely due to top-down impacts of predation by gray whales. Analysis indicated predation by an increasing population of gray whales and not climatic changes to be the cause of the amphipod decline (Coyle et al. 2007). Regardless of the cause, declines in benthic biomass

suggested by these Arctic studies would likely also affect walrus foraging success and could have adverse consequences on walrus energetics.

One notable feature of our data is the highest HTLP consumption by walruses was observed in samples reflecting walrus diets in 2007 and 2008 (Table 4.4). The normalized weighted mean Arctic sea ice minimum during this period exhibited the most extreme retreat (4.6 mil km^2) of all periods reflected by our samples (Table 4.4). The simultaneous decrease in Arctic ice extent and overall increased contribution of HTLP to walrus diets from 1999-2008, although not significant, is consistent with Fay (1960) and Rausch et al. (2007) hypothesis that decreased sea ice leads to an increased reliance of HTLP. However, while the normalized weighted average Arctic sea ice minimum in 2008-2009 is higher than 2007-2008 (Table 4.4, Fig. 4.3), the annual sea ice minimum continues to show increasingly severe ice loss (NSIDC 2012a; Stroeve et al. 2012). Whether a decrease in reliance on HTLP as seen in samples collected during the 2010 harvest is an indicator of future dietary trends, where ice-reliant HTLP species become less accessible in the absence of sea ice, remains to be seen. During the years covered by this study, Arctic sea ice minima decreased annually from 7 mil km^2 in 2001 to 4 mil km^2 in 2007; Pan-arctic sea ice minima remained low compared with historical data in both 2008 and 2009 (5 mil km^2). Over the same time period, mean annual sea ice extent in the Bering Sea increased from 0.3 mil km^2 in 2000 to 0.4 mil km^2 in 2009, compounding any data interpretations (NSIDC 2012a). If localized sea ice impacts (i.e., Bering Sea) have greater influence on walrus diet than pan-Arctic ice trends, the increased consumption of

HTLP reflected in the 2002-2009 samples (Table 4.3) is contrary to Fay and Rausch's hypothesis. However, the low contribution of HTLP to walrus diets in 2001 and 2010 samples, when weighted mean Bering Sea ice extent was highest, is consistent with Fay and Rausch's hypothesis and could indicate that expanding ice extent improves access to benthic prey. The opportunistic nature of walrus tissue sample collection creates the potential for biased results, particularly in later years of our study when more samples were available for analysis (Table 4.1). Interpretation of our data is also limited by the caveats of the SIAR program and SI analysis. Estimates produced by the SIAR program are precise; however, they only represent probable outcomes. SIAR also assumes that all stable isotopes are incorporated into the body equally and no routing occurs (Parnell et al. 2010). Substitution of tissue turnover rates when data specific to walruses are not available, could also lead to pointedly different model outputs (Bond and Diamond 2011). In addition, carbon isotope signatures can vary in response to carbon fluctuations in the food web. High organic carbon input into the Bering and Chukchi seas food web by primary producers is associated with high productivity as a result of the tight benthic-pelagic coupling characteristic of the ecosystem (Grebmeier 1993; Grebmeier et al. 1995; Grebmeier and Dunton 2000). Organic carbon availability would decrease the incorporation of the heavier carbon isotope (^{13}C) into consumer tissues, altering the carbon-13/carbon-12 ratio and thus the carbon isotopic signature of the tissue (Peterson and Fry 1987; Søreide et al. 2006). Changes in the isotopic signature of lower trophic level organisms from fluctuations in carbon availability would radiate though the food web, potentially alternating results of mixing models and preventing direct comparison of

modeling outputs from different studies. To fully explore the potential correlation between sea ice extent and consumption of HTLP by walruses, historical samples from previous decades as well as samples from recent sea ice minima years are needed.

Unfortunately, quality soft tissue samples, particularly from periods earlier in the century, are lacking, however, other hard tissues (e.g., teeth, whiskers) can provide dietary information over multiple years of an individual's life. To this effect, our opportunistic SI analysis of the whisker from the known seal-eating walrus provides a detailed timeline of the animal's reliance on HTLP. Our analysis showed periodic variations in foraging on HTLP in this individual (Fig. 4.4). The $\delta^{15}N$ values from the whisker ranged from 13.0‰ to 17.0‰. Correspondingly, oscillations in the $\delta^{13}C$ value inversely mirror those seen in the $\delta^{15}N$ values, ranging from -16.0‰ to -17.0‰. The negative correlation between $\delta^{13}C$ and $\delta^{15}N$ values is indicative of a walrus opportunistically preying on ice-associated pinnipeds (i.e., ringed seals) as decreased $\delta^{13}C$ values are typical of pelagic seal species (i.e., species that feed in open water as opposed to indirect association with the sea floor or benthos) compared to benthic feeders (Dehn et al. 2007; Herreman et al. 2008). For walruses, pelagically-sourced prey is limited to ringed seals, as this phocid also ranges nearshore and walruses are physiologically limited to shallow diving (Fay and Burns 1988). Alternatively or in addition, walruses are known to preferentially consume the blubber and muscle of seals (Fay 1982; Lowry and Fay 1984; Fay et al. 1990). Lipids typically have relatively lower $\delta^{13}C$ values compared to proteins (DeNiro and Epstein 1977); therefore, blubber has

relatively low $\delta^{13}C$ values compared to lean muscle. This can then also be observed in the association of low $\delta^{13}C$ values and high $\delta^{15}N$ values along the whisker length. SI studies on sea otter whiskers suggest that the carbon present in vibrissae is in fact representative of diet (i.e., sourced from ingested nutrients and not synthesized by the body, Newsome et al. 2009). We acknowledge that while the 2011 seal-eating walrus experienced obvious oscillations in diet composition, walrus whisker growth rate may vary seasonally and thus isotopic information may not be laid down at a constant rate. In many species, growth rates slow during periods of low food availability, hibernation, and other aspects of life history (Zhao and Schell 2004; Barboza et al. 2009). While male walruses do not exhibit a mandatory annual fasting period, it appears they decrease food intake during the December-March breeding season (Fay 1982). If food intake is not sufficient to meet energy demands and the animals own internal stores are metabolized to compensate for the energy deficit, a high $\delta^{15}N$ value can be reflected in any tissue formed during that period (Newsome et al. 2010). In addition to a higher $\delta^{15}N$ value, whisker growth rate may slow due to lack of nutrients or nutrient reallocation (Barboza et al. 2009). This scenario would result in a peak in $\delta^{15}N$ values within a small section of the timeline reflected by the whisker. Due to the combination of unknown variables affecting whisker growth rate, results should be interpreted cautiously. However, regardless of variation in growth rate, the sources of carbon and nitrogen to this seal-eater varied over time and have included both pinnipeds and bivalves, with the increase in $\delta^{15}N$ value and decrease in $\delta^{13}C$ value at the base of the whisker consistent with observed stomach content. This variation is harmonious with related SI findings by our laboratory indicating that the

majority of walruses are opportunistic foragers relying on prey of different trophic levels (Table 4.2). These changes in feeding on different trophic levels or different prey suggest that a series of external variables influence the proportion of walrus diet comprised of seals or similar HTLP (Fig. 4.4). Specifically, sea ice extent is likely not the sole primary factor controlling prey-switching in walruses, but larger climatic factors, such as regime changes may be influential. Fluctuations in HTLP reliance may be the result of changing environmental conditions affecting the prey base or alterations in the intensity of foraging competition. Alternately, these changes could occur due to the use of different foraging grounds across seasons and years. While male walruses primarily disperse to the Bristol Bay and Chukotka regions during summer months (Fay 1982), recent years have seen more mixed sex herds occupying summer habitat in the Chukchi Sea historically primarily used by females and calves (Garlich-Miller et al. 2011). Various foraging areas have different biodiversity and biomass of prey species, including seals, and will be differently affected by environmental and seasonal change (Bluhm et al. 2011; Weslawski et al. 2011). Furthermore, cyclical variation in prey populations are well known phenomena in both the terrestrial and marine environments (McCauley and Murdoch 1987; Norrdahl 1995; Krebs et al. 2001) and can have adverse effects on predator population health if prey abundance is reduced to levels below those necessary to sustain predator populations.

The wide summer dispersal range of walruses may result in variations in the magnitude to which walruses in different regions are exposed to diet-influencing

variables. We believe that geographic comparisons within years could be an appropriate direction in which to expand this work. However, the absence of samples from multiple locations (e.g., Cape Peirce and Barrow) within given years (2009 excepted) precludes us from pursuing this comparison. Furthermore, it can be assumed that, because the majority of animals migrate annually through the Bering Strait to summer grounds in the Chukchi Sea (Fay 1982) and because muscle tissue has a turnover rate of ~2 years, the SI signatures of muscle reflects foraging in both regions.

Changes in consumption of certain prey types can be the result of alterations in prey availability and accessibility and may lead to declines in individual and population-level health of the consumer (Kutz et al. 2005; Fischbach et al. 2007; Burek et al. 2008; Garlich-Miller et al. 2011). When assessing impacts of prey-switching, it is important to consider the energetic intake and expenditures associated with pursuing and processing different or uncommonly encountered prey (Rosen et al. 2007; Barboza et al. 2009). Born et al. (2003) estimated that an adult walrus, at 1,200 kg body mass, requires or the soft tissues of an estimated 4,000 bivalves/day (at 5g/bivalve wet weight), with each clam providing approximately 64 kJ dry weight (21 kJ/g dry weight) in absorbable nutrients (Hondolero et al. 2012). In comparison, ice seal blubber and muscle provide 34.5 kJ/g and 5.20 kJ/g dry weight, respectively (Fay 1982; Kuhnlein and Soueida 1992; Jorde and Owen 1988). While pinniped tissue, blubber in particular, may serve as a preferable source of nutrients, the mobile nature of these mammals compared to the relatively sessile sediment-dwelling invertebrate prey combined with the increased costs associated

with handling larger prey may negate the perceived benefits of preying upon seals and seabirds (Carbone et al. 2007).

Regardless of known predation on vertebrate marine life, morphological studies show walruses are adapted to prey on benthic invertebrates (Fay 1982). While multiple field observations of walruses in the vicinity of seal and bird carcasses exist (Fay 1982; Mallory et al. 2004; Fox et al. 2010), few eye-witness accounts describe the manner in which walruses capture seals and/or sea birds, (Breshin 1958; Fay 1960; Krylov 1971; Fay et al. 1977; Lowry and Fay 1984; Sease 1984; Burns et al. 1985; Merrick and Hills 1988; Fay et al. 1990; Gjertz 1990; Muir et al. 1995; Mallory et al. 2004; Wolkers et al. 2006; Fox et al. 2010). If reliance on HTLP still exists under more amenable ice conditions, as suggested by our analyses, it is likely that walruses depend on these prey opportunistically. The presence of large amounts of suitable sea ice habitat may reduce stressors, and result in easier access to HTLP prey and reduced foraging trip length. A reduction in nutritional stress and energetic demands encountered under optimal ice and foraging conditions may allow walruses to pursue prey that are energetically demanding to capture and process without adverse impacts to body condition. Similar trends in the pursuit and consumption of larger, higher trophic level organisms when energetic demands are low (and vice versa during periods of environmental stress, i.e., droughts, cold periods) have been documented in other marine and terrestrial species (Woodroffe et al. 2007; Beaulieu et al. 2009).

Future predictions suggest that walrus benthic invertebrate prey species will be increasingly adversely affected by early sea ice retreat and subsequent decline in primary productivity, resulting in declines in abundance and nutrient content of these prey types (Garlich-Miller et al. 2011). Atlantic walruses rely on seabirds and seals as an important food source, possibly due to a narrow continental shelf that limits both suitable bivalve-foraging habitat and walrus habitat near coastal regions, thus restricting access to benthic invertebrates within energetically-efficient foraging range and diving capabilities (Muir et al. 1995; Mallory et al. 2004; Fox et al. 2010). Fluctuations in reliance on HTLP have not been quantitatively reported in the Atlantic sub-species. Whether climate-induced declines in traditional benthic invertebrate prey press Pacific walruses to increase overall forage on HTLP cannot be concluded from the available data. It may be that walruses are able to adapt to changes in benthic prey types to offset changes in traditional prey base as has been shown from stable isotope analysis of bearded seal whiskers (Hindell et al. 2012). Additionally, impacts to any fluctuations in foraging may only be determined through more expansive efforts of investigation with robust sample sets representing years intermediate and prior to those examined in our study. We show that terminal growth tissues, such as whiskers, provide an individual-specific dietary timeline. This success in assessing diet history from a tissue that is relatively easily collected (and potentially by non-lethal means) holds substantial promise in the direction of future walrus dietary research.

For the purposes of management and conservation of the Pacific walrus population, a thorough investigation of seasonal variations and cycles in foraging should

be developed. We recommend future research pursue long-term dietary monitoring via tissue SI and SIAR modeling representing all seasons or the use of tissues characterizing long-term diet history (i.e., whiskers and teeth). However, SIAR modeling outputs should not be used in management decisions until walrus tissue turnover rates are determined from captive studies. Confirmation of walrus tissue turnover rates and whisker growth rates (via captive studies) will be a critical step in future research efforts. Concern for the status and abundance of ice seal and seabird populations, potential prey for walruses, further increases the need to understand trends and driving forces behind seal- and seabird-eating walruses.

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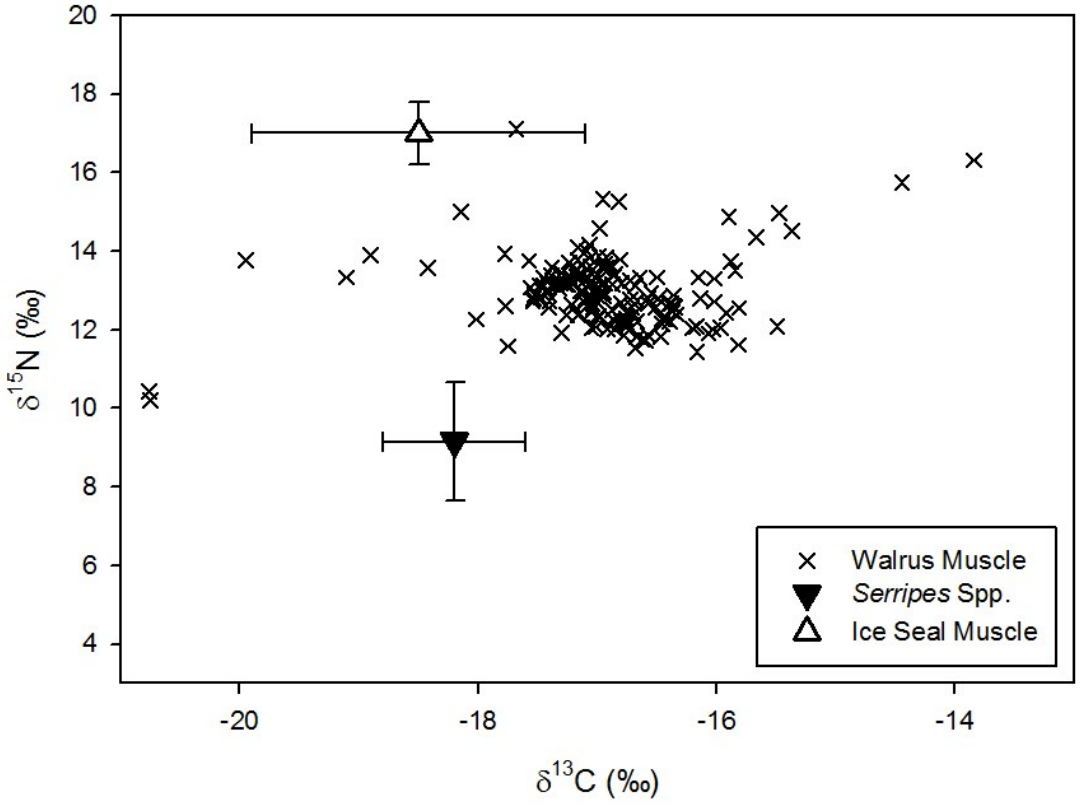

Fig. 4.1 Walrus muscle stable isotope ratios and stable isotope ratios of representative walrus prey

Scatter plot of Pacific walrus muscle stable nitrogen versus carbon isotope ratios (SI) and SI means and standard deviations for representative higher and lower trophic level walrus prey.

Note - *Serripes* spp. $\delta^{13}C$ and $\delta^{15}N$ values were provided by K. Iken, UAF (unpublished data). $\delta^{13}C$ and $\delta^{15}N$ values for ringed and bearded (*Erignathus barbatus*) seal muscle were provided by L. Horstmann-Dehn, UAF (Dehn et al. 2007). Both seal species consume benthic and pelagic fishes, crustaceans, and benthic invertebrates and both occupy a similar trophic level distinct from typical bivalve walrus prey (Dehn et al. 2007). We therefore used mean and standard deviation of $\delta^{13}C$ and $\delta^{15}N$ values for both ice seal species combined in the mixing model.

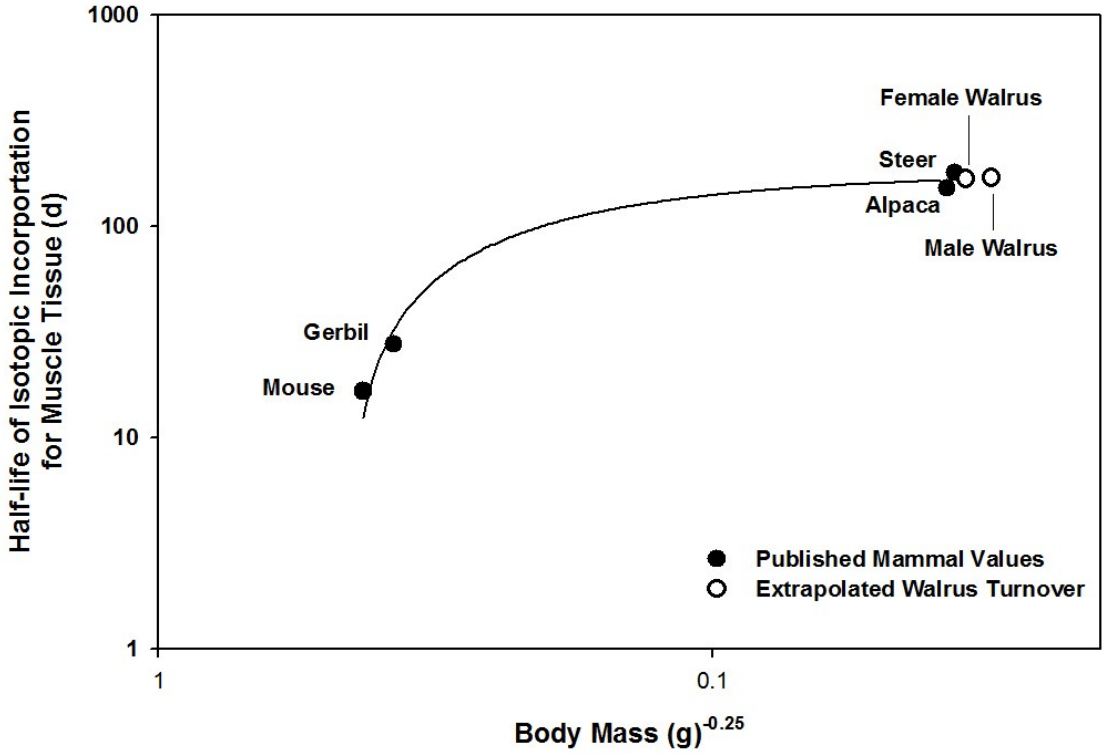

Fig. 4.2 Extrapolated half-life of isotopic incorporation for walrus muscle

Extrapolated half-life of isotopic incorporation for walrus muscle tissue (d) as calculated from a corrected curvilinear equation adapted from Carroll (2012) representing the relationship among body mass, metabolic rate, and tissue turnover rate (MacAvoy et al. 2005).

Note - Published values used to develop the equation are from the experimental studies of mouse (*Mus musculus*), gerbil (*Meriones unguiculatus*), alpaca (*Lama pacos*), and steer (*Bos primagenius*) (Tieszen et al. 1983, MacAvoy et al. 2005, Sponheimer et al. 2006, Bahar et al. 2009). Lean body mass (body mass minus estimated blubber weight: 15% for adult males, 19% for adult females) was calculated using values for Pacific walrus mass (from Fay 1982) and blubber values for Atlantic walruses from Knutsen and Born (1994) (blubber weight for Pacific walruses is not currently available). Body mass and turnover rates are detailed in Table 4.4.

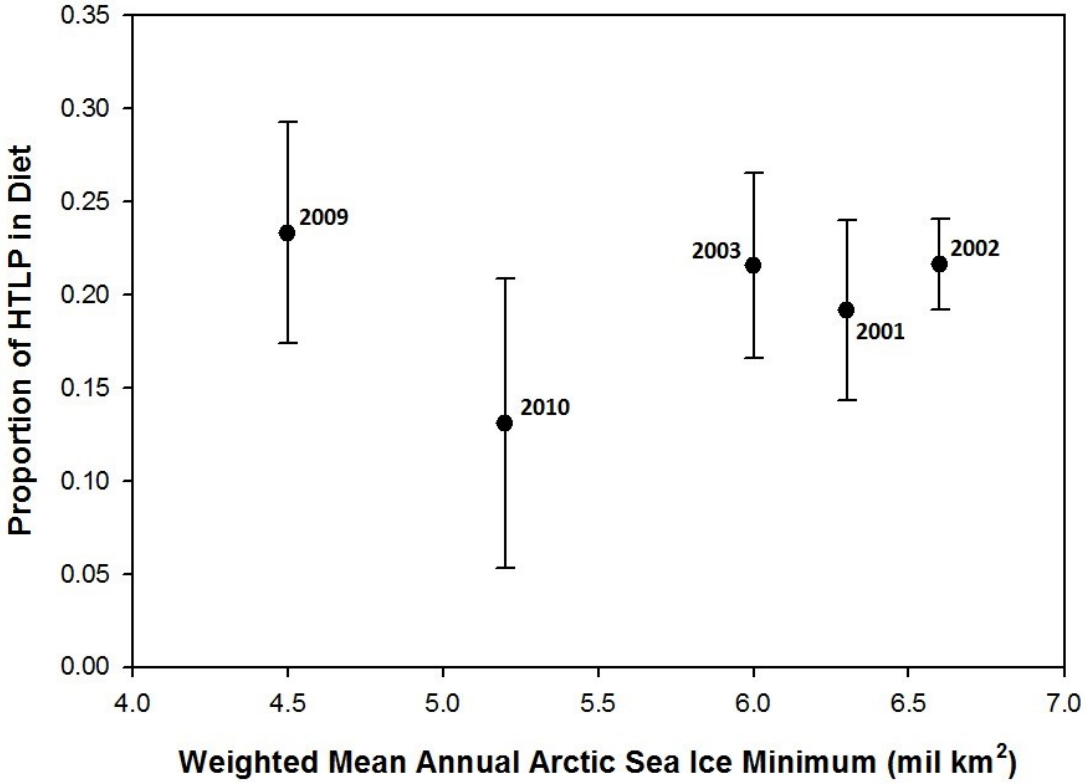

Fig. 4.3 Inter-annual variation in consumption of higher trophic level prey by walruses

Note - There was a slight increase in contribution of HTLP to walrus diet after 1999 (19% to 23%), and a significant decline in reliance on HTLP in samples collected in 2010 (P<0.05, 13%) compared to all other years except for samples collected in 2001 (P>0.05, 19%).

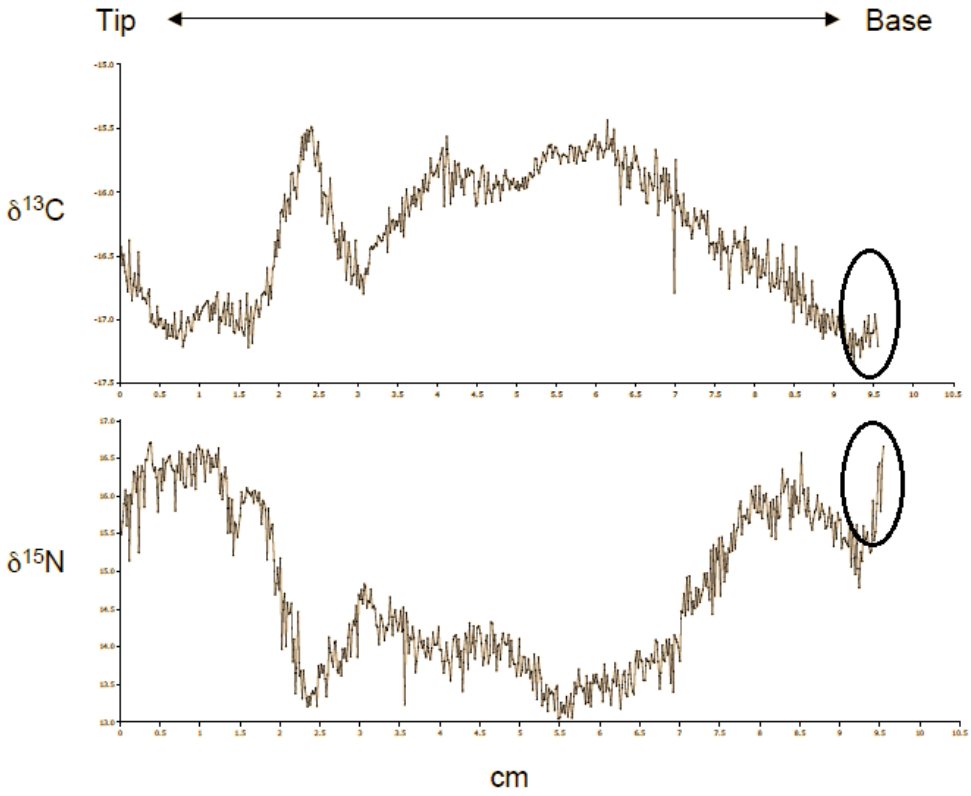

Fig. 4.4 Stable isotope values from a whisker of a seal-eating walrus

$\delta^{13}C$ (top) and $\delta^{15}N$ (bottom) values from stable isotope analysis of whisker subsections from a known seal-eating walrus (subsistence-harvested July 2012 in Barrow, Alaska).

Note - Based on Steller sea lion (*Eumetopias jubatus*) whisker growth rates (Hirons et al. 2001), the walrus whisker analyzed provides dietary information over the last two years of the animal's life. As new growth occurs at the whisker base, the base (circled in black) represents the most recent dietary information. Both $\delta^{13}C$ and $\delta^{15}N$ values at the base of the whisker are in accordance with a seal based diet as confirmed by stomach content analysis.

Table 4.1 Sampling year, locations, source, and sample sizes for muscle tissues examined

Location	Source	Type	Year	Sample (*n*)	
				Tongue Muscle	Lumbar Muscle
St. Lawrence Island	USFWS	Subsistence	2010	-	68
			2009	7	43
Barrow, Wainwright	NSB-DWM	Subsistence	2009	-	6
			2011	-	1
Cape Peirce	NPS[a]/Togiak NWR[b]	Mortality Event	2009	2	9
Various, Alaska	UA Museum of the North	Subsistence	2001-2003	-	19

Sampling year, locations (within Alaska), source, and sample sizes for muscle tissues examined (*n*=155 individuals).

[a] NPS: National Park Service

[b] NWF: National Wildlife Refuge

Table 4.2 Bulk stable isotope values and proportional contribution of higher trophic level prey to walrus diet

Species	Sample Size (*n*)	Mean $\delta^{15}N \pm SD$ [‰]	Mean $\delta^{13}C \pm SD$ [‰]
Serripes spp. soft tissue[b]	12	9.15 ± 1.5	-18.2 ± 0.6
Ice seal muscle[c]	137	17.0 ± 0.8	-18.5 ± 1.4
Ringed seal muscle[c]	82	16.0 ± 0.6	-18.5 ± 0.8
Bearded seal muscle[c]	55	16.8 ± 0.9	-17.1 ± 0.5

Mean ± standard deviation (SD) of bulk stable isotope values for walrus and representative prey (*Serripes* spp. for lower trophic level, mean value for bearded seals and ringed seals), and proportional contribution of higher trophic level prey (HTLP) to walrus diet by tissue, including enrichment factors for ringed seals[a] (mean diet-to-tissue isotope fractionation values) (Hobson et al. 1996).

[a] Enrichment factors are not available for walrus tissues. Values for ringed seal muscle were used, as ringed seals are the closest genetically-related animal for which enrichment factors have been determined

[b] Provided by K. Iken, UAF, unpublished data

[c] Provided by L. Horstmann-Dehn, UAF (ringed seal, *n*=82, bearded seal, *n*=55, Dehn et al. 2007)

Table 4.3 Tissue turnover rates for gerbil, alpaca, mouse, steer, and walrus muscle

Species	Mean Adult Body Mass (g)	Tissue Turnover Rate (d)
Mouse[b]	30	33
Gerbil[c]	50	55
Alpaca[d]	55,770	357
Steer[e]	493,000	302
Male Pacific Walrus	1,210,000	676
Female Pacific Walrus	832,000	671
Male Pacific Walrus (lean)[f]	1,029,000	674
Female Pacific Walrus (lean)[f]	674,000	669

Tissue turnover rates[a] (d) for gerbil (*Meriones unguiculatus*), alpaca (*Lama pacos*), mouse (*Mus musculus*), steer (*Bos primigenius*), and walrus muscle.

[a] Tissue turnover rate was calculated as four times the half-life (half-life equals 50%, 2x half-life equals 75%, 3x half-life equals 87.5%, 4x half-life equals 93.75%)

[b] MacAvoy et al. (2005)

[c] Tieszen et al. (1983)

[d] Sponheimer et al. (2006)

[e] Bahar et al. (2009)

[f] As Pacific walrus % blubber has not been documented, % blubber from Atlantic walruses was used (15% of body weight for adult males, 19% of body weight for adult females; Knutsen and Born 1994) and subtracted from total body mass values documented by Fay (1982)

Table 4.4 Contribution of higher trophic level prey to walrus diet by year and ice extent

Years Reflected by Sample	Normalized Weighted Mean Annual Sea Ice Minima (mil km^2)[a]	Normalized Weighted Mean Average Bering Sea Ice Extent(mil km^2)[a]	Tissue Sample Size (*n*)	HTLP (%)
2000, 1999	6.3	0.4	5	19.0 ± 4.8
2001, 2000	6.6	0.3	6	22.0 ± 2.3
2002, 2001	6.0	0.3	8	22.0 ± 5.0
2008, 2007	4.5	0.3	56	23.0 ± 5.7
2009, 2008	5.2	0.4	68	13.0 ± 7.9

Mode proportional contribution of higher trophic level prey (HTLP) to walrus diet ± standard deviation (SD) as estimated by SIAR mixing model by year, weighted mean annual sea ice minima for the Arctic Ocean (each September; in mil km^2), and weighted mean sea ice extent for the Bering Sea (in mil km^2).

[a] National Snow and Ice Data Center (NSIDC, 2012a)

Table 4.5 Comparison of time periods reflected by SIAR mixing model estimates

Comparison	P<0.05
2001 vs. 2002	no
2001 vs. 2003	no
2001 vs. 2009	no
2001 vs. 2010	no
2002 vs. 2003	no
2002 vs. 2009	no
2002 vs. 2010	**yes**
2003 vs. 2009	no
2003 vs. 2010	**yes**
2009 vs. 2010	**yes**

Comparison (one way ANOVA) of time periods reflected by SIAR mixing model estimates for proportional contribution of higher trophic level prey (HTLP) to the diet of walruses.

Chapter 5: Influence of Diet on Lipid Content and Cortisol Concentration in Blubber of Adult Pacific Walruses[4]

5.1 Abstract

Adverse changes in prey quality, quantity, and distribution can be stressors that lead to declines in body condition. We compared blubber concentrations of cortisol and total lipid with body condition in Pacific walruses (*Odobenus rosmarus divergens*) sampled during the 2009 and 2010 Alaskan subsistence harvests. We tested for correlations among these variables and estimates of dietary reliance on higher trophic level prey, sex, female reproductive status (presence or absence of peripartum lactation), and Native hunter visual assessment of body condition. Mean blubber cortisol concentration (n=38) was 13.60 ± 8.35 ng/g; mean %lipid of full-thickness blubber and skin (n=88) was 44.6 ± 12.4%, wet weight. The low %lipid of epidermis in the samples likely decreased overall %lipid; exclusion of the epidermis is recommended in future studies. Diet did not influence %lipid or cortisol concentration (P>0.05), nor were %lipid or cortisol concentrations correlated with visual body condition assessments (P>0.05). Females had higher %lipid than males (P<0.01); however, sex did not impact cortisol concentrations (P>0.05), nor did the female reproductive status influence %lipid or cortisol concentration (P>0.05), though stage of pregnancy/lactation may influence these

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[4] Seymour J, Horstmann-Dehn L-A, Rosa C, Atkinson S (Submitted to Mar Mamm Sci) Influence of Diet on Lipid Content and Cortisol Concentration in Blubber of Adult Pacific Walruses

results. Our findings suggest that, during the time period examined, moderate dietary changes did not appear to adversely affect the overall body condition of walruses.

Keywords: Blubber, cortisol, lipid content, Pacific walrus, diet

5.2 Introduction

The ability to quantify health (the presence or absence of injury and/or illness) of animals is a primary focus of animal science, from livestock to wildlife; however, determining the physical condition of animals is challenging, especially as the definition of health has evolved over time. Historically, research has relied on body condition as a proxy of health or fitness (hereafter used interchangeably), assessing condition by gross physical appearance (*e.g.*, Smith 1970; Lockyer *et al*. 1985; Jakob *et al*. 1996). More recently, biochemical methods have been developed that allow researchers to examine the microscopic conditions of tissues and to measure and produce biochemical ranges that are indicative of optimal body condition (*e.g.*, Brock *et al.* 2013; McKinney *et al.* 2014). For many species, however, development and validation of proxy measurements is still on-going, and are hampered by logistical difficulties. The remote ranges and legal protections of Arctic marine mammals make development and validation of methods for assessing health of these populations especially difficult. Currently, legal take, stranding events, catch-and-release work, non-lethal biopsy sampling, and photo documentation are the primary methods used to estimate body condition in marine mammals (*e.g.*, Lockyer *et al.* 1985; Arnould 1995; Pettis *et al*. 2004; Schulte-Hostedde *et al*. 2005; McKinney *et al*. 2014). Estimates of individual fitness are compared to established baselines and indices from captive or rehabilitative studies (*e.g.*, Beck *et al.* 1993; Castellini *et al.* 1993; Myers *et al*. 2006, 2010; Mashburn and Atkinson 2007; Trumble *et al*. 2013). These proxies are then extrapolated to predict overall marine mammal population health.

Innovative techniques now allow for the assessment of individual body condition proxies using minimally- or non-invasive methods: reproductive and stress hormones, contaminant concentrations, dietary information, and disease exposure can be evaluated from blood, muscle, saliva, blow, urine, and/or fecal samples (Thomson and Geraci 1986; Pietraszek and Atkinson 1994; Yoshioka *et al.* 1994; Fossi and Marsili 1997; Mashburn and Atkinson 2004; Pedernera-Romano *et al*. 2006; Hogg *et al*. 2009; Geiger *et al*. 2013). These techniques allow for a less biased sampling pool than necropsies and captive studies alone (Fossi and Marsili 1997). However, sample collection from free-ranging marine mammals is often logistically difficult and samples commonly used for health and stress assessment (*e.g.,* blood, urine) can be poorly representative due to capture stress or contamination (Ortiz and Worthy 2000; Romano *et al.* 2004). Collaborative efforts with Alaska Native subsistence communities allow for sample collection from animals hunted for food (Metcalf and Robards 2008). This method of obtaining tissues, while not without its own biases (*e.g.*, preferred harvest of healthy or younger/smaller animals), can provide large suites of tissues for analysis that aid in the establishment of much needed baselines (Garlich-Miller *et al*. 2006).

Cortisol, the primary biochemical indicator of the stress response in mammalian serum or plasma, is a lipophilic glucocorticoid hormone (Nelson 1995; Oki and Atkinson 2004; Myers *et al.* 2010) often measured to assess exposure to stressors, which, in high amounts can have adverse impacts to individual health (Thomson and Geraci 1986; Fair and Becker 2000; Mashburn and Atkinson 2004; Oki and Atkinson 2004; Romano *et al.* 2004). Other steroid hormones have been measured and validated successfully in the

blubber of free-ranging delphinids, showing that blubber can provide a minimally-invasive means of determining hormone concentrations associated with various aspects of life history and reproductive state (Kellar *et al.* 2006, 2009, 2013; Perez *et al.* 2011; Trego *et al*. 2013). Such studies show that secretions and tissues high in lipid can provide valuable information on steroid hormone production. Blubber may thus also be valuable for cortisol concentration measurements. Hormones and other lipid-soluble chemicals are sequestered in blubber longer than in tissues with shorter chemical residence time, such as blood (Liggins *et al*. 1993; Jarman *et al*. 1996; Zapol 2011). Cortisol concentrations in blood samples generally reflect adrenal activity over the past 30 min (St. Aubin and Geraci 1986). The short residence time of cortisol metabolites in blood (St. Aubin and Geraci 1986) diminishes their use in assessing a chronic stress response unless repeated sampling is used. Cortisol concentrations also normally fluctuate in response to various aspects of life history, including molting, pregnancy, and lactation (Oki and Atkinson 2004; Myers *et al.* 2010), thus interpretation should occur in conjunction with assessments of body condition and reproductive status, allowing other variables influencing the stress response to be considered.

Pacific walruses (*Odobenus rosmarus divergens*) are Arctic adapted pinnipeds with a thick blubber layer of up to 15 cm (Fay 1982). Walruses rely on sea ice for a substantial portion of their life history, thus increasing their susceptibility to habitat loss (an external stressor) through climate-induced sea ice retreat (Fay 1982; Garlich-Miller *et al*. 2011; Jay *et al*. 2011, 2012). The species' proclivity for close congregation on ice and land, and in adjacent open waters, renders blood and fecal sample collections logistically

difficult. Ongoing biopsy/satellite tagging and subsistence harvest bio-sampling programs allow access to blubber samples. Biopsy samples obtained from biopsy/satellite tagging studies, however, do not produce full-thickness epidermal and blubber cores (Hobbs *et al*. 2003). Blubber analyses provide information on aspects of individual health and life history, including reproductive status, contaminants, and diet composition (Norstrom and Muir 1994; Muir *et al.* 1995; Mansour *et al*. 2002; Kellar *et al.* 2006, 2009, 2013; Myers *et al*. 2012). Blubber thickness alone is not a reliable predictor of body condition due to a dense structural network of collagen that may act to restrict contraction of the blubber layer during lipolysis (Strandberg *et al*. 2008). Therefore, %lipid is generally considered a better gauge of condition than blubber thickness, particularly in species such as walruses, where sexes may undergo periodic but natural declines in blubber %lipid due to seasonal fasting or declines in food intake and/or lactation (West *et al*. 1979*a*, *b*; Beck *et al*. 1993). In marine mammals, blubber lipid content below the range in values associated with normal fluctuations in food intake or increased energetic demand (*e.g.*, lactation) is associated with late phase II fasting and/or phase III starvation (West *et al*. 1979*a*, *b*; Beck *et al.* 1993; Koopman *et al.* 2002; Gulland *et al.* 2005). Blubber %lipid, as well as blubber structural composition, varies by species (Strandberg *et al*. 2008), thus, inter-species health comparisons made *via* direct comparison of %lipid values are likely to be unreliable.

Walruses are considered benthic bivalve and gastropod specialist predators (Fay 1982); however, numerous other taxa have been described in their stomachs (Fay 1982; Sheffield and Grebmeier 2009). Changes in Arctic sea ice quality and extent are likely to

affect benthic invertebrate prey populations and prompt walruses to adopt alternative foraging strategies including opportunistic foraging on higher trophic level prey (HTLP) such as seals and seabirds (Rausch *et al.* 2007; Seymour *et al*. 2014*a*). Earlier and more extreme sea ice retreat results in "bottom-up" effects on the Arctic marine food web, such as decreased biomass input to the benthos and decreased prey quality (Grebmeier *et al.* 2006; Grebmeier 2012; Wang *et al.* 2013). Fay (1960) suggested that in nutritionally stressful situations Pacific walruses may prey on seals and incidence of this foraging strategy appears to be increasing in both the Pacific and Atlantic walrus (*Odobenus rosmarus rosmarus*) populations (Fay 1960; Lowry and Fay 1984; Wolkers *et al.* 2006). Previous mixing model analysis of walrus stable isotope (SI) data indicates that approximately 22% of walrus diet between 2000 and 2010 is HTLP (Seymour *et al.* 2014*b*).

In multiple marine mammal species prey shifts have been shown to result in an increased stress response (Rosen and Trites 2000), declining body condition (Thompson *et al*. 1997), increased disease susceptibility (Beck *et al*. 2004), decreased fecundity (Molnár *et al.* 2011), and altered contaminant exposure (McKinney *et al*. 2009); all of which can lead to population declines (Harwood *et al.* 2000; Burek *et al.* 2008). To assess potential impacts of prey shifting from benthic invertebrates to seals and seabirds on walrus population health, baseline data on "normal" body condition and stress response are needed. The ability to use biopsy samples to monitor diet (*via* SI analysis, Seymour *et al*. 2014*a*) and health proxies is invaluable; however, biopsy samples from live, darted animals are of relatively small size (10-60 cm^2, Thiemann *et al.* 2008) compared with

samples obtainable from harvested or deceased individuals. The limited amount of sample may restrict the number of analyses. While hormonal and SI analytical techniques require small amounts of tissue (0.3-0.5 mg, Seymour *et al*. 2014*a*), %lipid analyses are susceptible to mass-related measurement errors. Inclusion of the epidermis in %lipid blubber analyses may increase the amount of useable tissue from a biopsy, but could also skew absolute %lipid values (Hoekstra *et al*. 2002). Both blubber cortisol concentrations and body condition, as indicated by blubber %lipid, may provide insight into impacts of changes in the Arctic, including changes to the marine prey base and walrus population dynamics.

The objectives of this study were to 1) determine whether cortisol extraction techniques using enzymeimmunoassays (EIA) can be successfully applied to full-thickness walrus blubber cores, 2) develop a baseline range of species-specific values for cortisol concentrations in the blubber of walruses, 3) assess whether blubber biopsy samples with attached epidermis provide reliable %lipid data consistent with full-thickness blubber-only samples, 4) assess the potential association between quantitative evaluation of body condition *via* %lipid measures and qualitative evaluation *via* visual assessment by Alaska Native hunters, and 5) examine possible correlations among cortisol concentration, %lipid, sex, female reproductive status, and diet using previously estimated contributions of HTLP. We hypothesize that there will be sex- and diet-associated impacts on blubber %lipid and cortisol concentrations due to differences in reproductive energy demands and costs associated with processing large-bodied prey.

5.3 Materials and Methods

5.3.1 Sample Collection

Full-thickness sternal blubber samples (*n*=88) with attached epidermis were opportunistically collected during the Spring/Summer 2009 and 2010 Alaska Native walrus subsistence harvests on St. Lawrence Island, Alaska in the northern Bering Sea (April and May, *n*=81), and at Barrow and Wainwright (July, *n*=7), Alaska during 2009 and 2011 (Figure 5.1, Table 5.1). Samples were obtained in collaboration with community members, as well as the Eskimo Walrus Commission, the U.S. Fish and Wildlife Service (USFWS), and the North Slope Borough Department of Wildlife Management (NSB-DWM).

Sampling date, observed stomach contents, sex, and presence/absence of lactation (as an indication of peripartum reproductive status) were recorded for 81 of 88 walruses (with incomplete information for the remaining seven individuals), and subsequently each animal was given a unique identifier. Subsistence hunters also recorded their subjective perception of body condition for each harvested walrus. Categories were "very healthy", "average", and "unhealthy". Hunters generally based their assessment on blubber thickness and fat content which was built on personal experience from hunting and butchering walruses. Tissue samples were shipped frozen to the University of Alaska Fairbanks (UAF) Marine Mammal Laboratory and stored wrapped in aluminum foil in individual Ziploc^{TM} bags at -80 °C for 0-12 months prior to analysis.

5.3.2 Cortisol Extraction

Cortisol was extracted from (*n*=88) full-thickness blubber samples by the methods of Kellar *et al.* (2006). Briefly, subsamples ranging from 0.2-0.5 g in weight, depending on blubber thickness, were processed on a clean stainless steel or glass surface with individual, clean razor blades. Samples were homogenized in 1 mL of 100% ethanol using an automated, multiple-tube homogenization instrument (Disruptor Genie Digital, Scientific Industries) in 2 mL lysing matrix tubes with 1.4-mm ceramic spheres (FastPrep™ Lysing Matrix, MP Biomedicals) for eight 45 s intervals at a speed of 6.5 m/s. Cortisol was extracted and isolated through a succession of organic solvents (in order: ethanol; 4:1ethanol:acetone; diethyl ether; acetonitrile; and hexane), after each of which samples were evaporated to dryness under nitrogen. Cortisol extracts were then frozen at -20 °C until assays were performed.

5.3.3 Enzymeimmunoassay

Cortisol was determined *via* enzymeimmunoassays (EIA) using commercially available kits (Assay Designs Correlate-EIA™ Cortisol Enzymeimmunoassay Kit #900-071, validation data are included in following section). Frozen sample extracts were thawed and re-dissolved in 500 µL of phosphate buffered saline (pH 7.5) containing 1% bovine ɣ-globulin and vortexed for 15 min prior to EIA (Kellar *et al.* 2006). Plates were analyzed by a Spectramax 340PC plate reader at a wavelength of 405 nm with corrections between 570 and 590 nm using SOFTmax® Pro (version 4.0, Molecular Devices Corp. 2001) software. Standard concentrations were determined according to kit instructions.

Concentrations were as follows: 0.156, 0.313, 0.625, 1.250, 2.500, and 10.000 ng/g cortisol. Experimental concentrations were reported as mean ng of cortisol per gram wet-weight of sample.

Each sample was analyzed in duplicate and % bound values for each extract were calculated as follows:

(1) $$\%\ Bound = Net\ Optical\ Density / Net\ B_0 * 100$$

where net B_0 is the mean ng/g of the zero ng/g standard wells, and net optical density is the difference between the mean bound optical density and mean non-specific binding optical density. Blubber cortisol concentrations were then interpolated utilizing a four parameter logarithmic regression model of the % bound versus cortisol concentrations of the standards (SOFTmax® Pro (version 4.0, Molecular Devices Corp. 2001) software). Resulting concentrations were considered acceptable if the duplicate coefficient of variation (CV) was ≤20%, and if the concentration fell within 20-80% of the standard curve. If duplicate CV was >20% or samples did not fall within 20-80% of the standard curve, blubber was re-subsampled, re-extracted, and re-assayed until results were acceptable as defined above or until samples from a particular animal had been processed and analyzed four times (at which time it was removed from the sample pool).

5.3.3.1 Validation

Extraction efficiency was determined by spiking duplicate blubber samples with 0.01, 0.025, 0.050, and 0.075 mL of 10 ng/mL concentration of cold cortisol (the

equivalent of 1.0, 2.5, 5.0, and 7.5 ng of cortisol, respectively) prior to extraction and then calculating cortisol recovery. Estimated extraction efficiency of two sets of spiked samples was 75% and 85%. Parallelism was tested by comparing serial dilutions of pooled blubber extracts from five individuals with the known standard controls. The slope of these diluted samples was not significantly different from known standards (Mann-Whitney Rank Sums Test, P=0.32). Test sensitivity was calculated as 2 SD from the zero standard and was 0.33 ng/mL, linearity (slope of 1.05, correlation coefficient 0.999), and cross-reactivity were determined by the kit manufacturer using human and porcine samples and are in accordance with guidelines by the National Committee for Clinical Laboratory Standards Evaluation Protocols. Cross-reactivity for this EIA kit was as follows: cortisol (100%), prednisolone (122.35%), corticosterone (27.68%), 11-deoxycortisol (4.0%), progesterone (3.64%), prednisone (0.85%), testosterone (0.12%), androsterone (<0.1%), cortisone (<0.1%), and estradiol (<0.1%, Assay Designs 2001). No correlation was found between cortisol measurements and specific assay runs (Spearman's rank order correlation, P=0.22), indicating no interference dependent on which plate was used for sample analysis or order in which samples were analyzed. Inter-assay CV was 26%.

5.3.4 Lipid Extraction

Blubber samples (n=88) were stored at -80 ˚C until analysis. Subsamples of full-thickness blubber of approximately 1 cm^3 were cut from the center of each piece using sterilized knives and a sterilized glass cutting board to prevent contamination. Epidermis

was included to simulate the type of samples available during potential future walrus biopsy sampling. Samples were sub-sectioned lengthwise to produce two duplicate full-thickness samples for lipid extraction. Samples were freeze-dried on a VirTis Sentry™ freeze-dryer, re-weighed to determine loss of water, and then analyzed for lipid content at the UAF Marine Mammal Lab using chloroform: methanol (2:1) in a modified Soxhlet procedure after Schlechtriem *et al.* (2003). Each duplicate sample was weighed into a cellulose thimble of known mass and lipid-extracted for 24 h. If the CV of duplicates was >20%, blubber was re-subsampled and re-extracted, until data had a CV of ≤20% or until samples from a particular animal had been processed and analyzed four times (at which time it was removed from the sample pool) and %lipid (wet weight) was calculated.

To compare %lipid from full-thickness blubber samples including epidermis to values without epidermis, samples of full-thickness blubber (no epidermis) from randomly selected females and males from both 2009 and 2010 (for a total of 12 individuals) were lipid-extracted as described above. To assess potential variation in lipid content with blubber depth, a second set of full-thickness blubber cores from this subsection of samples was cut into equal halves ("outer blubber", closer to epidermis, and "inner blubber", closer to the internal musculature) and also lipid-extracted, as was full-thickness epidermis from these animals. Outer and inner blubber layers were determined by bisecting each full-thickness blubber (without epidermis) core sample in half horizontally. Depth of the resulting sample varied dependent on the full-thickness of the individual's blubber.

5.3.5 Estimation of the Contribution of Higher Trophic Level Prey

Briefly, Stable Isotope Analysis in R (SIAR), a Bayesian mixing model within R (version 2.12.2, Parnell *et al.* 2010; R Development Core Team 2011), was used to estimate proportional contribution of prey (*i.e.,* clam, seabird, and ice seal) to the diet of walruses (Seymour *et al*. 2014*a*). SI analyses were performed at the University of Alaska Fairbanks Stable Isotope Facility. Mode, standard deviation (SD), and range of $\delta^{13}C$, $\delta^{15}N$, and %HTLP are summarized in Table 5.2 for reference. Walrus $\delta^{13}C$ and $\delta^{15}N$ signatures for lumbar muscle, along with mean isotopic values for *Serripes* spp. (a common benthic bivalve prey, Fay 1982; Sheffield *et al*. 2001; Sheffield and Grebmeier 2009), ringed seal (*Pusa hispida*), and bearded seal (*Erignathus barbatus*) muscle, and spectacled eider (*Somateria fischeri*) muscle were input into SIAR (Seymour *et al*. 2014*a*). As tissue enrichment factors for walrus muscle are unknown, tissue enrichment factors for ringed seal (*Pusa hispida*) muscle were applied from Hobson *et al.* (1996) to estimate contributions of walrus prey that are isotopically distinct.

5.3.6 Statistical Analyses

Statistical analyses were performed in SigmaPlot (version 10.0, TE Sub Systems Inc and Sax Software 2006) and R (version 2.12.2, R Development Core Team 2011). MANOVA was used to assess differences in lipid content and cortisol concentrations with sex, female reproductive status, visually-assessed body condition, and proportional contribution of HTLP. Wilk's multivariate test was applied to MANOVA results to assess the probability that one or more variables influenced another. As %lipid and

cortisol data failed normality ($P<0.05$), one-way ANOVA on ranks (Iman and Conover 1979) was applied to combinations of all independent and dependent variables to determine higher level interactions that might not be uncovered by a MANOVA. Dunn's pairwise multiple comparison tests were used when ANOVAs indicated statistically significant differences to determine which groups differed. Spearman rank correlations were used to determine correlations among variables. An alpha of ≤0.05 was considered significant for all tests.

5.4 Results

5.4.1 Visually Assessed Body Condition

Of the samples provided, visually-assessed body condition ("very healthy", "average", or "unhealthy") was recorded for 81 of 88 animals. Fifty five individuals were described as "very healthy", 30 were "average", and three were noted by hunters to be in "unhealthy" condition (Table 5.3).

5.4.2 Blubber Cortisol

Cortisol concentrations with acceptable results were determined for 38 of 88 individuals. Mean cortisol concentration was 13.60 ± 8.35 ng/g with a wide range of concentrations (2.77 to 34.04 ng/g, Table 5.3, Figure 5.2). There was no significant association among cortisol concentration and any variable examined (ANOVA, $P>0.05$ each for sex, peripartum lactation, and visually-assessed body condition; MANOVA $F=0.50$, Wilk's $P=0.46$ for %lipid, %HTLP). Mean cortisol concentrations and concentration ranges arranged by categorical variable are summarized in Table 5.3.

5.4.3 Blubber Lipid Content

Mean %lipid of walrus full-thickness blubber with attached epidermis (*n*=88) was 44.6% ± 13% (wet weight, Table 5.4). There was no significant difference (ANOVA, *P*=0.23) between %lipid of the outer and inner blubber layers regardless of sampling year or sex (Table 5.5), thus %lipid of outer and inner blubber layers were averaged for comparison with %lipid of epidermis. Percent lipid in epidermis versus the combined outer and inner blubber layers showed significant differences (ANOVA, *P*<0.01, Dunn's Pairwise Multiple Comparison test, *P*<0.05) between %lipid of epidermis and that of blubber regardless of sampling year or sex (Table 5.5). Mean %lipid of epidermis (only) was lower than that of averaged combined outer and inner blubber: %lipid of epidermis was 9.1% ± 12% wet weight, while %lipid of mean combined outer and inner blubber without epidermis was 65.0% ± 11% wet weight (Table 5.5).

Full-thickness blubber and attached epidermis of females (*n*=63) had significantly higher %lipid compared with males (*n*=18) (ANOVA, *P*<0.01, Dunn's Pairwise Multiple Comparison test, *P*<0.05, Table 5.4). The full-thickness blubber with attached epidermis of peripartum female walruses (*n*=53, 47.4% ± 8.7%) had slightly lower, although not significantly different, %lipid than non-lactating females (*n*=9, 48.1% ± 14.9% wet weight, ANOVA, *P*=0.89, Table 5.4). The proportion of HTLP in walrus diet had no significant effect on blubber %lipid (MANOVA, *P*=0.50; Wilk's, *P*=0.46). Body condition, as visually-assessed by subsistence hunters, was not correlated with %lipid (ANOVA, *P*=0.06, Table 5.4).

5.5 Discussion

5.5.1 Blubber Cortisol

This is the first study to measure cortisol concentrations in Pacific walrus blubber. Thus, our analysis provides researchers with a baseline range for blubber cortisol concentrations in healthy, adult walruses, a proxy by which long-term stress responses can be assessed. Much of the difficulty with measuring cortisol concentrations in full-thickness walrus blubber relates to its greater thickness (up to 15 cm, Fay 1982) relative to more temperate marine mammal species (1-3 cm, Struntz *et al*. 2004). The thick blubber layer of walruses makes it difficult to subsample a full-thickness, uniform sample of small enough size for extraction and EIA. While the lack of %lipid stratification in walrus blubber suggests that cortisol concentration does not vary across blubber depth, further EIAs, RIAs, and/or histochemical tests are recommended to confirm that cortisol concentrations are uniform throughout the blubber layer. However, our findings of wide concentration ranges for this species may be typical for blubber cortisol of walruses and may reflect natural individual variability. Research on other marine mammal tissues has shown that cortisol concentrations can vary significantly among individuals of the same species (Gardiner and Hall 1997; Mashburn and Atkinson 2004, 2007) resulting in a broad spectrum of cortisol concentrations across a population. These broad ranges reflect individual variation resulting from individual susceptibility to various biological and environmental stressors. Thus, the environment surrounding each animal should be considered when interpreting results. For example, Oki and Atkinson (2004) found that

harbor seal (*Phoca vitulina*) plasma cortisol concentrations varied diurnally only in summer months. This seasonal difference may have been a reflection of increased exposure to sunlight in summer and/or differing demands on metabolic processes during winter months. Likewise, serum cortisol concentrations ranged widely in other marine mammals species examined according to season, age, sex, and body condition (Gardiner and Hall 1997; Oki and Atkinson 2004; Myers *et al.* 2006; 2010; Petrauskas *et al*. 2008). To date, only one published study examined cortisol in walruses (Tryland *et al.* 2009). These authors reported a cortisol concentration range from <10 to 78 ng/mL in plasma of two juvenile male Atlantic walruses (Tryland *et al.* 2009); however, in addition to the small sample size used in their study, the results of Tryland *et al*. (2009) were obtained in different laboratories and using different methods (RIAs in the case of Tryland *et al*. 2009). Because of the difference in laboratories and methods, the results from Tryland *et al.* (2009) are not directly comparable with our own results. Whether and how plasma cortisol concentrations correspond with measurements obtained from other sources (*e.g.*, blubber, urine, saliva) has not been investigated for walruses. It is likely that cortisol concentrations across different walrus tissues follow a similar pattern of variation as that of progesterone concentrations measured in bowhead whales (*Balaena mysticetus*), where hormone concentrations peaked first in serum, then in urine, and lastly, in blubber (Kellar *et al*. 2013). Confirmation of the difference in concentrations of cortisol among tissues with different hormone residence times will require captive studies similar to Gardiner and Hall (1997, harbor seal) and Mashburn and Atkinson (2004, Steller sea lion, *Eumetopias jubatus*), to gain an understanding of the influence of biological,

physiological, and ecological variables on cortisol concentrations. Captive studies, however, do not necessarily provide an accurate representation of free-ranging populations (Atkinson *et al*. 2009). For example, Gardiner and Hall (1997) found no effect of age, sex, or season on plasma cortisol concentrations of captive harbor seals, but all three variables influenced plasma cortisol concentrations in wild seals (156-176 ng/mL in captive animals versus 329-380 ng/mL in free-ranging animals). We therefore recommend that future research on cortisol in walruses include measurements of concentrations in different tissues and secretions (*e.g.*, serum, saliva, blubber) of both captive and free-ranging animals from different age classes, sexes, reproductive stages, season, and health status.

5.5.1.1 Effect of Sex and Female Reproductive Status

Cortisol concentrations may differ based on sex and reproductive status of the individual (Mashburn and Atkinson 2004, 2007; Kellar *et al.* 2006, 2009; Myers *et al*. 2006). While we interpret our findings cautiously, the lack of a significant difference in blubber cortisol concentrations between male and female walruses was initially unexpected. Cortisol and other hormones are often elevated in lactating marine mammals compared to non-lactating females due to biochemical processes associated with reproduction (Yoshioka *et al.* 1994; Atkinson and Yoshioka 2007). Peripartum females constituted the majority of female walruses analyzed (n=33 versus n=5 non-peripartum females, respectively) due to the opportunistic nature of our sample collection. It is likely that the range of cortisol concentrations in our study results from a cumulative effect of

steroid sequestration in blubber over multiple months as blubber biochemical residence times are longer than those of blood or feces (Hobson *et al.* 1996; Welle 1999). Short-term changes in hormone levels from reproductive events may thus not be detectable in blubber as blubber cortisol concentrations represent an average of the organism's cortisol production over time, possibly several months. In addition, lactating females may not accumulate additional blubber stores due to the increased energetic costs of lactation, possibly resulting in no new cortisol incorporation into blubber or increased concentration of mobilized cortisol in the blood. Cortisol can be passed from mother to offspring *via* milk in the same manner as organic contaminants (Addison and Brodie 1987; Brummelte *et al.* 2010; Wang *et al*. 2011, 2012). The broad range of cortisol concentrations in this study may also be the result of averaging cortisol concentrations across the depth of the blubber layer when the inner layer of blubber is hypothesized to be more metabolically active due to its proximity to muscle tissue (West *et al.* 1979*a*; Liwanag *et al*. 2012). Mobilization often occurs from the demands of reproduction (Wheatley *et al*. 2007, 2008) and during periods of decreased food intake such as the partial fasting period exhibited by male walruses during the mating season (West *et al*. 1979*a*). Blubber lipid deposition in marine mammals occurs at the inner layer (Ackman *et al*. 1975; West *et al*. 1979*a*). Fatty acid (FA) gradients occur through the depth of the blubber, with FAs in the inner layers (Ackman *et al*. 1975; West *et al*. 1979*a, b*; Wheatley *et al*. 2007; Skoglund *et al*. 2010) being most characteristic of recent dietary input (West *et al*. 1979*a*; Skoglund *et al*. 2010). Because different depths within blubber contain steroid concentrations that may reflect different time periods and/or different

deposition/mobilization events, blubber depth hormone profiles beyond those in the present study are needed to ascertain the degree of resolution *via* blubber hormone analysis.

Among other stressors, prolonged fasting can be associated with elevated cortisol concentrations in marine mammals (Exton *et al.* 1972; Ortiz *et al.* 2001) in association with lipolysis. Whether walrus males undergo a breeding-related fasting period during the mating season (January through March) has not been determined (Fay 1982), but decreases in foraging are likely (West *et al*. 1979*a*). As will be discussed below in the context of blubber %lipid, females may fast for brief periods after birth, but resume foraging shortly thereafter (Fay 1982). Any males fasting during the breeding season may exhibit higher cortisol concentrations in tissues such as serum relative to the non-breeding season (Ortiz *et al*. 2001, 2006). These higher concentrations may potentially be sequestered in remaining lipid tissues, as blubber acts as a reservoir for steroids circulating in the bloodstream (Deslypere *et al*. 1985). The degree to which cortisol is sequestered in inner blubber layers may be dependent on the rate of cortisol production and on the intensity of blubber mobilization to meet energy requirements. Outer blubber layers more likely reflect the "steady-state" of steroid hormones produced by the body over long time periods (Koopman *et al*. 2002). While all males analyzed in this study were adults, the blubber cortisol concentrations in this small sample (n=5) may not completely represent the male population segment.

5.5.1.2 Effects of Diet and Correlation with Visual Assessment

A transition to foraging on novel prey could result in a stress response to novel chemical and physical demands on the digestive system (Rosen and Trites 2000; Rosen *et al*. 2007; Rosen 2009) as well as a stress response from the increased energetic cost of pursuing and processing novel prey (Rosen and Trites 2000; Barboza *et al*. 2009). The absence of a correlation of diet with cortisol concentrations (Figure 5.3) provides preliminary support for the hypothesis that walruses opportunistically consume HTLP with little or no increased stress response.

As a hormonal indicator of stress, cortisol concentrations can be expected to increase after a change to a novel prey type (Boonstra 2013). As mentioned above, the period over which cortisol concentrations are reflected in blubber is longer than that of blood (Hobson *et al*. 1996). Thus, hormone concentrations in tissues with longer residence times, such as blubber (Hobson *et al*. 1996; Welle 1999), are less prone to show short-term fluctuations in cortisol production. As such, long-term dynamics reflecting changes in the prey base could be correlated in future studies, while short-term dietary changes may not be detectable in blubber.

The majority of walruses included in this study were “very healthy” or “healthy” based on the reports of experienced hunters. Thus, as the body condition of targeted animals was sufficient enough for them to be targeted for harvest, ranges in blubber cortisol concentration are likely due more to individual biochemical variation (*i.e.*, differences in hormone production or clearance), and/or individual variation in reproductive cycle or fasting period than to overall “unhealthy” body condition.

Likewise, “very healthy” and “healthy” individuals varied greatly in blubber %lipid. For example, the walrus with the highest blubber cortisol concentration (34.04 ng/g, an adult male harvested in 2010 and described by hunters as “very healthy”) had 23.6 %lipid wet weight value for full-thickness blubber with epidermis, which is at the low end of the range for males (Table 5.4). The lack of correlation between visually-assessed body condition and blubber %lipid indicates that perhaps neither method by itself is reliable for estimating the health of an individual walrus. Ideally, future investigations of blubber cortisol concentration and %lipid will include greater proportion of animals categorized as “unhealthy” in body condition, allowing further investigation of correlations that may exist between overall health and a diet-induced stress response.

5.5.2 Blubber Lipid Content

The lipid content of Pacific walruses determined in this study was lower than measurements in Atlantic walruses (~65% wet weight for combined inner and outer blubber with skin, this study versus ~83%, Muir *et al*. 1995) and other Arctic marine mammals, (75% for bowhead whales (Hoekstra et al. 2002) and 91-97% for ringed seals (Addison and Smith 1974). Marine mammal body condition assessments have historically relied on blubber depth measurements (Pitcher *et al*. 2000); however, blubber %lipid and fatty acid analysis may provide more detailed information of the body condition of marine mammals (Beck *et al*. 1993). Inclusion of epidermis in our samples, with its lower %lipid content, likely decreased the absolute %lipid values of each individual. While trends in %lipid of the population can still be monitored over time

when the epidermis is included, we recommend that this tissue be excluded from blubber %lipid assessments of body condition. In fact, our results may represent the lower end of annual %lipid as walruses rely on their fat stores during the energetically-demanding mating and calving season, which occurs in spring and coincides with the subsistence hunt on St. Lawrence Island (Fay 1982). Higher %lipid is common among female pinnipeds compared with males due to energetically costly reproductive events (*i.e.*, gestation, lactation, Boness and Bowen 1996). Additionally, differences in reproductive hormones (*e.g.*, estrogen or progesterone versus testosterone) between the sexes may result in greater accumulation of lipids in females than in males (Boness and Bowen 1996). The lack of significant variation in blubber %lipid of lactating and non-reproductive females was unexpected. The perceived similarity may be an artifact of the sampling period, because our tissue samples were collected during the peri- and postpartum period (Fay 1982), when many individuals were undoubtedly in the late stages of pregnancy or early stages of lactation.

Alaska Native traditional knowledge indicates that walruses with calves are fattest and thus most desirable when hunting. However, it is important to note that %lipid refers to quantity of lipid per gram of blubber and not total blubber weight. Our findings do not exclude the possibility that pregnant and recently post-partum females have thicker blubber layers and thus %lipid should not be considered a substitute for visually-assessed body condition as an indicator of health status. A more comprehensive approach to assessing body condition would be a combination of blubber %lipid, blubber depth, and visual assessment.

The uniform distribution of %lipid between outer and inner blubber layers indicates that %lipid can be obtained from non-full-thickness samples collected during spring (depth and composition of blubber may vary seasonally, Fay 1982; West *et al*. 1979*a*; Skoglund *et al*. 2010). Full-thickness blubber samples are still preferable for some types of analysis (*e.g.*, contaminant profiles, fatty acid profiles) due to chemical and physical stratification (Budge *et al.* 2008; Strandberg *et al*. 2008), and FA gradients that can occur through the blubber depth in other marine mammal species (Ackman *et al*. 1975; West *et al*. 1979*a*; Skoglund *et al*. 2010).

Prey-switching from one type of prey to another, can be accompanied by decreases in body condition as the energetic cost of foraging and prey handling may increase and eventually outweigh the predator's energy gain (Rosen and Trites 2000; Rosen *et al.* 2007; Barboza *et al.* 2009; Rosen 2009). The low variability in %lipid of walrus blubber regardless of prey consumption from different trophic levels suggests that walruses are physiologically adapted to manage and assimilate energy from both higher and lower trophic level prey. It is feasible that walruses relying largely on seals as prey would be able to consume large enough quantities to meet energy requirements (Seymour *et al.* 2014*b*); particularly as the blubber of ice-associated seals is calorically dense compared with the soft tissue of benthic invertebrates (Kuhnlein and Soueida 1992; Hondolero *et al.* 2012). Alternatively, the lack of correlation of HTLP and %lipid may indicate that walruses switch between invertebrate and vertebrate prey with high enough frequency that there is no measureable effect on energy stores by substituting prey. Walruses may be opportunistic predators, as indicated by stomach content analysis of a

known seal-eating walrus subsistence-harvested in Barrow, AK in summer 2011. This animal contained large quantities of *Mya* spp. periostracums in addition to muscle and blubber of two ringed seals (Seymour *et al.* 2014*b*). Not surprisingly, given %lipid and cortisol concentrations in this study, the individual was a young adult in good body condition (48 %lipid wet weight, blubber with attached epidermis; 15.78 ng/g cortisol). The lack of a correlation of dietary changes in prey of different trophic levels on body condition seen in this seal-eating walrus, and from our analyses of blubber %lipid, cortisol concentration, and diet suggest that prey-switching and/or prey substitution may not have an adverse effect on overall Pacific walrus health.

5.6 Conclusion

Our investigation indicates that multiple minimally-invasive methods (*i.e.,* analysis of blubber biopsy samples) can be used to assess walrus body condition. Analysis of blubber %lipid shows it to be uniform through sternal blubber depth. Determination of %lipid from biopsy may therefore be useful to on-going walrus monitoring programs; however, epidermis should be removed prior to analysis if absolute %lipid values are desired. Our research also shows that, regardless of reproductive status, the blubber of adult female walruses has higher %lipid compared with males. The higher lipid content in females is consistent with higher energy requirements associated with reproduction. Our study presents the first extraction of cortisol from walrus blubber, and the prospect of a minimally-invasive manner to track proxies of the stress response will serve as an invaluable tool for management, though further development and validation

of this method is needed before wide spread application. The absence of any statistical correlation of %lipid and cortisol concentrations with annual diet (*i.e.*, mode annual proportional consumption of HTLP) is a preliminary indication that the current walrus population is not experiencing adverse impacts to body condition from moderate degrees of prey switching.

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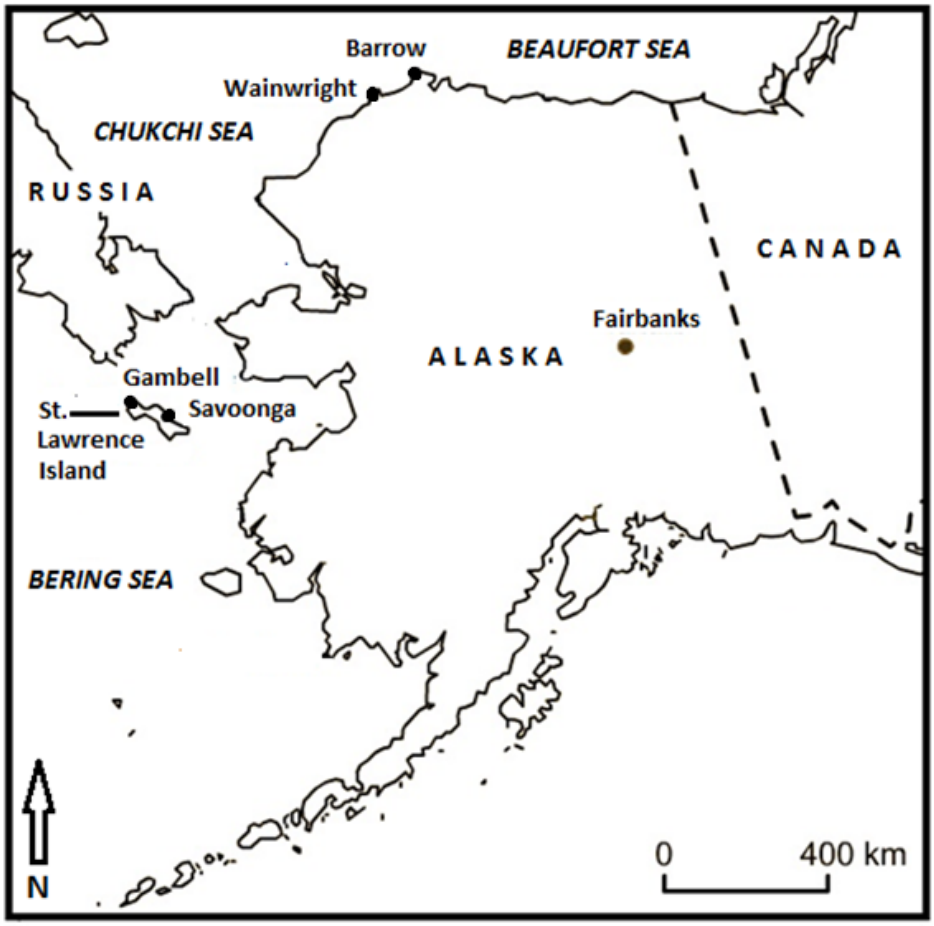

Fig. 5.1 Sampling locations for walrus tissues

Sampling locations for walrus tissues (Fairbanks is identified as a point of reference).

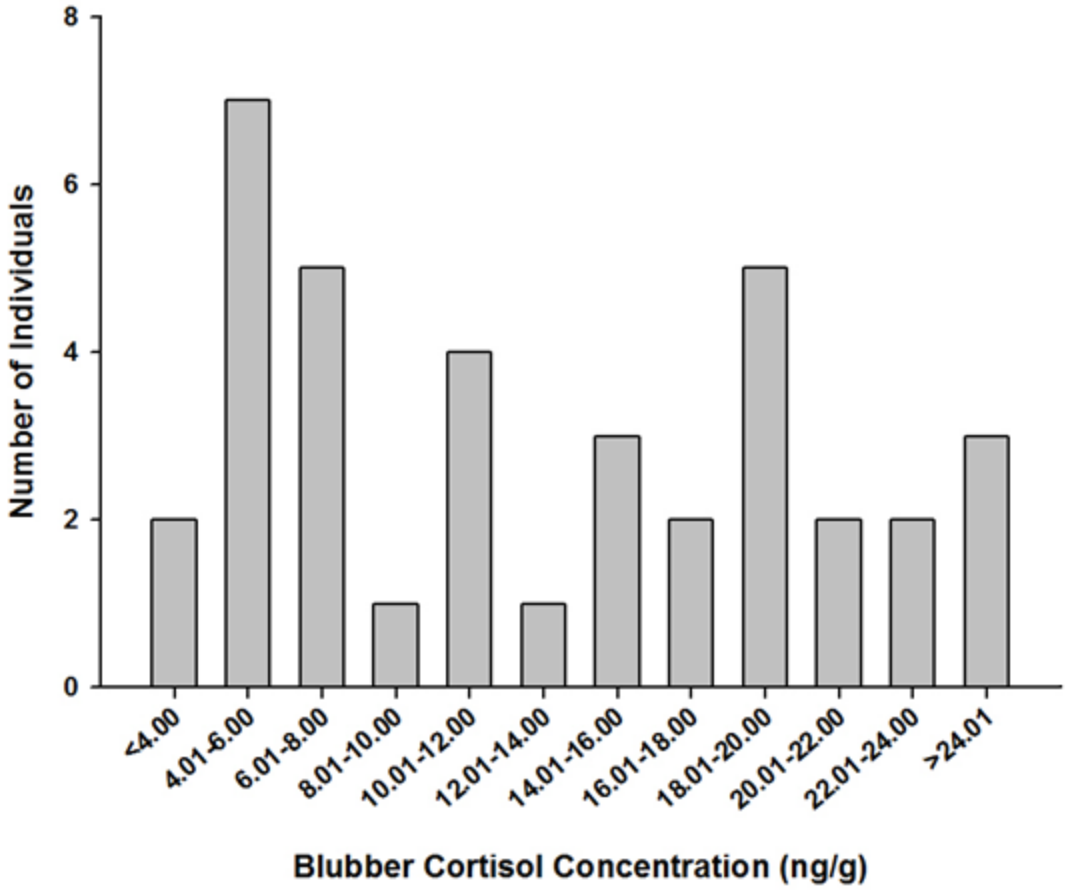

Fig. 5.2 Range and frequency of cortisol concentrations in walrus blubber

Range and frequency of distribution of cortisol concentrations (in ng/g) determined by enzymeimmunoassay of full-thickness walrus blubber (*n*=38).

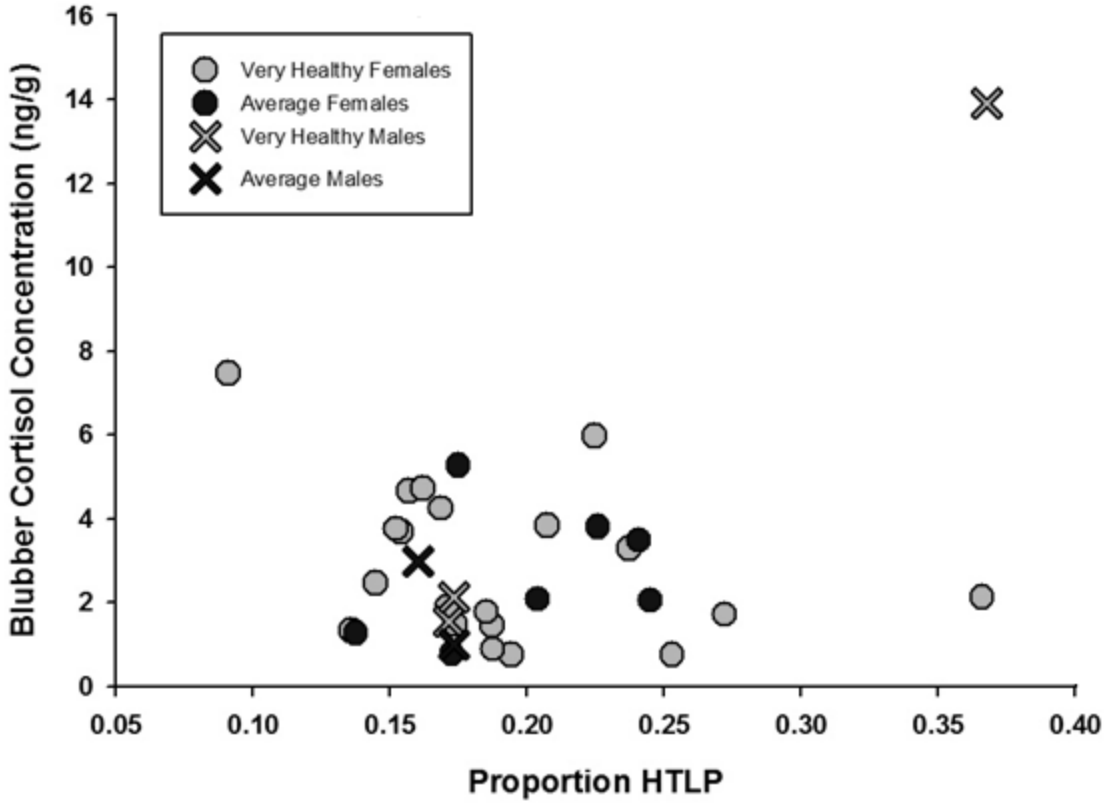

Fig. 5.3 Blubber cortisol concentrations relative to consumption of higher trophic level prey

Blubber cortisol concentrations of female and male walruses relative to proportion of higher trophic level prey in diet.

Table 5.1 Sampling year, location, and sample sizes for walrus blubber examined in this study

Location	Date	Available Sample (*n*)		
		Male	Female	Unknown Sex
Barrow	2009	1	-	2
	2011	1	-	-
St. Lawrence Island	2009	7	17	-
	2010	11	46	-
Wainwright	2009	-	-	3

Sampling year, location, and sample sizes for walrus blubber examined in this study (*n*=88). Samples from St. Lawrence Island were collected in April and May; samples from Barrow and Wainwright, Alaska, were collected in July.

Table 5.2 Walrus muscle bulk stable isotope values and contribution of higher trophic level prey to diet

	Sample Size (*n*)	Mean $\delta^{15}N \pm SD$ [‰]	Mean $\delta^{13}C \pm SD$ [‰]	HTLP (NE Model)	HTLP (EI Model)	
					Seal	Eider
Overall	163	13.0 ± 0.9	-17.0 ± 0.8	22.0% ± 10%	15.0% ± 10%	9.0% ± 10%
Female	111	13.0 ± 0.6	-17.0 ± 0.6	21.0% ± 7.2%	14.0% ± 6.2%	11.0% ± 5.4%
Male	46	13.0 ± 1.4	-17.0 ± 1.2	23.0% ± 12.3%	19.0% ± 12.1%	5.0% ± 11.9%

Mean ± standard deviation (SD) of walrus lumbar muscle bulk stable isotope (SI) values and mode proportional contribution of higher trophic level prey (HTLP) to diet by using both inclusion (EI) and exclusion (NE) of spectacled eiders in the mixing model (SIAR, adapted from Seymour *et al*. 2014*a*).

Table 5.3 Cortisol concentrations in walrus blubber by sex, lactation, and body condition

Category	Sample Size (*n*)	Mean Cortisol Concentration (ng/g)	H_0[3]
Overall	*38*	13.60 ± 8.35 (*2.77 to 34.04*)	
Male	*5*	12.77 ± 3.25 (*10.04 to 16.64*)	*P*=0.93
Female	*32*	13.70 ± 8.78 (*2.77 to 34.04*)	
Peripartum Female[1]	*33*	17.15 ± 11.36 (*5.60 to 34.04*)	*P*=0.63
Non-Reproductive Female[1]	*5*	13.16 ± 8.52 (*2.77 to 31.38*)	
"Very Healthy"[2]	*27*	14.49 ± 8.56 (*2.77 to 34.04*)	*P*=0.97
"Average"[2]	*12*	11.11 ± 7.76 (*4.00 to 23.93*)	

Mean ± standard deviation (SD) and range (in parentheses) of cortisol concentrations (ng/g) of walrus full-thickness blubber by sex, lactation, and visual assessment of body condition[1].

[1] Reproductive status was not known for all females. Peripartum females were defined as those exhibiting lactation

[2] As assessed by Alaska Native hunters as "very healthy", "average", or "unhealthy". No animals from the "unhealthy" category (*n*=3) had blubber cortisol concentrations measured within the acceptable 20% range

[3] H_0 testing lack of difference between categorical variables (*i.e.*, sex, peripartum lactation, body condition

Table 5.4 Percent lipid in walrus blubber and skin by sex and lactation

Category	Sample Size (*n*)	Mean %Lipid (Wet)	H_0[4]
Overall	*88*	44.6 ± 12.4 (*10.0 to 88.9*)	
Male[2]	*20*	35.8 ± 8.8 (*19.4 to 51.7*)	*P*=0.05
Female[2]	*63*	47.9 ± 14.8 (*23.8 to 61.9*)	
Peripartum Female[2]	*53*	47.4 ± 8.7 (*23.8 to 61.9*)	*P*=0.89
Non-Reproductive Female[2]	*9*	48.1 ± 14.9 (*42.8 to 58.3*)	
"Very Healthy"[3]	*55*	44.1 ± 15.2 (*23.6 to 61.9*)	
"Average"[3]	*30*	43.2 ± 5.7 (*19.1 to 58.3*)	*P*=0.06
"Unhealthy"[3]	*3*	30.0 ± 11.3 (*22.7 to 37.3*)	

Mean ± standard deviation (SD) of %lipid in walrus full-thickness blubber and skin (excepting hair and pigmented epidermal layer) by sex and female reproductive status[1]. Percent lipid is based on wet weight.

[1] Reproductive status was not known for all females. Peripartum females were defined as those exhibiting lactation

[2] Numbers may not add to total samples analyzed as not all walruses sampled were of known sex and reproductive status

[3] As assessed by Alaska Native hunters as "very healthy", "average", or "unhealthy"

[4] H_0 testing lack of difference between categorical variables (*i.e.*, year, sex, peripartum lactation, body condition)

Table 5.5 Percent lipid in blubber layers and skin from 12 walruses

Category	Sample Size (*n*)	Mean %Lipid (Epidermis, wet)	Mean %Lipid (Outer Blubber, wet)	Mean %Lipid (Inner Blubber, wet)
Overall	12	9.1 ± 11.3	67.1 ± 11.4	62.9 ± 12.2
2009	5	6.2 ± 2.3	71.4 ± 13	61.2 ± 1.1
2010	7	9.6 ± 13.1	65.4 ± 7.2	63.3 ± 13.6
Male	5	6.9 ± 2.1	68.8 ± 10.8	53.4 ± 7.4
Female	7	10.6 ± 3.3	69.1 ± 8.2	68.1 ± 0.3

Mean ± standard deviation (SD) of %lipid in outer and inner blubber layers[1], and skin (excluding hair and pigmented epidermis) from 12 walruses. Percent lipid is based on wet weight.

Note - %lipid did not change with sex (P=0.23).

[1] Outer and inner blubber layers were determined by bisecting each full-thickness blubber core in half horizontally. Depth of the resulting sample varied dependent on the full-thickness of the individual's blubber. "Outer" refers to the blubber layer closer to epidermis, while "inner" refers to the layer closer to muscle

Chapter 6: General Conclusion

Climate change impacts, including increased Arctic sea ice minima, may cause an uncoupling of the benthic and pelagic foodwebs in the Arctic Ocean and Bering Sea (Grebmeier et al. 2006; Grebmeier 2012). This uncoupling may result in decreased nutrient advection to the benthic system (Hunt et al. 2002; Grebmeier et al. 2006; Brown and Arrigo 2012) and lead to declines in benthic productivity, potentially triggering bottom-up effects to marine consumers (Grebmeier et al. 2006; Grebmeier 2012). Pacific walruses (*Odobenus rosmarus divergens*), a large Arctic generalist carnivore, are primarily benthic foragers, feeding on bivalves and other soft-tissued invertebrate prey (Fay 1982; Sheffield and Grebmeier 2009). However, the diet of walruses includes over a hundred different prey taxa (Sheffield et al. 2001; Sheffield and Grebmeier 2009) including occasional consumption of seals and seabirds (Fay 1960; Krylov 1971; Fay et al. 1977; Lowry and Fay 1984; Sease 1984; Burns et al. 1985; Merrick and Hills 1988; Fay et al. 1990; Muir et al. 1995; Sheffield et al. 2001; Mallory et al. 2004; Wolkers et al. 2006; Fox et al. 2010). Furthermore, Fay (1960), Rausch et al. (2007), and Jay and Fischbach (2008) hypothesized that reliance on higher trophic level prey (HTLP) by walruses may increase during unfavorable ice conditions or other circumstances where benthic invertebrates are unavailable or inaccessible.

Consumption of HTLP can be associated with adverse health impacts, including increased energetic demands, increased disease and contaminant exposure that result in declines in body condition, reproductive success, and overall survival (Muir et al. 1995;

Kutz et al. 2005; Burek et al. 2008). However, our findings suggest that walruses can prey on HTLP with little to no apparent increase in exposure to the zoonotic parasite *Trichinella*, and that predation on HTLP does not result in declines in body condition, or increases in foraging-related exposure to stressors. Furthermore, walruses appear to opportunistically consume both HTLP and benthic invertebrates. This diverse prey base may allow walruses to adapt to declines in benthic invertebrate prey availability as a consequence of the effects of benthic-pelagic uncoupling, decreased sea ice extent (used by walruses as a diving platform), and other effects of environmental change (Sheffield and Grebmeier 2009).

The importance of HTLP to the current Pacific walrus population is not well understood because dietary studies have historically relied on stomach content analysis, a method biased towards hard-bodied organisms (Sheffield et al. 2001; Pierce et al. 2004; Sheffield and Grebmeier 2009). To develop a better understanding of the contribution of HTLP to walrus diet, we applied a Bayesian mixing model to stable carbon and nitrogen isotope values of walrus muscle, skin, and liver to determine the proportional contribution of each source to the diet. While stable isotope analysis does not provide high taxonomic resolution, it is not hampered by the bias associated with stomach content analysis (i.e., differential digestion, Sheffield et al. 2001; Pierce et al. 2004; Sheffield and Grebmeier 2009). Depending on fractionation and cellular turnover of different tissues, and the metabolic rate of the predator, stable isotope signatures can reflect the integrated diet of a consumer from days to years (Hobson et al. 1996; Newsome et al. 2010). Our extrapolations suggested that stable isotope signatures of walrus muscle reflect the

average integrated diet over a two year period. Stable isotope mixing model results of muscle indicate that approximately 22% of the Pacific walrus integrated diet is HTLP. This was higher than anticipated based on historical stomach content analyses (~10%, Fay 1982; Lowry and Fay 1984; Sheffield and Grebmeier 2009), but consistent with Atlantic walruses (*Odobenus rosmarus rosmarus*, 25.0%, Muir et al. 1995). Mixing model analysis of tissues with higher turnover rates (i.e., liver and skin) yield a broader range in %HTLP (0-60%, mode 10%) suggesting that liver has a faster cellular turnover rate than muscle (supported by Hobson et al. 1996; Newsome et al. 2010), and that walruses pursue an opportunistic foraging strategy based on prey availability. That walruses may feed occasionally on HTLP was further supported by stable isotope analysis of a whisker from a known seal-eating walrus. The continual growth of vibrissae provides a sequential timeline of isotopic information, and thus diet (Schell et al. 1989; Hobson et al. 1996; Cherel et al. 2009). Consumption of seal muscle and blubber by walruses is indicated by the association of relatively low $\delta^{13}C$ and high $\delta^{15}N$ values along the whisker length, as elevated $\delta^{15}N$ signatures are associated with a higher trophic level diet, while relatively low $\delta^{13}C$ values are indicative of consumption of lipid-rich tissue (DeNiro and Epstein 1977). Alternatively or in addition, the relatively low $\delta^{13}C$ values periodically present along the walrus vibrissa could be indicative of consumption of offshore rather than benthic organisms (Dehn et al. 2007; Herreman et al. 2008). $\delta^{13}C$ values can also be a broad-scale geographic identifier of foraging grounds (Kelly 2000; Kurle and Worthy 2002). Muscle $\delta^{13}C$ values of male and female walruses suggest use of separate foraging areas based on sex, although overall reliance on HTLP is similar

between the sexes. This spatial sexual separation is in agreement with traditional migratory patterns of the species (Fay 1982; Garlich-Miller et al. 2011). However, the ability to determine geographical influences on diet will come from analysis of tissues that record dietary stable isotope input continuously. Future studies focusing on tissues with sequential, determinate growth (e.g., whiskers, nails) should be conducted to uncover more subtle environmental and geographical influences on walrus diet. Investigations should also be made into whether some individuals (identified by experts in specific walrus morphological characteristics, such as Alaska Native coastal community members) may be obligate seal-eaters, as suggested by traditional ecological knowledge from the Bering Strait region (Fay 1960; 1982). We also suggest the use of skin biopsies as a minimally-invasive approach to analyze walrus diet as our tissue type comparisons indicated that stable isotope and mixing model analysis provided comparable dietary information. We caution, however, that data from whiskers and skin should not be considered robust until captive studies confirm species-specific whisker growth rates, fractionation factors, and turnover rates for walrus muscle and skin.

Comparison of %HTLP from 1999 to 2009 with annual Arctic sea ice minima revealed no clear correlation between sea ice extent and walrus diet. The lowest HTLP contribution to walrus diet occurred during 2008-2009 (13%), one of two sampling periods that experienced great seasonal loss of pan-arctic sea ice (the other being 2007-2008 with mode HTLP of 23%). Prior to 2008, there was a gradual, but not significant increase in HTLP consistent with the opportunistic foraging hypothesis by Fay (1960), Rausch et al. (2007), and Jay and Fischbach (2008). Future stable isotope analyses using

more complete and longer temporal samples are needed to understand trends in HTLP and the proposed influence of sea ice.

Trichinella was present in ringed seals (*Pusa hispida*), polar bears (*Ursus maritimus*), and Arctic foxes (*Vulpes lagopus*), but absent from walrus samples. Walruses are a known source of community-wide outbreaks of trichinellosis (caused by the nematode parasite *Trichinella* spp. Proulx et al. 2002; Moller et al. 2005). Fay (1960) also hypothesized that consumption of pagophilic seals was the primary method by which walruses contract *Trichinella*. Historical analysis of Alaska marine mammal species using trichinoscopy has shown the presence of *Trichinella* at sporadic and low levels (<2%, reviewed by Forbes 2000) with the exception of polar bears (*Ursus maritimus*), which have high (>70%) prevalence of infection (Fay 1960; Weyermann et al. 1993). This study is the first application of a validated artificial digestion method (Forbes and Gajadhar 1999; Leclair et al. 2003) and is the first multi-species survey examining prevalence in Alaska marine mammals in 50 years. Genetic analysis confirmed the species present in all positive samples to be the freeze-tolerant *T. nativa.* While a definitive link between predation on ice-associated seals and *Trichinella* infection in walruses could not be established in this study, successful extraction and genotypic analysis of larvae from a decade-old polar bear muscle sample confirmed that archived and degraded samples can provide information on the prevalence of *Trichinella* in the Arctic. The moderate reliance of walruses on HTLP (~22% mode integrated diet over two years) and the overall low prevalence of *Trichinella* in marine and marine-associated mammals in Alaska suggest that the threat of transmission from walruses to humans is

currently low. Monitoring prevalence of *Trichinella* infection in host species can assess the risk associated with this parasite to Alaskan communities and determine trends associated with environmental change (Burek et al. 2008; Hueffer et al. 2013). In addition, few studies have examined *Trichinella* infection in avian species; however, quails (*Corturnix corturnix* spp.) experimentally infected with the parasite have been shown to expel the larvae in a form that remains infective (Odevskaia 2013) suggesting that seabirds could potentially play a role in *Trichinella* transmission in the marine environment. Utilization of antibody tests (as a non-lethal method of monitoring, Gajadhar et al. 2008) to detect exposure to *Trichinella* in animals should be validated in various marine mammal species in combination with digestion assays (when assessing the infection status of carcasses). To elucidate the transmission cycle of *Trichinella*, monitoring of multiple species, including rodents, birds, and potential marine invertebrate prey of walruses (via artificial digestion) is imperative.

The consumption of HTLP can increase energetic costs of the predator associated with capture, handling, and processing (Carbone et al. 2007; Rosen et al. 2007). Over months or years, these increased costs may result in adverse impacts to health and body condition (Rosen and Trites 2000; Rosen et al. 2007; Rosen 2009). Assessing individual and population-level health in free-ranging walruses, however, has been hampered by logistical constraints and unstandardized measurement techniques (e.g., measurements of blubber depth). Innovative techniques now allow for the measurement of individual health proxies with minimally-invasive methods to measure reproductive and stress hormones, contaminants, dietary information, and disease exposure (Thomson and Geraci

1986; Fossi and Marsili 1997; Pedernera-Romano et al. 2006; Hogg et al. 2009; Hunt et al. 2013). Cortisol, the primary biochemical indicator of stress response in mammals, is commonly measured to determine presence and impacts of external stressors (Thomson and Geraci 1986; Fair and Becker 2000; Oki and Atkinson 2004; Mashburn and Atkinson 2004; Romano et al. 2004; Myers et al. 2010). Following successful quantification of reproductive hormones in lipid-rich marine mammal tissues, including blubber (Kellar et al. 2006; 2009; 2013; Trego et al. 2013; Trumble et al. 2013), we extracted and measured cortisol concentrations in blubber of free-ranging walruses in a first attempt to develop a baseline for cortisol concentrations in this species. Our analysis shows a wide range in cortisol concentrations. However, research on other marine mammals has indicated that cortisol concentrations vary substantially among individuals of the same species with sex, season, and reproductive status (Gardiner and Hall 1997; Mashburn and Atkinson 2004; 2007). Our findings suggest that blubber cortisol concentrations are not influenced by sex, reproductive status, or proportion of HTLP in walrus diet; however, these findings should be accepted cautiously as the lack of significant impact by these variables may be the result of averaging over a small sample size. If the lack of a correlation between cortisol concentrations and diet holds, it indicates that the portion of the walrus population considered healthy enough for subsistence harvest is not nutritionally-stressed at levels detectable from the assessment of blubber cortisol concentrations. As samples were obtained primarily from subsistence harvests targeting apparently healthy animals, the initial conclusion that no walruses are adversely impacted by foraging on HTLP should be cautiously considered until cortisol measurements can be conducted on

samples representative of the entire population. Future investigation of cortisol concentrations in walruses should focus on further development and validation of the blubber enzymeimmunoassay (EIA) technique. To understand the difference and correlations of concentrations of sequestered cortisol among tissues with different turnover rates, captive studies should also be conducted. While this study found no influence of sex or reproductive status on the blubber cortisol concentrations of walruses, research on captive animals can provide confirmation of whether these variables influence long-term cortisol concentrations. Overall, further refinement of measuring cortisol as an indicator of external stressors is needed before its wide application to marine mammal blubber. Our research provides a minimally-invasive technique to track and monitor proxies of a long-term stress response. After refinement and validation, assays of blubber cortisol and other steroid hormones could provide researchers with a minimally-invasive sampling method. Success in extracting cortisol from keratinous tissues (Bryan et al. 2013) holds promise that this technique may be applied to walrus vibrissae in tandem with SI analysis to provide direct comparisons between cortisol concentrations and prey source. In addition, once a confirmed baseline of cortisol concentrations for walruses is fully developed, cortisol and stable isotope analysis of bone lipids and collagen, respectively, can be conducted to examine the influence of walrus diet composition to stress response over longer time scales, including archeological samples (Yarrow et al. 2010; Mark et al. 2011).

Blubber thickness has historically been used to quantify body condition although it has not always been found to be a reliable predictor of body condition (Strandberg et al.

2008), and blubber %lipid is generally considered a better gauge of condition (West et al. 1979a; b; Beck et al. 1993). We measured %lipid of full-thickness walrus blubber with attached skin to develop a baseline of %lipid in full-thickness biopsy samples and assessed whether presence of skin in the sample impacts qualitative comparisons. The lipid content of blubber samples with attached skin (~45% wet weight) in this study is lower than measurements in Atlantic walrus blubber (~83%, Muir et al. 1995) and other Arctic marine mammals (Addison and Smith 1974; Hoekstra et al. 2002), although these previous studies were conducted on samples of blubber only (no skin included). These comparatively lower measurements may be the result of the inclusion of skin in our samples. In our tissue-specific study, skin contained minimal amounts of lipid. Inclusion of skin likely diluted the absolute %lipid values of each individual. While trends in %lipid of the population can still be monitored over time when the skin is included, we recommend that this tissue be excluded from future blubber %lipid assessments of body condition. For walruses, %lipid of blubber and attached skin was higher in females than males, consistent with the need for greater energy stores in females of reproductive age (Boness and Bowen 1996). The %lipid of walrus blubber and attached skin varied little with contribution of HTLP to the diet suggesting that walruses are physiologically adapted to manage and assimilate energy from both higher and lower trophic level prey. It is feasible that walruses relying largely on seals or seabirds as prey are able to consume large enough quantities of HTLP prey to meet energy requirements. Alternatively, the lack of correlation of HTLP and %lipid may indicate that walruses switch between invertebrate and vertebrate prey with high enough frequency that there is no measureable

effect on energy stores when substituting prey. Future studies of walrus blubber lipids should include the development of a time series to compare %lipid across years and variations in annual Arctic sea ice minima similar to recent time series generated for whales by Williams et al. (2013) and Truchon et al. (2013). Analysis of fatty acid profiles (which provide information on nutrient sources and their mobilization, Beck et al. 1993), and comparisons with the contribution of HTLP will also provide finer details regarding the influence of diet on walrus blubber lipids.

Research on population health (or even population trends) of walruses and other Arctic marine mammals is often impeded due to logistical challenges. However, data on the health of these species are sought after by Arctic scientists, industry, government, and environmental advocates to determine impacts of industrial development and climate change on ecosystem health (McCarthy 2001). This dissertation examined the reliance of Pacific walruses on HTLP on temporal scales and relative to annual sea ice minima. Our research also investigated the influence of HTLP on transmission of the zoonotic parasite *Trichinella* spp., stress response, and body condition.

Ultimately, the ability of Pacific walruses to forage on a diverse assemblage of prey types (Sheffield and Grebmeier 2009) does not appear to result in measureable physiological changes relative to the proportional contribution of HTLP in their diet. Their diverse diet may mitigate effects of climate change on walrus habitat and prey populations, including bottom-up effects of early sea ice retreat and benthic-pelagic uncoupling on the Arctic marine food web (Grebmeier et al. 2006; Bluhm et al. 2011), diminishing sea ice (i.e., foraging platform), and increased use of terrestrial haulouts

away from high density foraging areas (Jay and Fischbach 2008; Jay et al. 2011; 2012). Only continued monitoring of walruses will reveal whether long term reliance on HTLP will sustain a healthy walrus population as environmental changes persist.

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Appendices

Appendix A: Sample collection map

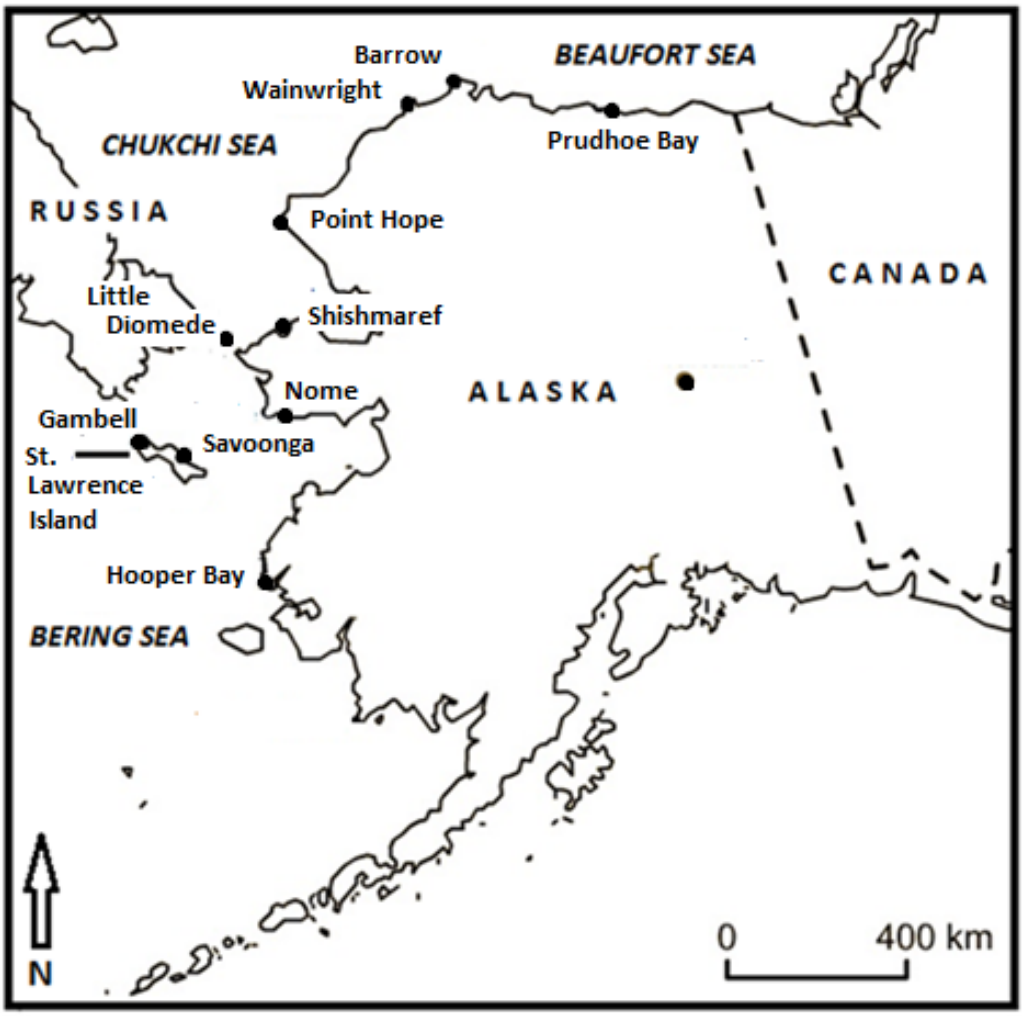

Fig A-1: Sampling locations for walrus, seal, and fox tissues

Sampling locations for walrus, seal, and fox tissues (Fairbanks is identified as a point of reference)

Appendix B: Stable $\delta^{13}C$ and $\delta^{15}N$ isotope values and proportion of higher trohic level prey in the diet of individual walruses by tissue type and sex

Table B-1: Stable isotope signatures and proportion of higher trophic level prey in the diet of walruses

Tissue Type	Sex[a]	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	%HTLP (NE)[b]	%HTLP (EI)[b]
Liver	F	-19.6	13.0	17.7%	6.9%
Liver	F	-12.0	14.9	12.8%	14.5%
Liver	F	-18.7	14.1	15.0%	5.7%
Liver	F	-18.0	12.3	9.1%	15.7%
Liver	F	-18.3	13.4	9.5%	12.4%
Liver	F	-17.7	12.2	12.6%	12.1%
Liver	F	-17.6	13.3	18.5%	14.2%
Liver	F	-17.4	12.4	17.8%	7.3%
Liver	F	-16.8	11.8	11.7%	12.3%
Liver	F	-18.8	15.0	15.0%	11.9%
Liver	F	-18.5	13.5	17.5%	16.3%
Liver	F	-18.5	14.4	21.1%	13.2%
Liver	F	-18.6	14.2	11.3%	6.7%
Liver	F	-19.0	11.8	15.0%	6.1%
Liver	F	-18.7	13.1	12.2%	8.8%
Liver	F	-18.6	13.7	18.6%	6.7%
Liver	F	-18.6	14.5	12.1%	11.2%
Liver	F	-18.8	13.2	9.3%	6.6%
Liver	F	-18.8	12.2	9.0%	13.8%
Liver	F	-18.2	13.5	6.3%	12.1%
Liver	F	-18.5	15.0	5.1%	9.1%
Liver	F	-18.3	11.1	10.8%	12.7%
Liver	F	-18.5	12.9	7.3%	11.6%
Liver	F	-18.7	13.5	13.2%	8.8%
Liver	F	-18.0	13.6	15.5%	7.2%
Liver	F	-17.4	12.5	14.3%	16.3%
Liver	F	-18.3	11.7	3.7%	19.3%
Liver	F	-18.7	11.0	4.5%	18.1%

Table B-1, continued

Tissue Type	Sex[a]	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	%HTLP (NE)[b]	%HTLP (EI)[b]
Liver	F	-17.3	12.1	10.6%	7.9%
Liver	F	-17.3	12.9	9.7%	15.2%
Liver	F	-18.3	13.7	3.8%	16.8%
Liver	F	-18.4	11.9	4.8%	6.8%
Liver	F	-18.8	13.1	9.2%	16.5%
Liver	F	-18.6	11.1	14.1%	12.5%
Liver	F	-18.0	14.0	11.1%	11.6%
Liver	F	-17.6	13.1	13.2%	9.9%
Liver	F	-17.6	11.8	8.1%	15.4%
Liver	F	-17.6	13.0	12.6%	13.7%
Liver	F	-18.1	12.3	10.9%	10.0%
Liver	F	-17.8	12.9	8.6%	9.5%
Liver	F	-18.7	11.4	7.7%	11.1%
Liver	F	-17.7	12.5	10.4%	13.5%
Liver	F	-17.4	12.9	5.4%	16.3%
Liver	F	-17.6	12.1	6.4%	19.1%
Liver	F	-18.2	12.3	11.4%	12.1%
Liver	F	-18.6	12.7	1.7%	14.6%
Liver	F	-18.3	11.6	14.2%	12.7%
Liver	F	-19.5	11.7	8.9%	8.8%
Liver	F	-18.3	13.1	4.7%	10.7%
Liver	F	-17.1	13.4	5.8%	16.7%
Liver	F	-17.5	13.0	9.1%	14.0%
Liver	F	-17.5	13.1	7.9%	19.3%
Liver	F	-18.0	12.7	12.1%	19.3%
Liver	F	-17.4	12.8	9.6%	18.1%
Liver	F	-17.4	13.6	8.5%	11.9%
Liver	F	-17.8	13.3	5.1%	5.7%
Liver	F	-17.7	13.1	3.2%	5.2%
Liver	F	-17.3	14.4	5.7%	6.6%
Liver	F	-17.7	14.5	6.8%	3.4%
Liver	F	-17.1	14.3	2.0%	12.3%
Liver	F	-16.8	15.1	9.3%	5.6%
Liver	F	-17.0	13.4	1.7%	14.1%
Liver	F	-18.0	14.0	9.4%	5.8%

Table B-1, continued

Tissue Type	Sex[a]	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	%HTLP (NE)[b]	%HTLP (EI)[b]
Liver	F	-16.4	9.8	1.3%	8.3%
Liver	M	-19.1	12.8	17.4%	5.3%
Liver	M	-17.8	11.0	8.2%	16.6%
Liver	M	-18.6	13.4	21.5%	17.9%
Liver	M	-18.4	13.7	15.3%	14.2%
Liver	M	-17.6	12.2	14.9%	15.3%
Liver	M	-19.4	16.0	16.8%	9.2%
Liver	M	-18.7	14.2	8.9%	8.8%
Liver	M	-18.2	14.7	5.6%	16.5%
Liver	M	-16.3	13.1	10.1%	15.1%
Liver	M	-17.00	13.7	12.5%	13.5%
Liver	M	-19.3	14.0	7.5%	6.7%
Liver	M	-16.8	13.8	14.0%	6.3%
Liver	M	-17.5	11.6	9.8%	10.3%
Liver	M	-17.5	11.2	6.7%	15.2%
Liver	M	-17.8	12.9	9.9%	10.6%
Liver	M	-19.0	13.2	12.8%	8.2%
Liver	M	-17.9	13.3	6.9%	18.0%
Liver	M	-17.8	14.4	4.5%	17.2%
Liver	M	-17.4	15.0	14.3%	17.6%
Liver	M	-17.5	12.8	0.8%	7.9%
Liver	M	-17.9	12.7	5.1%	4.3%
Liver	M	-15.9	14.5	3.8%	5.1%
Liver	M	-18.6	14.3	6.1%	7.4%
Liver	M	-16.0	11.2	1.6%	9.6%
Liver	M	-15.6	10.0	11.5%	4.6%
Liver	M	-16.5	10.4	3.1%	6.6%
Liver	U	-18.7	11.3	13.9%	12.5%
Liver	U	-18.5	12.7	13.5%	7.1%
Liver	U	-17.0	10.6	13.4%	10.7%
Liver	U	-12.0	14.1	16.2%	18.2%
Liver	U	-18.7	14.1	15.6%	19.7%
Liver	U	-17.9	15.4	14.3%	16.8%
Liver	U	-18.4	14.5	14.8%	12.8%
Liver	U	-18.4	15.0	6.8%	8.9%

Table B-1, continued

Tissue Type	Sex[a]	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	%HTLP (NE)[b]	%HTLP (EI)[b]
Liver	U	-17.8	12.0	9.6%	14.7%
Liver	U	-17.8	11.8	6.7%	16.7%
Liver	U	-18.8	13.3	13.2%	18.3%
Liver	U	-18.2	13.5	7.4%	13.0%
Liver	U	-18.9	12.8	0.8%	13.6%
Liver	U	-16.3	13.8	8.5%	15.4%
Liver	U	-17.3	11.3	10.4%	9.9%
Liver	U	-17.8	13.6	5.9%	17.3%
Liver	U	-17.5	14.7	5.4%	6.4%
Muscle	F	-17.0	13.6	32.3%	29.9%
Muscle	F	-17.5	13.1	35.4%	22.0%
Muscle	F	-17.0	13.9	36.0%	27.3%
Muscle	F	-17.1	13.6	32.2%	27.8%
Muscle	F	-17.1	13.2	37.5%	25.1%
Muscle	F	-16.5	12.6	27.6%	24.1%
Muscle	F	-16.5	12.8	32.6%	27.0%
Muscle	F	-17.1	13.3	33.6%	26.6%
Muscle	F	-17.2	13.4	35.7%	27.4%
Muscle	F	-17.2	13.3	27.2%	25.7%
Muscle	F	-17.1	12.5	34.2%	27.8%
Muscle	F	-17.0	12.9	36.6%	27.8%
Muscle	F	-17.5	12.8	36.6%	25.6%
Muscle	F	-17.1	12.8	36.0%	26.0%
Muscle	F	-17.3	13.5	9.1%	17.2%
Muscle	F	-17.0	12.6	17.2%	24.2%
Muscle	F	-16.5	12.3	13.3%	22.1%
Muscle	F	-16.9	13.2	13.6%	19.4%
Muscle	F	-16.9	12.0	19.0%	15.6%
Muscle	F	-16.4	12.7	17.0%	18.1%
Muscle	F	-16.1	11.9	17.4%	16.9%
Muscle	F	-16.9	13.7	10.2%	21.6%
Muscle	F	-16.2	12.0	17.1%	17.4%
Muscle	F	-16.4	12.9	18.8%	23.0%
Muscle	F	-16.4	12.5	15.8%	20.7%
Muscle	F	-17.0	12.7	17.7%	16.7%

Table B-1, continued

Tissue Type	Sex[a]	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	%HTLP (NE)[b]	%HTLP (EI)[b]
Muscle	F	-16.0	12.1	16.4%	16.1%
Muscle	F	-16.7	11.8	15.2%	26.1%
Muscle	F	-16.5	12.2	16.5%	24.3%
Muscle	F	-17.0	13.0	19.4%	23.1%
Muscle	F	-17.3	12.4	15.4%	22.2%
Muscle	F	-17.1	13.7	13.4%	19.6%
Muscle	F	-19.1	13.3	16.2%	22.1%
Muscle	F	-17.8	12.6	17.2%	23.1%
Muscle	F	-17.0	12.4	15.6%	22.0%
Muscle	F	-16.6	12.7	14.2%	20.9%
Muscle	F	-16.4	12.3	17.3%	17.3%
Muscle	F	-17.3	13.2	33.1%	28.6%
Muscle	F	-16.3	12.4	32.0%	27.2%
Muscle	F	-16.9	12.5	32.3%	33.2%
Muscle	F	-16.7	13.3	25.8%	32.0%
Muscle	F	-16.6	12.8	33.1%	33.0%
Muscle	F	-17.8	13.9	20.4%	33.5%
Muscle	F	-17.4	13.6	18.2%	32.9%
Muscle	F	-17.1	13.2	21.2%	29.8%
Muscle	F	-16.4	12.7	16.0%	30.0%
Muscle	F	-16.8	12.1	15.9%	33.1%
Muscle	F	-17.2	13.5	18.2%	36.0%
Muscle	F	-17.5	13.1	23.7%	38.0%
Muscle	F	-17.3	13.2	23.1%	38.4%
Muscle	F	-17.2	13.7	16.3%	36.9%
Muscle	F	-17.0	12.1	15.6%	36.8%
Muscle	F	-16.7	12.2	20.4%	29.4%
Muscle	F	-17.3	13.2	17.6%	36.4%
Muscle	F	-17.0	12.7	16.8%	36.4%
Muscle	F	-17.6	13.1	20.7%	32.8%
Muscle	F	-17.1	12.8	22.6%	30.00%
Muscle	F	-17.0	12.3	20.4%	28.9%
Muscle	F	-17.5	13.3	15.6%	32.3%
Muscle	F	-17.3	13.1	18.7%	33.2%
Muscle	F	-17.1	13.4	19.0%	33.3%

Table B-1, continued

Tissue Type	Sex[a]	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	%HTLP (NE)[b]	%HTLP (EI)[b]
Muscle	F	-16.9	13.6	15.9%	37.1%
Muscle	F	-17.5	12.9	16.8%	31.2%
Muscle	F	-17.2	12.4	17.8%	35.6%
Muscle	F	-16.8	11.9	13.7%	34.7%
Muscle	F	-16.6	12.9	23.1%	34.2%
Muscle	F	-16.0	12.7	21.8%	32.2%
Muscle	F	-17.0	13.1	25.3%	20.4%
Muscle	F	-17.0	12.9	24.1%	20.7%
Muscle	F	-16.4	12.6	22.4%	21.5%
Muscle	F	-17.2	12.7	19.4%	22.1%
Muscle	F	-16.2	11.4	17.3%	19.6%
Muscle	F	-16.6	11.7	21.6%	18.5%
Muscle	F	-15.8	12.6	24.5%	20.4%
Muscle	F	-17.1	13.8	19.7%	19.0%
Muscle	F	-16.7	12.4	17.9%	24.3%
Muscle	F	-17.0	13.2	17.8%	23.6%
Muscle	F	-17.4	13.2	18.2%	26.1%
Muscle	F	-18.0	12.3	14.5%	24.4%
Muscle	F	-20.0	13.8	17.5%	21.4%
Muscle	F	-17.4	13.4	18.7%	17.5%
Muscle	F	-16.8	12.2	18.7%	22.3%
Muscle	F	-16.8	12.7	23.5%	18.6%
Muscle	F	-17.0	14.6	20.5%	24.8%
Muscle	F	-17.1	13.0	22.4%	22.4%
Muscle	F	-17.5	12.8	22.2%	21.9%
Muscle	F	-17.1	14.2	24.1%	21.3%
Muscle	F	-17.2	13.8	23.0%	23.1%
Muscle	F	-17.1	12.1	23.4%	18.0%
Muscle	F	-17.2	14.1	20.0%	21.0%
Muscle	F	-17.4	13.2	17.3%	17.4%
Muscle	F	-16.8	12.4	17.1%	18.8%
Muscle	F	-15.8	11.6	16.2%	21.7%
Muscle	F	-17.0	12.9	16.4%	18.0%
Muscle	F	-16.7	12.8	25.4%	17.8%
Muscle	F	-16.9	13.4	20.8%	20.7%

Table B-1, continued

Tissue Type	Sex[a]	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	%HTLP (NE)[b]	%HTLP (EI)[b]
Muscle	F	-17.1	13.0	19.2%	16.0%
Muscle	F	-17.1	13.3	21.8%	20.8%
Muscle	F	-15.9	13.7	18.5%	16.9%
Muscle	F	-17.2	13.4	22.0%	23.4%
Muscle	F	-16.9	13.6	21.6%	24.6%
Muscle	F	-16.9	13.2	17.3%	18.4%
Muscle	F	-17.1	14.0	25.1%	21.2%
Muscle	F	-17.5	12.7	25.0%	20.7%
Muscle	F	-16.1	12.8	26.9%	22.1%
Muscle	F	-15.9	12.4	21.3%	7.3%
Muscle	F	-17.0	13.3	25.6%	9.8%
Muscle	M	-16.0	13.3	33.8%	25.0%
Muscle	M	-18.9	13.9	26.7%	33.6%
Muscle	M	-18.2	15.0	34.4%	30.7%
Muscle	M	-16.9	13.8	32.6%	32.9%
Muscle	M	-14.5	15.8	30.9%	30.8%
Muscle	M	-15.5	15.0	35.9%	32.1%
Muscle	M	-16.8	13.8	32.7%	28.2%
Muscle	M	-15.7	14.4	35.7%	30.5%
Muscle	M	-18.4	13.6	35.9%	30.0%
Muscle	M	-16.6	11.9	18.2%	20.4%
Muscle	M	-17.0	12.9	13.8%	18.2%
Muscle	M	-17.2	12.4	17.3%	23.1%
Muscle	M	-17.8	11.6	16.1%	22.0%
Muscle	M	-16.7	12.7	32.1%	28.5%
Muscle	M	-16.8	13.2	36.8%	28.7%
Muscle	M	-16.4	12.6	28.2%	29.9%
Muscle	M	-17.3	13.2	25.3%	34.0%
Muscle	M	-16.9	12.0	16.1%	30.6%
Muscle	M	-16.0	12.0	17.1%	34.1%
Muscle	M	-16.8	12.1	16.5%	25.0%
Muscle	M	-16.6	11.8	13.8%	21.3%
Muscle	M	-16.7	13.1	14.4%	22.0%
Muscle	M	-16.7	13.3	20.7%	15.4%
Muscle	M	-17.4	12.7	23.1%	20.8%

Table B-1, continued

Tissue Type	Sex[a]	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	%HTLP (NE)[b]	%HTLP (EI)[b]
Muscle	M	-15.5	12.1	20.1%	21.4%
Muscle	M	-16.5	11.8	17.3%	22.2%
Muscle	M	-16.9	12.3	18.5%	19.3%
Muscle	M	-16.2	12.1	17.3%	17.3%
Muscle	M	-16.8	12.3	17.1%	21.3%
Muscle	M	-16.7	11.5	25.2%	21.4%
Muscle	M	-13.8	16.3	16.2%	18.7%
Muscle	M	-15.9	13.5	21.0%	18.4%
Muscle	M	-15.4	14.5	16.3%	23.5%
Muscle	M	-16.9	13.4	18.0%	17.8%
Muscle	M	-17.1	12.8	16.6%	19.7%
Muscle	M	-17.3	11.9	17.9%	19.4%
Muscle	M	-15.9	14.9	23.8%	22.3%
Muscle	M	-16.9	13.7	19.6%	19.5%
Muscle	M	-16.5	13.3	17.5%	22.4%
Muscle	M	-20.8	10.2	20.7%	18.7%
Muscle	M	-20.8	10.4	12.4%	4.4%
Muscle	U	-16.8	12.2	34.3%	22.4%
Muscle	U	-17.1	12.7	34.2%	27.7%
Muscle	U	-17.0	15.3	24.3%	18.6%
Muscle	U	-16.8	12.3	20.2%	4.3%
Muscle	U	-16.8	15.3	13.1%	1.6%
Skin	F	-18.6	15.1	31.1%	29.5%
Skin	F	-17,0	15.2	30.8%	30.7%
Skin	F	-17.2	13.8	31.8%	30.7%
Skin	F	-19.0	13.2	28.3%	34.6%
Skin	F	-16.4	12.9	34.0%	29.5%
Skin	F	-16.2	13.9	35.9%	33.8%
Skin	F	-19.1	13.7	28.3%	30.3%
Skin	F	-17.2	14.3	33.8%	30.1%
Skin	F	-16.8	14.0	35.5%	30.3%
Skin	F	-16.0	13.7	31.2%	32.6%
Skin	F	-14.5	14.6	30.8%	34.6%
Skin	F	-15.6	15.8	30.4%	33.3%
Skin	F	-16.6	14.5	28.4%	33.3%

Table B-1, continued

Tissue Type	Sex[a]	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	%HTLP (NE)[b]	%HTLP (EI)[b]
Skin	F	-15.9	14.6	28.8%	30.6%
Skin	F	-17.3	15.4	31.9%	26.0%
Skin	F	-16.1	13.7	32.2%	27.9%
Skin	F	-18.0	14.2	30.1%	35.7%
Skin	F	-16.4	13.3	30.4%	33.1%
Skin	F	-17.5	14.2	33.1%	32.00%
Skin	F	-15.7	13.4	31.8%	32.2%
Skin	U	-16.7	13.5	30.9%	31.5%
Skin	U	-16.9	14.3	33.2%	25.1%
Tongue	F	-16.4	12.2	36.4%	23.4%
Tongue	F	-17.1	12.8	34.1%	27.9%
Tongue	F	-17.2	13.3	12.7%	17.8%
Tongue	F	-17.4	12.9	8.8%	20.0%
Tongue	F	-17.6	13.8	16.1%	17.1%
Tongue	F	-17.4	12.6	18.5%	36.5%
Tongue	M	-16.2	13.3	30.5%	33.1%
Tongue	M	-17.00	13.7	33.8%	23.2%
Tongue	M	-16.7	12.6	14.9%	31.4%
Tongue	M	-17.7	17.1	33.9%	25.3%

Bulk stable isotope signatures and estimated proportion of higher trophic level prey in the diet of walruses by tissue type and sex.

[a] F=female, M=male, U=unknown sex

[b] NE=SIAR mixing model results without spectacled eider input, EI=SIAR mixing model results with spectacled eider input

Appendix C: Blubber cortisol enzymeimmunoassay standard curves and unknown walrus blubber samples

Pacific walrus blubber cortisol concentrations were determined via enzymeimmunoassays (EIA) using commercially available kits (Assay Designs Correlate-EIA™ Cortisol Enzymeimmunoassay Kit #900-071, validation data included in following section) of a subset of samples (n=39). Each sample was analyzed in duplicate and % bound values for each extract were calculated as follows:

(1) $$\% \, Bound = Net \, Optical \, Density / Net \, B_0 * 100$$

where net B_0 is the mean ng/g of the zero ng/g standard wells and net optical density is the difference between the mean bound optical density and mean non-specific binding optical density. Blubber cortisol concentrations were then interpolated utilizing a 4 parameter logistic nonlinear regression model of the % bound versus cortisol concentrations of the standards. Resulting concentrations were considered acceptable if the duplicate coefficient of variation (CV) was ≤20% and if the concentration fell within 20-80% of the standard curve. Plots of each EIA plate (n=6), including standard curves and acceptable unknown samples, are presented below.

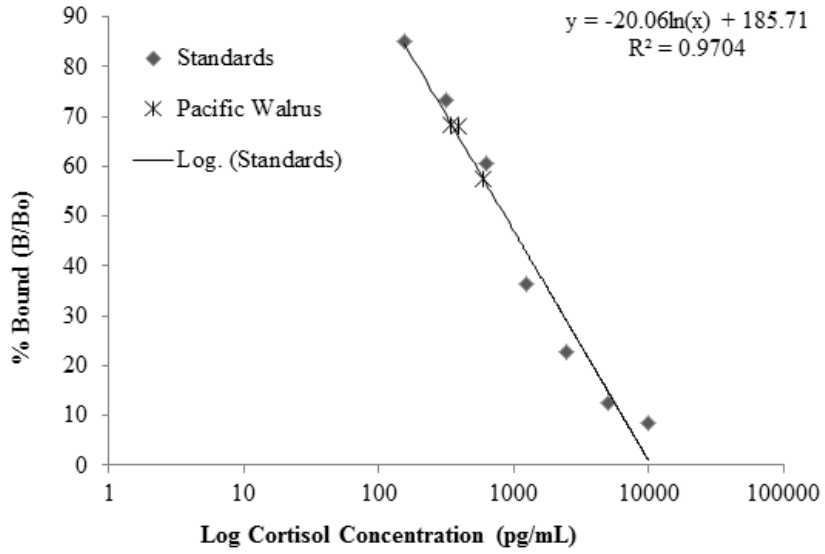

Fig. C-1 Enzymeimmunoassay plate #1 standard curve and unknown walrus blubber samples

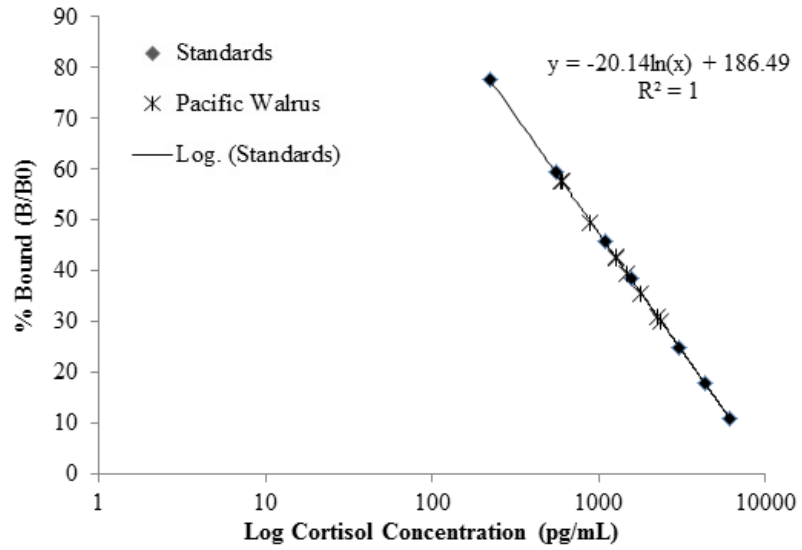

Fig. C-2 Enzymeimmunoassay plate #2 standard curve and unknown walrus blubber samples

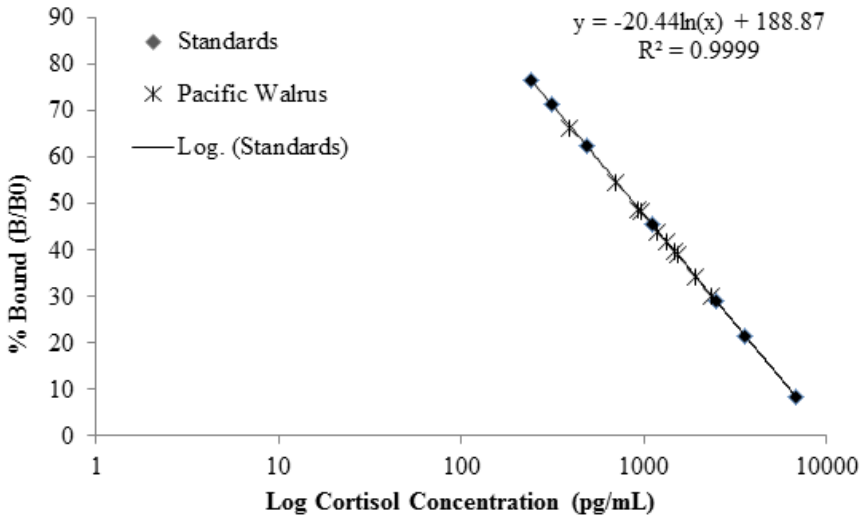

Fig. C-3 Enzymeimmunoassay plate #3 standard curve and unknown walrus blubber samples

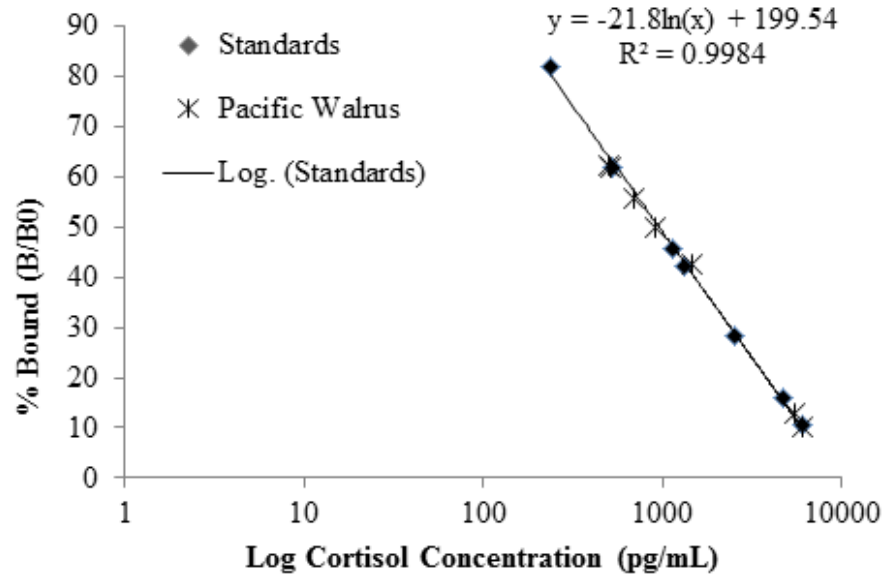

Fig. C-4 Enzymeimmunoassay plate #4 standard curve and unknown walrus blubber samples

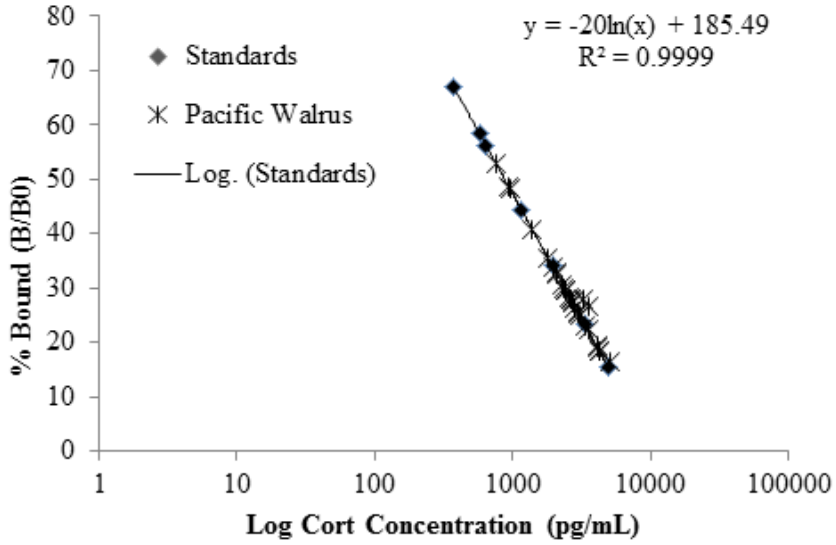

Fig. C-5 Enzymeimmunoassay plate #5 standard curve and unknown walrus blubber samples

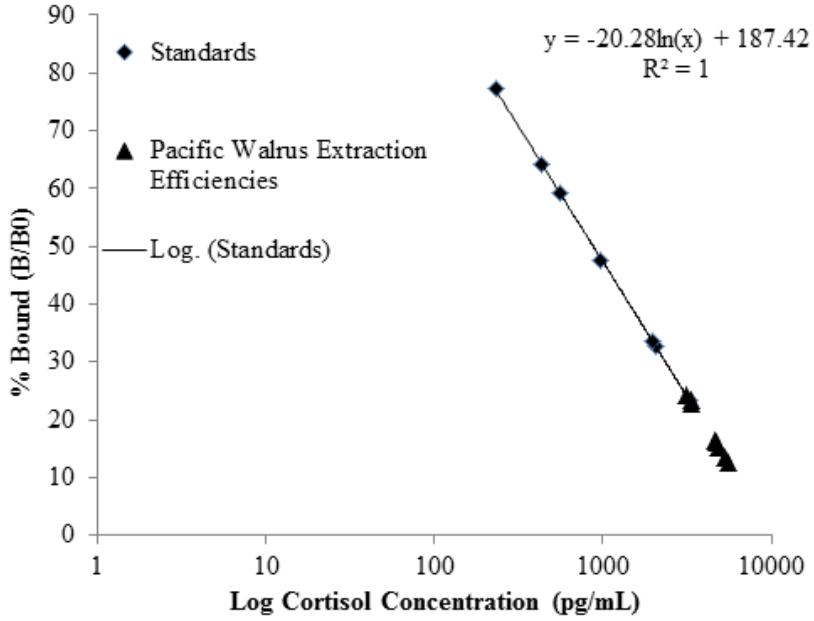

Fig. C-6 Enzymeimmunoassay plate #6 standard curve and extraction efficiency samples

Appendix D: Comparison of walrus blubber and skin %lipid wet and dry weight measurements

Mean %lipid of walrus full-thickness blubber with attached skin (*n*=88) was determined using chloroform:methanol (2:1) in a modified Soxhlet procedure after Schlechtriem *et al.* (2003). Each duplicate sample was weighed into a cellulose thimble of known mass and lipid-extracted for 24 h. If the CV of duplicates was >20%, blubber was re-subsampled and re-extracted, until results were acceptable CV ≤20% or until samples from a particular animal had been processed and analyzed four times (at which time it was removed from the sample pool). Percent lipid (wet and dry weight) were calculated. Mean %lipid of walrus blubber with attached skin was 75.4% ± 13.1% (dry) or 44.6% ± 12.4% (wet). Statistical tests showed that sex, lactation, proportional contribution of higher trophic level prey (HTLP) (represented by ringed seal (*Pusa hispida*), bearded seal (*Erignathus barbatus*), and spectacled eider (*Somateria fischeri*), did not influence %lipid, regardless whether wet or dry weight were used (ANOVA, $P>0.05$). There was no correlation between visually assessed body condition and %lipid (Spearman's Rank Order Correlation, $P>0.05$, Table C-1).

References

Schlechtriem C, Focken U, Becker K (2003) Effect of different lipid extraction methods on $\delta^{13}C$ of lipid and lipid-free fractions of fish and different fish feeds. Isot Environ Health Stud 39:135-140

Table D-1 Percent lipid in walrus blubber and skin by sex and lactation

Category	Sample Size (*n*)	Mean %Lipid (Dry)	Mean %Lipid (Wet)	H_0[4]
Overall	88	75.4 ± 14.1 (*42.0 to 92.2*)	44.6 ± 12.4 (*10.0 to 88.9*)	
Male[2]	20	65.9 ± 6.7 (*42.0 to 85.2*)	35.8 ± 8.8 (*19.4 to 51.7*)	*P*=0.05 dry, *P*=0.05 wet
Female[2]	63	77.4 ± 11.6 (*53.0 to 92.2*)	47.9 ± 14.8 (*23.8 to 61.9*)	
Peripartum Female[2]	53	78.1 ± 7.7 (*53.9 to 92.2*)	47.4 ± 8.7 (*23.8 to 61.9*)	*P*=0.31 dry, *P*=0.89 wet
Non-Reproductive Female[2]	9	73.5 ± 12.4 (*56.6 to 87.5*)	48.1 ± 14.9 (*42.8 to 58.3*)	
"Very Healthy"[3]	55	76.7 ± 12.6 (*46.2 to 92.2*)	44.1 ± 15.2 (*23.6 to 61.9*)	*P*=0.09 dry, *P*=0.06 wet
"Average"[3]	30	74.1 ± 8.9 (*42.0 to 87.5*)	43.2 ± 5.7 (*19.1 to 58.3*)	
"Unhealthy"[3]	3	59.8 ± 4.3 (*57.2 to 62.4*)	30.0 ± 11.3 (*22.7 to 37.3*)	

Mean ± standard deviation (SD) of %lipid in walrus full-thickness blubber and skin (without hair and pigmented epidermal layer) by sex and female reproductive status[1]. Percent lipid is based on both dry and wet weight.

[1] Peripatum females are defined as lactating and/or in the presence of a calf/fetus

[2] Numbers may not add to total samples analyzed as not all walruses sampled were of known sex and reproductive status

[3] As assessed by Alaskan Native hunters as "very healthy", "average", or "unhealthy"

[4] H_0 testing lack of difference between categorical variables (i.e., sex, lactation, body condition)