MILK FATTY ACID COMPOSITION OF PERINATAL AND FORAGING STELLER SEA LIONS: EXAMINATION FROM PUP STOMACHS

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A

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ABSTRACT

To investigate the relationship of milk fatty acid composition between perinatal and foraging Steller sea lions and within each maternal state (i.e., perinatal or foraging), milk samples were collected in 2010 and 2011 via gastric intubation from Steller sea lion pups on a small rookery in the central Gulf of Alaska. Subsamples of initial milk samples were taken over four hours post-collection to examine changes of fatty acids within milk over time. Maternal states of lactating females of sampled pups were determined via remotely operated video cameras on the rookery. Fatty acid composition within milk, collected from Steller sea lion pup stomachs, did not change over the four hour post-collection period, and thus milk fatty acids were not modified within milk over time. Milk fatty acid composition between Steller sea lion maternal states was different, and thus can be utilized to distinguish between perinatal and foraging Steller sea lions of the same geographic region. In the absence of direct observations, this study demonstrated the use of a viable method to determine maternal state. Milk fatty acid composition remained relatively constant within perinatal Steller sea lions, suggesting steady mobilization of fatty acids from blubber to milk, and within foraging Steller sea lions, implying females forage in the vicinity of the rookery and on similar prey species. Differences in milk fatty acid composition between maternal states, including differences in the relative percentages of polyunsaturated fatty acids, may have implications for growth and development of offspring. For lactating Steller sea lions, foraging after the perinatal period is important for continued delivery of fatty acids needed by young pups.

DEDICATION

To my amazing and thoughtful family and friends...

My mom, dad, brothers, and sister for their support, encouragement, patience, and wisdom while pursuing my Master's degree. Thank you!

Also to my wonderful sister for numerous late night talks, comedic relief, and always knowing the right thing to say.

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PREFACE

Steller Sea Lion Life History

Steller sea lions (SSL, *Eumetopias jubatus*) are the largest living species of the Otariidae family and range along the North Pacific Ocean rim from northern Japan to southern California, including the Gulf of Alaska (Kenyon & Rice 1961, Loughlin et al. 1984, 1987). Declines of over 80% in SSL abundance since the 1970's resulted in the western distinct population segment (i.e., west of 144º W) of SSLs being listed as endangered under the United States Endangered Species Act in 1997 (Sease et al. 2001, U.S. Federal Register 1997). Although the eastern distinct population segment (i.e., east of 144º W) was recently delisted from a threatened status (U.S. Federal Register 2013), the western population has still not recovered.

In both distinct population segments, SSLs are opportunistic foragers and concentrate their foraging efforts on densely aggregated prey nearshore and over the continental shelf (Merrick & Loughlin 1997, Sinclair & Zeppelin 2002). In the Gulf of Alaska, year-round SSL diet consists of primarily walleye pollock (*Theragra chalcogramma*), Pacific sand lance (*Ammodytes hexapterus*), and Pacific herring (*Clupea pallasi*) (Pitcher 1981, Merrick et al. 1997, McKenzie & Wynne 2008). Seasonal and geographic shifts in SSL diets occur and are thought to reflect changes in prey distribution (Merrick et al. 1997, Womble & Sigler 2006).

Parturition occurs for female SSLs from mid-May to mid-July on rookeries, areas where females give birth and adult breeding males actively defend breeding territories (Pitcher & Calkins 1981, Loughlin et al. 1984, Pitcher et al. 2001). Reproductive female SSLs may give birth every year, usually to a single pup (Pitcher & Calkins 1981). Pregnant SSL females show moderately strong natal rookery fidelity, tending to return to the rookery where they were born to give birth (Raum-Suryan et al. 2002). Breeding takes place from late-May to late-July, with individual females copulating (i.e., mating) 6 to 14

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days after parturition (Gentry 1970, Sandegren 1970, Pitcher & Calkins 1981, Maniscalco et al. 2006). After copulation, the blastocyst does not implant in the uterus until late September or October (i.e., embryonic diapause), resulting in a nine month gestation period (Pitcher & Calkins 1981, Pitcher et al. 2001).

Generally, SSL mothers nurse their pups for one year, weaning them just prior to the next breeding season (Pitcher & Calkins 1981). However, mothers may continue to nurse their offspring for over two years (Gentry 1970, Sandegren 1970, Pitcher & Calkins 1981, Trites et al. 2006). Lactating females feed in close proximity to their birthing rookery, foraging on average 17 to 20 km away (Merrick et al. 1994, Merrick & Loughlin 1997) for 18 to 23 h (Merrick & Loughlin 1997). Steller sea lion pups start supplementing their milk diet with fish well before weaning (Raum-Suryan et al. 2004, Rehberg & Burns 2008), as young as three months old (Raum-Suryan et al. 2004). Pups begin diving for short durations at shallow depths well before weaning, averaging less than 10 m in depth and for less than 2 min (Merrick & Loughlin 1997, Pitcher et al. 2005, Rehberg & Burns 2008). However, six month old pups are able to dive to nearly 100 m (Pitcher et al. 2005). Round trips of pups are short in distance and duration, averaging less than 15 km for less than 20 h (Raum-Suryan et al. 2004).

Lipids and Fatty Acids

Milk produced through lactation in sea lions, as well as other marine mammals, is composed primarily of lipids (Jenness & Sloan 1970, Trillmich & Lechner 1986, Iverson 1988, Adams 2000). Fatty acids (FA) comprise the majority of neutral lipids including triacylglycerols and complex lipids such as phospholipids (Jenness 1974, Iverson 1993). Lipids are a heterogeneous group of compounds insoluble in water but soluble in organic solvents. When FAs are stored in fat (e.g., blubber), they are preferentially stored as energy in the form of triacylglycerols, which consist of three FA molecules esterified to a glycerol backbone. Fatty acids incorporated in structural elements of the body, such as cellular membranes, are preferentially integrated in the form of phospholipids, which have two FAs attached to a glycerol molecule with a polar derivative of phosphatidic acid (Budge et al. 2006). The most abundant

lipid class in milk is triacylglycerols, which represent 98-99% total lipids in several species of mammals, including seals and sea lions (Iverson 1988, Iverson et al. 1992, Iverson 1993).

Fatty acids, composed of long-chains of carbon and hydrogen atoms with a carboxyl group (COOH) on one end and a methyl group (CH_3) at the other, have many structural arrangements. Individual FAs may have no double bonds between carbon atoms (i.e., saturated FAs, SAFA), a single double bond between two carbon atoms (i.e., monounsaturated FAs, MUFA), or multiple double bonds between different carbon atoms (i.e., polyunsaturated FAs, PUFA). For FAs with geometric isomers, i.e., FAs with the same molecular formula and order of atoms, but different spatial arrangement of the atoms, and restricted rotation about a double bond, the notation of *cis* indicates that hydrogen atoms are on the same side with respect to the double bond (e.g., oleic acid: 18:1n-9*cis*, linoleic acid: 18:2n-6*cis*). The notation of *trans* indicates that hydrogen atoms are on different sides with respect to the double bond. The majority of FAs exist naturally in their *cis* forms, but can be altered to their *trans* forms, which are thermodynamically more stable and have higher melting points (Gunstone 2004). Fatty acids, according to the shorthand nomenclature of the International Union of Pure and Applied Chemistry, are designated as carbon chain length: number of double bonds and location (n–x) of the double bond nearest the terminal methyl group.

Essential Fatty Acids

Two families of PUFAs, the n-6 and n-3 FAs, cannot be synthesized de novo in mammals and therefore must be supplied in the diet (Tinoco 1962, Innis 1991). Linoleic acid (18:2n-6*cis*) and alpha-linolenic acid (α-18:3n-3), the parent FAs of the n-6 and n-3 families, respectively, are essential fatty acids because they have no endogenous precursors and thus only arise from dietary intake (Tinoco 1962, Yamanaka et al. 1981). Through enzymatic chain elongation and desaturation, linoleic acid and alpha-linolenic acid are converted to their long-chain PUFA derivatives (Cook 1991, Sprecher et al. 1995, 1999, Ferdinandusse et al. 2001). Linoleic acid is the precursor of arachidonic acid (20:4n-6), whereas alpha-linolenic acid is the precursor of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA,

22:6n-3) (Cook 1991, Sprecher et al. 1995, 1999, Ferdinandusse et al. 2001). Enzymes in these metabolic pathways are shared by n-6 and n-3 FAs, and therefore, there is competition among these FAs for the same metabolic enzymes (Mohrhauer & Holman 1963, Innis 1991, Spector 1999), which then affects synthesis of n-6 and n-3 eicosanoids (Bell et al. 1995, Whelan 1996, Koletzko & Rodriguez-Palmero 1999).

Specific FAs within the n-6 and n-3 PUFA families are essential for normal growth and neurological functioning (Connor & Neuringer 1988, Innis 1991, 2005). Arachidonic acid is essential for normal growth and a precursor to prostaglandins and leukotrienes, which are important in synaptic transmission (Needleman et al. 1986, Piomelli 1994). In addition, arachidonic acid is an integral structural component of phospholipids in cellular membranes (Sastry 1985, Innis 2007). Docosahexaenoic acid is also an important structural component of phospholipids of biomembranes, including cellular membranes of the brain and retina (Neuringer & Connor 1986, Neuringer et al. 1988, Innis 2007). Docosahexaenoic acid, as well as EPA, are associated with phospholipids of hormone precursors and thus are involved in many physiological processes (Innis 2005). Deficiencies in n-6 FAs result in several abnormalities, including growth retardation, skin lesions, and reproductive failure in humans (*Homo sapiens*) and other mammals (Brown et al. 1938, Holman 1971, Yamanaka et al. 1981, Innis 1991). Deficiencies in n-3 FAs lead to altered learning behavior, impaired vision, and abnormal electroretinograms in various mammalian species (Neuringer & Connor 1986, Neuringer et al. 1988, Innis 2000).

Milk Lipid and Fatty Acid Composition

Marine mammal milk is higher in lipid (i.e., fat) than milk of terrestrial mammals (Jenness & Sloan 1970, Dils 1986). Milk lipid, on a percentage basis, varies among terrestrial mammals including 1.9% in horses (*Equus caballus*), 12.9% in domestic dogs (*Canis lupus familiaris*), and 3.8% in humans (Jenness & Sloan 1970). Milk lipid percent also varies among marine mammal species including 26.9% lipid in beluga whales (*Delphinapterus leucas*) sampled in July (Lauer & Baker 1969), 47.0% lipid in New Zealand fur seals (*Arctocephalus forsteri*) sampled March to October (Baylis & Nichols 2009),

54.4% lipid in northern elephant seals (*Mirounga angustirostris*) sampled in December (Le Boeuf & Ortiz 1977), and 47.5% to 69.0% lipid in hooded seals (*Cystophora cristata*) sampled in March (Oftedal et al. 1988). California sea lion (*Zalophus californianus*) milk consists of 31.7% lipid first month postpartum (Iverson 1988), Australian sea lion (*Neophoca cinerea*) milk of 25.4% lipid averaged across austral summer and winter (Kretzmann et al. 1991), and SSLs in Alaska of 21.6% milk lipid first month postpartum (Adams 2000). Overall milk composition changes over part of the lactation period in many marine mammals, including an increase in total percent lipids and decrease in total percent water (Riedman & Ortiz 1979, Iverson 1988, Arnould & Boyd 1995, Lang et al. 2005). Marine mammal milk, with a high amount of lipid, serves to conserve water in the lactating female (Riedman & Ortiz 1979, Oftedal et al. 1987a) and rapidly transfer energy to the offspring to promote offspring growth and offset offspring heat loss in an aqueous environment (Jenness et al. 1981, Dils 1986).

Milk FA composition differs among mammals (Glass et al. 1967, Dils 1986, Iverson 1988, 1993). Ruminants have an abundance of short-chain FAs (e.g., 4-6 carbons) in their milk, whereas non-ruminants have a larger amount of medium-chain FAs (e.g., 8-12 carbons) in their milk (Glass et al. 1967, Dils 1986). Lagomorph (e.g., rabbits) milk has high concentrations of FAs with 8 and 10 carbons, whereas primate milk is characterized by high concentrations of FAs with 8 to 18 carbons (Glass et al. 1967). Milk FA composition of marsupials, insectivores, and carnivores consists of high concentrations of FAs with 16 and 18 carbons (Glass et al. 1967). Milk of marine mammals is distinguished by large amounts of monounsaturated FAs with 16 carbons and long-chain (i.e., ≥20 carbons) monounsaturated and polyunsaturated FAs (Glass et al. 1967). Studies on milk FA composition in pinnipeds have found high relative percentages of myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1n-7), oleic acid (18:1n-9*cis*), vaccenic acid (18:1n-7), eicosenoic acid (20:1n-9), EPA, and DHA (Jangaard & Ke 1968, Iverson 1988, Brown et al. 1999, Lea et al. 2002).

Milk FAs arise from circulating FAs from recent dietary intake, de novo synthesis within the mammary gland, and circulating FAs from mobilization of stored fat (Bauman et al. 1970, Jenness 1974, Grigor & Warren 1980, Iverson 1993). In phocids (i.e., true seals), milk FA composition is thought to

reflect diet during the fattening period before birth due to a brief (4-50 days, Oftedal et al. 1987a) dedicated lactation period, whereas in otariids (i.e., fur seals, sea lions) it appears to indicate diet both before or after birth due to a prolonged (4-12 months, Oftedal et al. 1987a) lactation that includes foraging during lactation (Iverson 1993). However, some selective mobilization of FAs from blubber to milk, and mammary gland de novo synthesis, occurs resulting in multiple influences on milk FA composition (Riedman & Ortiz 1979, Grahl-Nielsen et al. 2000). Research has been conducted on milk FAs of several pinniped species, including sea lions (Iverson 1988, Baylis et al. 2009), but information on milk FA composition of SSL milk, and its relation to pup health and nutrition, is limited (Adams 2000). Furthermore, the relationship of milk FA composition between fasting and feeding SSLs has not been fully determined.

Milk Lipid Digestion via Gastric Lipase

Milk fat, which is secreted in globules from the mammary gland, is made up of a core of triacylglycerol molecules within a lipid membrane (Patton & Keenan 1975). As milk fat enters a pup's stomach, gastric lipase, the major digestive enzyme acting on milk fat before it enters the small intestine, penetrates the membrane of the milk fat globule and rapidly hydrolyzes triacylglycerol molecules within the globule (i.e., releases individual FAs from their carbon backbone, Patton et al. 1982, Iverson 1988). However, gastric lipase does not disrupt the globule membrane, and therefore released products of hydrolysis, including free fatty acids, remain within the globule until reaching the small intestine where they are then absorbed into the body (Patton et al. 1982, Iverson 1988).

This thesis explores milk FA composition of fasting and feeding SSLs to better understand similarities and differences in milk FA composition over the fasting period (perinatal), over early feeding bouts (foraging), and between maternal states (i.e., perinatal and foraging). Furthermore, this thesis examines how differences in milk FA composition between perinatal and foraging SSLs, if any, relate to pup nutrition. Information on milk FA composition of SSLs can then be used to investigate maternal physiology and milk FAs crucial for pup nourishment.

INTRODUCTION

Lactation is energetically demanding for mammals as mothers transfer vital nutrients and energy via milk to their offspring (Pond 1977, Iverson 1993). Milk of pinnipeds (i.e., true seals, fur seals, sea lions, and walruses) is energy and lipid rich, with milk composition and energy density dependent on the reproductive strategy of each species (Oftedal et al. 1987a). Energy is stored in adipose tissue (e.g., blubber) primarily in the form of triacylglycerols (Iverson 1988, Iverson et al. 1992, Iverson 1993). Lactating females require energy intake to meet their own metabolic demands and those of their offspring. If dietary intake is not sufficient to meet energetic demands of lactation, a female must reduce milk output and/or catabolize her body tissues to supply substrates for continued milk synthesis and secretion (Iverson 1993, Oftedal 2000). If lactating pinnipeds are experiencing a low plane of nutrition, milk proximate composition (e.g., percent lipid) does not change, but rather overall milk output is reduced (Ono et al. 1987, Oftedal et al. 1987b, Iverson 1993). Reduced milk output can negatively affect offspring maturation and survival due to their dependence on milk for neonatal growth and development (Bonner 1984, Oftedal 2000).

One animal in particular where focus on lactation is important, is the Steller sea lion (SSL, *Eumetopias jubatus*), specifically SSLs of the western distinct population segment (i.e., west of 144º W). Declines of over 80% in SSL abundance since the 1970's resulted in the western distinct population segment of SSLs being listed as endangered under the United States Endangered Species Act in 1997 (Sease et al. 2001, U.S. Federal Register 1997). When examining recovery potential of the western distinct population segment of SSLs, it is important to address incoming energy resources of lactating females, as these resources are critical for maternal energetics and care, including successful reproduction and lactation (Trites & Donnelly 2003). Furthermore, it is necessary to understand how incoming resources from female foraging influence pup provisioning. Lactating SSLs rely on incoming nutrients and energy from prey for continued milk production throughout lactation because females do not have large blubber reserves (Adams 2000, Pitcher et al. 2000, Davis et al. 2002). Thus, foraging success of

lactating SSLs of the western distinct population segment is essential for pup development and survival, and ultimately recovery of this endangered population.

Foraging success of lactating SSLs may be at risk due to competition with commercial fisheries for food resources near rookeries. Three primary year-round prey items of SSLs in the Gulf of Alaska are walleye pollock (*Theragra chalcogramma*), Pacific sand lance (*Ammodytes hexapterus*), and Pacific herring (*Clupea pallasi*) (Pitcher 1981, Merrick et al. 1997, McKenzie & Wynne 2008). If direct competition for these resources is occurring between lactating females and commercial fisheries and indirectly causing insufficient foraging by lactating females, fishing regulations may need to be revised to alleviate competition. Less competition would indirectly promote sufficient energy intake of lactating females. Research regarding maternal attendance of lactating SSLs in the western distinct population segment suggests that females are not having difficulty obtaining prey (Milette & Trites 2003, Maniscalco et al. 2006, Trites et al. 2006).

Sea lions, like other pinnipeds within the family Otariidae (i.e., fur seals, sea lions), fast while lactating during the perinatal period, i.e., time between parturition (birth) and the next foraging trip. After approximately one week, sea lions switch to foraging trips alternating with onshore fasting bouts (Gentry 1970, Higgins et al. 1988, Trillmich 1990, Maniscalco et al. 2006). Throughout the perinatal period, females depend on energy stored prior to parturition to support their own metabolic demands and produce milk for their offspring (Oftedal et al. 1987a). Obtaining sufficient food during foraging after the perinatal period is crucial to replenish body stores and continued milk production (Ono et al. 1987).

Fatty acids (FA), the main constituents of most lipids, in milk of otariids reflect diet either during the fattening period prior to parturition or the foraging trip intervals following it (Iverson 1993). Dietary FAs are deposited in blubber with minimal modification during the fattening period and then mobilized from blubber into milk during the perinatal period (Iverson 1993, Iverson et al. 1995b). Lipoprotein lipase, a tissue-bound enzyme that controls tissue uptake of circulating FAs, has higher activity in blubber compared with the mammary gland before parturition, which causes deposition of incoming dietary FAs into blubber during the fattening period (Robinson 1963, Hamosh et al. 1970, Iverson 1993). After

parturition, lipoprotein lipase activity has higher activity in the mammary gland compared with blubber, which causes uptake of mobilized FAs by the mammary gland (Robinson 1963, Hamosh et al. 1970, Iverson 1993). Therefore, milk FAs secreted during the perinatal period are similar to blubber stores and reflect diet over the fattening period (Iverson 1993). During feeding bouts following parturition, lipoprotein lipase activity remains higher in the mammary gland compared with blubber, and incoming dietary FAs are directed to the mammary gland rather than to maternal blubber (Iverson 1993, Iverson et al. 1995a, Mellish et al. 1999). As a result, incoming dietary FAs secreted into milk between foraging trips resemble recent dietary intake (Iverson 1993, Iverson et al. 1997a, Smith et al. 1997). This study focuses on milk FA composition, not overall milk composition (i.e., total carbohydrate, protein, lipid, and water).

Prey availability impacts milk FA composition of lactating marine mammals, including SSLs (Iverson 1993, Iverson et al. 1997a). Dietary milk FAs are a result of prey consumption and, because FA composition of fishes differ among species (Iverson et al. 1997b, Budge et al. 2002, Iverson et al. 2002), changing prey intake from one species to another will change milk FA composition of lactating females (Iverson 1993, Iverson et al. 1997a, 2001). Furthermore, FA compositions of fishes differ within species based on age, maturity, body size, and geographic location (Iverson et al. 1997b, Budge et al. 2002, Iverson et al. 2002), and thus differences in fish age, maturity, body size, and location within prey species can influence milk FA composition.

Although diet influences milk FA composition, selective mobilization of FAs from blubber to milk and mammary gland FA synthesis and selective uptake can also affect milk FA composition (Iverson 1993, Grahl-Nielsen et al. 2000, Wheatley et al. 2008). In several marine mammal species, relative percentages of certain FAs in milk do not match with those in blubber, indicating some degree of selective mobilization of FAs and/or mammary gland activity (Riedman & Ortiz 1979, Grahl-Nielsen et al. 2000). Higher relative percentages of saturated fatty acids (SAFA) myristic acid (14:0), palmitic acid (16:0), and stearic acid (18:0) are found in milk compared with blubber, but percentages of monounsaturated fatty acids (MUFA) with 14, 16, and 18 carbons are lower (Ackman & Jangaard 1965,

Ackman et al. 1968, Iverson et al. 1995b, Grahl-Nielsen et al. 2000, Birkeland et al. 2005). Percentages of arachidonic acid (20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3) are higher in milk relative to blubber as opposed to linoleic acid (18:2n-6*cis*) and alpha-linolenic acid (α-18:3n-3) which are lower (Grahl-Nielsen et al. 2000, Birkeland et al. 2005, Wheatley et al. 2008). In lactating SSLs, milk FA composition differs from blubber FA composition, including higher relative percentages of palmitic acid, oleic acid (18:1n-9*cis*), stearidonic acid (18:4n-3), arachidonic acid, and EPA in milk compared with blubber (Adams 2000). In contrast, relative percentages of monounsaturated FAs with 20 and 22 carbons, and DHA, are lower in milk than blubber of lactating SSLs (Adams 2000). Due to the selective mobilization of FAs from blubber to milk and mammary gland activity, there are changes in milk FA composition over the course of lactation unrelated to dietary consumption. Non-dietary influences on milk FA composition are likely in part due to the changing energetic demands and developmental needs over the course of lactation of the growing offspring (Staniland & Pond 2005, Wheatley et al. 2008).

In the wild, studies comparing body condition and physiology between otariid females in their perinatal periods (i.e., perinatal females) and those who have started foraging (i.e., foraging females) are limited due to difficulties determining whether foraging has started. Although direct observation of females has been used to differentiate between maternal states (i.e., perinatal period or foraging) (Iverson et al. 1997a, Smith et al. 1997), it is not always possible to conduct real-time observations. It is important to identify differences between perinatal and foraging females to understand maternal changes that occur due to physiological demands of the perinatal period (e.g., using limited energy reserves for both maternal metabolism and milk production), and replenishment of a female's body while feeding.

Milk FA composition may provide a means to determine the maternal states of lactating otariid females of the same species in the same area, when milk FA composition of multiple individuals is compared. Based on studies of harbor seals (*Phoca vitulina*) and black bears (*Ursus americanus*), milk FA composition of perinatal and foraging otariid females should be different, creating two distinct groups of females that are differentiated by differences in their milk FA composition (Iverson & Oftedal 1992,

Smith et al. 1997, Iverson et al. 2001). When milk FAs of several females of the same species are examined, two general milk FA compositions should be apparent if perinatal and foraging females are both sampled, and individual females will be characteristic of one of two groups, indicating their maternal state. Comparing the same species is necessary because variations in diet among otariid species will lead to species-specific differences in milk FA composition based on diet (Brown et al. 1999, Debier et al. 1999), which may be problematic in differentiating perinatal and foraging females of different species. Likewise, comparing lactating females of the same geographic region is necessary due to within species variation of prey FAs based on geographic region (Iverson et al. 1997b, Budge et al. 2002), which may lead to milk FA composition differences in sampled individuals who are consuming the same prey species but in different regions. If milk FA composition of females within the same species and geographic region differs between maternal states, it can be used to distinguish maternal states and facilitate comparisons between perinatal and foraging females of that species and location to better understand differences in body condition and physiology between maternal states.

Milk FA composition can either stay similar or change over the course of lactation (Luukkainen et al. 1994, Debier et al. 1999, Birkeland et al. 2005, Wheatley et al. 2008). Milk FA composition remains relatively constant over the course of lactation in beluga whales (*Delphinapterus leucas*, Birkeland et al. 2005) and Old World fruit bats (Chiroptera: Pteropodidae, Hood et al. 2001). Changes in milk FA composition with progression of lactation have been noted in mammals that feed throughout lactation (human (*Homo sapiens*): Luukkainen et al. 1994, Makrides et al. 1995, cow (Holsteins): Kay et al. 2005, ewe (Tsigai, improved Valachian, and Lacaune): Pavlíková et al. 2010) and in Antarctic fur seals (*Arctocephalus gazella*, Iverson et al. 1997a, Staniland & Pond 2005) and black bears (*Ursus americanus*, Iverson & Oftedal 1992), who go through a fasting period before feeding during lactation. Phocids (i.e., true seals) fast throughout lactation, and milk FA composition either remains relatively constant (Stull et al. 1967, Riedman & Ortiz 1979, Debier et al. 1999), or varies (Iverson et al. 1995b, Wheatley et al. 2008, Fowler et al. 2014) over the course of lactation. For females that feed throughout or during a part of lactation, variation in milk FA composition over the course of lactation may result from maternal dietary

changes over the course of lactation (Iverson & Oftedal 1992, Iverson et al. 1997a, Smith et al. 1997). For all females, variation in milk FA composition over the course of lactation may be a result of mothers delivering FAs required by offspring at different stages of their development (Staniland & Pond 2005, Wheatley et al. 2008).

Saturated FAs, MUFAs, and PUFAs are important for young offspring in different ways. Saturated FAs store more chemical energy per unit mass than do MUFAs or PUFAs (Maillet & Weber 2006), and therefore are an important energy source for pups, especially blubber-poor neonates (Wheatley et al. 2008). One SAFA, palmitic acid, is an abundant FA in marine mammal milk and likely a means of transferring energy reserves of a readily catabolizable type to offspring (Bryden & Stokes 1969). Monounsaturated FAs have lower melting points when compared to SAFAs of the same chain length, and therefore remain fluid at lower temperatures (Sinensky 1974), which is important for maintaining membrane fluidity and heat conservation in cold marine environments (Best et al. 2003). Polyunsaturated FAs, although not as energy dense as SAFA or MUFA, are more easily mobilized than SAFA or MUFA (Raclot & Groscolas 1995, Herzberg & Farrell 2003) and more prone to oxidation when compared with SAFA (DeLany et al. 2000). Polyunsaturated FAs, specifically n-6 and n-3 FAs, are required for growth and development (Connor & Neuringer 1988, Innis 1991, 2005). Sea lion pups rely solely on milk for infant nutrition (Bonner 1984), and therefore varying relative percentages of milk FAs over the course of lactation, including changes during the transition from fasting to foraging, may influence energy reserves, thermoregulation, growth, and development of offspring (Fowler et al. 2014).

Examining milk FA composition of lactating SSLs on Chiswell Island, a small rookery in the Gulf of Alaska, offers a means to compare milk FA composition between perinatal and foraging females, examine changes in milk FA composition within the perinatal period and within early lactation foraging trips, and investigate resources (i.e., milk FAs) provided to offspring. The remote-monitoring system at Chiswell Island presents a unique scenario to determine maternal state, length of perinatal period, and number of foraging trips taken, if any, for individual females by the time milk is collected. Researchers at the Alaska SeaLife Center (ASLC) conduct long-term monitoring studies of SSLs within the western

distinct population segment at the Chiswell Island rookery using remotely-operated cameras (Maniscalco et al. 2006). Through repeated behavioral observations (daily every other hour), individual females' attendance patterns (i.e., presence/absence on the rookery) are monitored, thereby establishing females in their perinatal periods and those who have started foraging, and the number of foraging trips females have taken. The lactation period for Chiswell females begins with a brief perinatal fast of approximately 10.7 days (range: 1.3 – 17.1 days) followed by alternating foraging trips at sea with nursing at the rookery (Maniscalco et al. 2006).

Milk collection directly from SSL female teats is very limited because the western distinct population segment of SSLs is currently considered endangered. However, milk can be collected from the stomachs of recently suckling pups to examine maternal milk FA composition because gastric milk FA composition is reflective of maternal milk FA composition (Iverson 1988, 1993). Gastric milk FA composition has been shown to be relatively constant in California sea lion (*Zalophus californianus*) pups for at least eight hours post-suckling (Iverson 1988); however, this has never been verified for SSL pups. If modification (e.g., elongated, desaturated, oxidized) of milk FAs occurs while milk remains in the stomach of a SSL pup, it would lead to biased comparisons of FAs in milk collected from SSL pup stomachs at different times post-suckling.

This study investigates milk FA composition of perinatal and foraging SSLs through examination of milk collected from pup stomachs. To this aim, there are two objectives: 1) verify that FA composition in milk that has been collected from SSL pup stomachs does not change over time, and 2) determine if milk FA composition differs between perinatal and foraging SSLs and within each maternal state.

MATERIALS & METHODS

Sample Collection

Milk samples were collected from the stomachs of 20 SSL pups on July 1, 2010 (with milk, *n*=7) and June 30, 2011 (with milk, *n*=13) at the Chiswell Island rookery (59°36.13'N, 149°34.05'W, Fig. 1). Chiswell Island is located in the northern Gulf of Alaska within the range of the endangered western distinct population segment of SSLs and is within the United States Fish and Wildlife Service Alaska Maritime National Wildlife Refuge. Steller sea lion pups were randomly captured using 62-cm diameter hoop nets and then physically restrained while a milk sample was withdrawn from the stomach via gastric intubation with a 10-mm veterinary stomach tube. In this study, all milk samples from pup stomachs were collected at least five days post-parturition, past the timeframe in which colostrum is produced (Boersma et al. 1991, Csapó et al. 1995, Macias & Schweigert 2001, Pavlíková et al. 2010). In addition, all milk samples had a coloration of white or off-white rather than yellow as is observed in colostrum (Macias & Schweigert 2001). Thus, all milk samples collected were considered mature milk, not colostrum. Milk samples, ranging in volume between 2 to 51.5 ml, were collected from each pup and put into separate 50-ml falcon tubes (VWR International, LLC, Radnor, PA, U.S.A.). Milk samples were kept at ambient temperature, approximately 10.5ºC, in both sampling years. For 19 of 20 pups, 1 ml aliquots (i.e., subsamples) of the original milk sample were taken at 0, 2, and 4 hours (h) post-collection. For the remaining pup, a 1 ml aliquot was taken at initial collection only (i.e., 0 h) due to low milk volume collected from its stomach. Each subsample was immediately placed into a 4-ml glass vial, with a teflon-lined cap, containing 2 ml chloroform ($CHCl_3$, OmniSolv grade, EMD Millipore, Billerica, MA, U.S.A.) with 0.01% butylated hydroxytoluene (v/w) (BHT, 2,6-bis(1,1-dimethylethyl)-4-methylphenol, Sigma-Aldrich Co. LLC, St. Louis, MO, U.S.A.). All milk subsamples preserved in $CHCl_3$ with 0.01% BHT were stored at -20°C until processed. Pups were either permanently or temporarily marked using hot-iron branding (2010) or hair shaving (2011), respectively, to identify sampled pups via remotely-operated cameras once mothers and pups reunited following collections. Collections for this project were performed under National Marine Fisheries Service permit number 14324 and under the ASLC

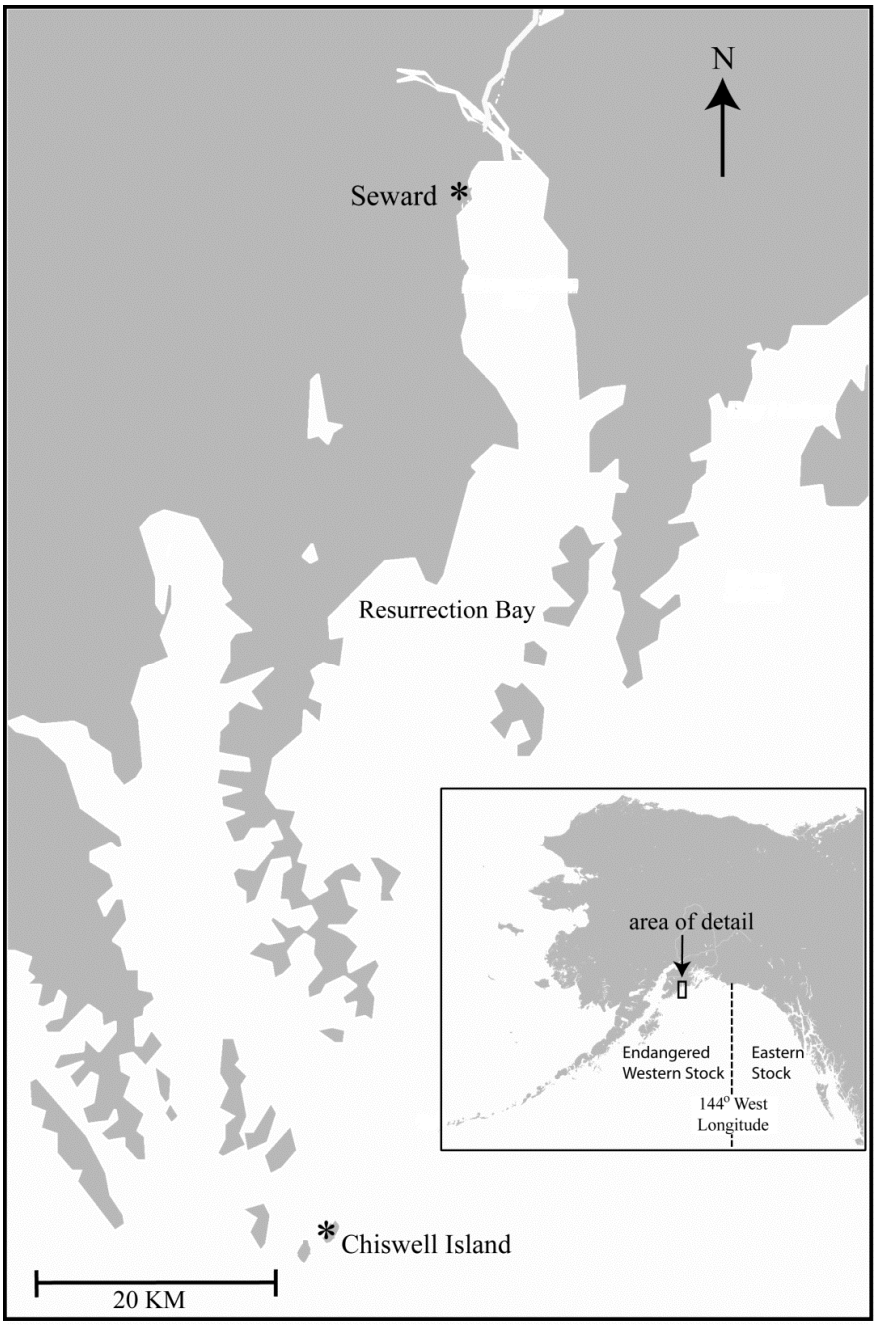

Figure 1: Location of Chiswell Island. Location of Chiswell Island (59°36.13'N, 149°34.05'W) outside of Resurrection Bay in the northern Gulf of Alaska within the range of the endangered western distinct population segment (endangered western stock) of Steller sea lions.

Institutional Animal Care and Use permit number R10-03-01. See Appendix A for a detailed description of sample collection.

Behavioral Observations

Reunions of mothers and their pups were observed via remotely-operated cameras at the ASLC after researchers departed the rookery following each collection trip. Female SSLs with marked pups were identified by their distinctive natural markings (e.g., scars, fungal patches, Maniscalco et al. 2006, Parker et al. 2008). Their presence/absence on the rookery from birth to the sampling day was determined from multiple daily visual observations as described in Maniscalco et al. (2006). Daily presence/absence data were used to establish if a female was in her perinatal period on the sampling day or had been foraging prior to the sampling day. A female was classified as 'perinatal' if she was continually observed on the rookery every day since birth. A female was classified as 'foraging' if she was not observed on the rookery for over four hours on any day since birth. Length of the perinatal period for perinatal females was based on the number of days between parturition and milk collection. The minimum number of foraging trips observed for foraging females was determined using presence/absence data, with a foraging trip being indicated by an absence from the rookery for over four hours.

Lipid Extraction and Fatty Acid Analysis

Sample processing was conducted at the University of Alaska Fairbanks Kodiak Seafood and Marine Science Center. Each milk subsample in $CHCl_3$ with 0.01% BHT was thawed and homogenized using a vortexer (VWR Mini Vortexer, VWR International, LLC) until milk and $CHCl_3$ with 0.01% BHT were visually indistinguishable, and 50% of the homogenate, by weight, was removed. Lipids were extracted using the Folch method (Folch et al. 1957) as modified by Budge et al. (2006). See Appendix B for lipid percentages of SSL milk samples and a sub-study examining lipid recovery of known lipid percentages. Fatty acids in the extracted lipids were converted to their methyl ester counterparts using an acid catalyzed esterification as described in Budge et al. (2006). Briefly, 1.5 ml of methylene chloride (HPLC grade, Honeywell Burdick & Jackson, Morristown, NJ, U.S.A.) and 3.0 ml of Hilditch reagent (0.5 N sulfuric acid (ACS grade, VWR International, LLC) in methanol (HPLC grade, Avantor Performance Materials, Center Valley, PA, U.S.A.)) were added to all extracted lipids in each subsample.

Each sample was flushed with nitrogen (Airgas USA, LLC, Nor Pac region, Vancouver, WA, U.S.A.), capped, and heated at 100ºC for 1 h (Budge et al. 2006). Fatty acid methyl esters (FAME) were extracted into hexane (practical grade, EMD Millipore), concentrated, and brought up to volume with hexane (50 mg FAME per ml hexane, practical grade, EMD Millipore). See Appendix A for detailed description of lipid extraction.

Fatty acid methyl esters were analyzed by gas chromatography using an Agilent model 6850N Series II (Agilent Technologies, Wilmington, DE, U.S.A.) coupled to a flame ionization detector (Agilent Technologies) and fitted with a DB-23 (60 m × 0.25 mm i.d., 0.25 µm film) capillary column (Agilent Technologies). Fatty acid methyl ester peaks were identified based on retention times of known standard mixtures 189-19 FAME (Sigma-Aldrich Co. LLC), PUFA 1 (marine source, Sigma-Aldrich Co. LLC), PUFA 2 (animal source, Sigma-Aldrich Co. LLC), PUFA 3 (Menhaden oil, Sigma-Aldrich Co. LLC), Bacterial Acid Methyl Esters (Sigma-Aldrich Co. LLC), and 189-13 FAME (Sigma-Aldrich Co. LLC). Authentic standards of docosatrienoic acid (22:3n-3, Nu-Check-Prep, Inc., Elysian, MN, U.S.A.), adrenic acid (22:4n-6, Nu-Check-Prep, Inc.), and osbond acid (22:5n-6, Nu-Check-Prep, Inc.) were also used. To ensure accurate identification of FAs, six subsamples were analyzed using a gas chromatography model 6890 interfaced with a mass spectrometer detector model 5973N (Agilent Technologies) fitted with a DB-23 (60 m × 0.25 mm i.d., 0.25 µm film) capillary column (Agilent Technologies). Mass spectra of fatty acid methyl esters in standards, listed above, were compared to spectra available in the NIST/EPA/NIH Mass Spectral Library (NIST 05 v.2.0; National Institute of Standards and Technology, Gaithersburg, MD, U.S.A.). In parallel, mass spectra of fatty acid methyl esters in samples were also compared to library spectra available and/or spectra of FAME standards. Fifty-six FAs were identified in SSL milk (Table 1). See Appendix A for a detailed description FA analysis.

Table 1: Fatty Acids in Steller Sea Lion Milk. Identified fatty acids (FA) in Steller sea lion milk. Dominant FAs (>0.5% of total FAs) are indicated in bold. Source of origin based on Iverson et al. (2004), B: biosynthesized, B/D: biosynthesized and/or dietary, D: dietary. [1]: FAs used in multivariate analysis of variance (MANOVA) of most abundant dominant dietary FAs, [2]: FAs used in MANOVA of dominant biosynthesized/dietary FAs.

FA	FA common name	Source
SATURATED FAs		
12:0	lauric acid	B
13:0	tridecanoic acid	B
iso 14:0	13-methyl-tetradecanoic acid	B
14:0[2]	myristic acid	**B/D**
iso 15:0	14-methyl-pentadecanoic acid	B
ante iso 15:0	13-methyl-pentadecanoic acid	B
15:0	pentadecanoic acid	B
iso 16:0	15-methyl-hexadecanoic acid	B
16:0[2]	palmitic acid	**B/D**
iso 17:0	16-methyl-heptadecanoic acid	B
17:0	margaric acid	B/D
18:0	stearic acid	**B/D**
20:0	arachidic acid	B
MONOUNSATURATED FAs		
14:1n-9	physeteric acid	B
14:1n-7	*cis*-7-tetradecenoic acid	B
14:1n-5	myristoleic acid	B
16:1n-11	*cis*-5-hexadecenoic acid	**B**
16:1n-9	hypogeic acid	B
16:1n-7[2]	palmitoleic acid	**B/D**
16:1n-5	*cis*-11-hexadecenoic acid	B
17:1n-9	*cis*-8-heptadecenoic acid	B
18:1n-13	*cis*-5-octadecenoic acid	B
18:1n-11	*cis*-7-octadecenoic acid	**B**
18:1n-9*cis*[2]	oleic acid	**B/D**
18:1n-7[2]	vaccenic acid	**B/D**
18:1n-5	*cis*-13-octadecenoic acid	B
20:1n-11[1]	gadoleic acid	**D**
20:1n-9[1]	gondoic acid	**D**
20:1n-7	paullinic acid	D
22:1n-11[1]	cetoleic acid	**D**
22:1n-9	erucic acid	D
22:1n-7	*cis*-15-docosenoic acid	D
24:1n-9	nervonic acid	B

Table 1 continued:

FA	FA common name	Source
POLYUNSATURATED FAs		
16:2n-4	*cis*-9,12-hexadecadienoic acid	D
16:3n-4	*cis*-6,9,12-hexadecatrienoic acid	D
16:4n-1	*cis*-6,9,12,15-hexadecatrienoic acid	D
18:2n-7	*cis*-8,11-octadecadienoic acid	B
18:2n-6*cis*[1]	linoleic acid	**D**
18:2n-4	*cis*-11,14-octadecadienoic acid	D
18:3n-6	gamolenic acid	D
18:3n-4	pseudoeleostearic acid	D
18:3n-3	alpha-linolenic acid	**D**
18:4n-3[1]	stearidonic acid	**D**
18:4n-1	*cis*-8,11,14,17-octadecatetraenoic acid	D
20:2n-6 and/or 20:2n-9	eicosadienoic acid and/or *cis*-8,11-eicosadienoic acid	D
20:3n-6	*cis*-8,11,14-eicosatrienoic acid	D
20:4n-6	arachidonic acid	**D**
20:3n-3	*cis*-11,14,17-eicosatrienoic acid	D
20:4n-3[1]	*cis*-8,11,14,17-eicsoatetraenoic acid	**D**
20:5n-3[1]	eicosapentaenoic acid	**D**
21:5n-3	*cis*-6,9,12,15,18-heneicosapentaenoic	D
22:4n-6	adrenic acid	D
22:5n-6	osbond acid	D
22:4n-3	*cis*-10,13,16,19-docosatetraenoic acid	D
22:5n-3[2]	docosapentaenoic acid	**B/D**
22:6n-3[1]	docosahexaenoic acid	**D**

A five-point calibration curve was determined for all FAs in the Supelco 189-19 standard mixture. The curve covered the range of concentrations these FAs were found in the samples investigated. Response factors (RF) for these FAs were established for the system and determined relative to the RF of 18:0 (RF=1) as indicated by Ackman & Sipos (1964). The RF correlating to each quantified FA was multiplied by the FA's chromatogram peak area to yield a corrected area. Corrected peak areas of FAs and chromatogram peak areas of unidentified compounds were summed to yield a total area value, and each FA and unidentified compound was expressed as percent of total FAs and unidentified compounds. Chromatogram peak areas of unidentified compounds were removed from samples (range 0.46-2.04%

total FAs and unidentified compounds) and corrected peak areas of FAs were summed to yield another total area value. The corrected area of each FA was divided by the total area of all FAs and was expressed as percent of total FAs. Fatty acids were designated according to the shorthand nomenclature of the International Union of Pure and Applied Chemistry for carbon chain length: number of double bonds and location (n–x) of the double bond nearest the terminal methyl group.

Milk Fatty Acid Stability Over Time

Because milk samples were taken from SSL pup stomachs at different times post-suckling to compare milk FA composition between and within perinatal and foraging females, it was necessary to verify that SSL pup gastric milk FA composition remains unchanged over time, as found for California sea lions (Iverson 1988), to rule out modification of FAs while milk is in the stomach. Modification of FAs in the stomach would lead to biased comparisons because each milk sample was not in a pup's gastric system for the same amount of time post-suckling. Due to permit stipulations, however, only one gastric milk sample per pup was allowed to be collected, making true verification unattainable. Nevertheless, these gastric milk samples, which were put into 50-ml falcon tubes (VWR International, LLC) and the tubes were closed, likely contained gastric lipase, a digestive enzyme found in milk of seals and sea lions (Iverson 1988). Consequently, milk samples were susceptible to composition modifications due to gastric lipase activity similar to milk contained in the stomach (Iverson 1988). Thus, serial aliquots taken from original milk samples at 0, 2, and 4 h post-collection were analyzed for FA composition before statistical analyses of maternal states to verify that FA composition in milk remained unchanged over time.

Changes in FA composition within milk over a four-hour period were investigated using 55 of the 56 identified FAs (Table 1) in 19 of the 20 milk samples via a one-way repeated measures multivariate analysis of variance (RM-MANOVA) test on transformed data. Stearic acid (18:0) was used as the standardization FA in the log transformation, which caused all milk samples to have values of zero for stearic acid post-transformation, and thus stearic acid was not included in the analysis. Nineteen of 20

milk samples were used because one milk sample had enough volume for only one subsample, which was taken at initial collection. Data were log transformed to meet the assumptions of normality using:

[1] $$x_{trans} = \ln(x_i/c_r)$$

recommended by Aitchison (1986) for compositional data, where x_{trans} is the transformed FA percentage expressed as percent of total FAs, x_i is the original FA percent (% of total FAs), and c_r is the original percent of stearic acid (% of total FAs) (Budge et al. 2002, 2006). Stearic acid is often selected as the reference FA in the log transformation because it can be reliably quantified and gives little information about diet due to origins from biosynthesis (Budge et al. 2002, 2006, Beck et al. 2007). For the analysis, time was considered the independent variable, and individual FA percentages were considered dependent variables. Stearic acid was tested for a change over time via the distribution-free Friedman test using untransformed data. Relative percentages of each FA over time for each pup (n=19) were averaged and used in statistical analyses of maternal states.

Interannual Comparison

Milk from perinatal and foraging SSLs was collected in both sampling years (2010 and 2011). Before milk FA compositions from both sampling years for each maternal state could be combined and used in further analyses, all milk FAs (n=56) were individually compared between years for both perinatal and foraging females to verify that year did not influence milk FA composition for either maternal state. Comparisons were done using Mann-Whitney U tests on untransformed data, and included stearic acid as it was not used as a standardization FA in a log transformation.

Statistical Analyses of Maternal States

All 20 milk samples were incorporated into statistical analyses of maternal states. The milk sample with a single subsample at initial collection was included in statistical analyses of maternal states

to increase sample size. Fatty acids found at <0.5% of total FAs in more than 10 of the 20 milk samples were not included in multivariate statistical analyses because the "noise" introduced due to low precision of their determination may adversely affect results (Dahl et al. 2003, Falk-Petersen et al. 2004). Remaining FAs (i.e., FAs found at >0.5% of total FAs in more than 10 of the 20 milk samples), consisting of 19 out of 56 identified FAs (Table 1), were classified as 'dominant FAs' and used in multivariate statistical analyses. Prior to multivariate statistical analyses, FA percentages were normalized using the log transformation as shown in Equation 1 (Beck et al. 2007, Meynier et al. 2008, Wang et al. 2009). Stearic acid was used as the standardization FA in data transformation, which caused all milk samples to have values of zero for stearic acid post-transformation, and therefore stearic acid, although a dominant FA, was not included in multivariate analyses.

Multidimensional scaling (MDS) and principal component analysis (PCA) were used to examine differences in milk FA composition between maternal states. Multidimensional scaling based on a Bray-Curtis similarity matrix (Bray & Curtis 1957) of dominant FAs, except stearic acid (i.e., n=18 FAs) was used to two-dimensionally visualize variation in milk FA composition between perinatal and foraging females. Principal component analysis was utilized to determine dominant milk FAs, not including stearic acid (i.e., n=18 FAs), primarily causing separation of maternal states. Principal component analysis takes the original correlated variables (i.e., FA percentages) and derives linear combinations (i.e., principal components, PC) of these variables that are uncorrelated but still represent information and variation in the original variables (Wold et al. 1987, Walton et al. 2000, Staniland & Pond 2004). The first two PCs (PC1 and PC2) describe the largest and second largest variance in the original correlated variables. The four FAs most influential on each of the first two components were determined based the on magnitude of their loadings (i.e., degree of variation accounted for by that FA) to that component.

Multivariate analysis of variance (MANOVA) is commonly used to analyze FA compositions (Kirsch et al. 2000, Iverson et al. 2001, Budge et al. 2002, Beck et al. 2007). However, MANOVA requires the number of samples in each sample group to be greater than the number of variables (i.e., FAs) used in the analysis, which gives some assurance that the covariance matrices are homogeneous

(Stevens 1986, Budge et al. 2002, Beck et al. 2007). Sample size of perinatal females (*n*=9) limited the number of FAs that could be used in MANOVA to eight FAs (i.e., *n*-1 where *n* is the number of samples), and thus subsets of dominant FAs, excluding stearic acid, were used in MANOVA tests (Type III-sum of squares) to examine differences in milk FA composition between maternal states. Previous studies on FAs have limited the number of analyzed FAs due to small sample size and selected abundant FAs for multivariate analyses (Kirsch et al. 2000, Iverson et al. 2001, Beck et al. 2007, Wang et al. 2007). Based on Iverson et al. (2004), fatty acids within each MANOVA were chosen based on their primary source as either solely from diet or biosynthesized but likely from diet (i.e., biosynthesized/dietary). Percentages of the eight most abundant dominant dietary FAs and all six dominant biosynthesized/dietary FAs, not including stearic acid, were each analyzed by MANOVA (Table 1).

Given that multivariate methods are restricted by sample size and data must be transformed prior to analysis, univariate tests on data not transformed prior to analysis were conducted to explore differences between maternal states in individual FA percentages (% of total FAs) and combined percentages of FAs sharing the same molecular characteristics. Molecular characteristics consisted of degree of saturation or position of the first double bond relative to the terminal methyl group. Stearic acid was included in univariate tests because data were not log transformed prior to analysis. Each identified milk FA (*n*=56) was examined via a Mann-Whitney U test to determine if the relative percentage of each FA differed between perinatal and foraging females. Student's t-tests (two-tailed) were used to investigate differences in total percentages of milk FA classes (i.e., SAFAs, MUFAs, and PUFAs) between maternal states. Ratios of total percent PUFAs to total percent SAFAs and total percent PUFAs to total percent MUFAs were compared between perinatal and foraging SSLs through student's t-tests (two-tailed) to examine if differences in milk FA classes between perinatal and foraging SSLs collectively led to differences in milk FA composition between maternal states. The ratio of total percent n-3 FAs to total percent n-6 FAs was compared between perinatal and foraging SSLs using a Mann-Whitney U test to explore if changes in n-6 and n-3 FAs collectively led to a difference in milk FA composition between maternal states, and also how the influx of dietary FAs while foraging influenced the n-3 to n-6 ratio.

For analyses of milk FA composition within each maternal state, data were not log transformed prior to analyses, and therefore stearic acid was included (i.e., stearic acid included as a SAFA). Milk FAs were compared individually within each maternal state as well as compared via groupings, according to their molecular characteristics, to assess overall milk FA composition within each maternal state. The relative percentages of EPA and DHA were combined and considered a grouping because EPA can be metabolized to DHA. For perinatal females, Kruskal-Wallis one-way analysis of variance tests were used to examine if differences existed among days in perinatal period (3 days: n=3, 8 days: n=4, 9 days: n=1, 13 days: n=1) for each individual FA, total percent EPA+DHA, the ratio of total percent PUFAs to total percent MUFAs, and total percent n-3 FAs to total percent n-6 FAs. For foraging females, Kruskal-Wallis one-way analysis of variance tests were used to examine if differences existed among the minimum number of foraging trips observed (1 trip: n=4, 2 trips: n=1, 3 trips: n=2, 4 trips: n=3, 7 trips: n=1) for each individual FA, total percent EPA+DHA, the ratio of total percent PUFAs to total percent MUFAs, and total percent n-3 FAs to total percent n-6 FAs.

Statistical analyses were performed using Systat Version 13 (Systat Software Inc., Chicago, IL, U.S.A.). An alpha of 0.05 was considered significant. Outliers, i.e., values smaller than the lower quartile (25% of data are less than this value) or larger than the upper quartile (25% of data are greater than this value) by at least 1.5 times the interquartile range (difference between upper and lower quartiles), if present in data, were excluded from statistical analyses. All data are reported as mean ± standard deviation.

RESULTS

Milk Fatty Acid Stability Over Time

A total of 56 FAs were identified and quantified in all SSL milk subsamples (Table 1). Milk FA composition, consisting of 55 FAs (i.e., 56 FAs minus stearic acid), did not change significantly from initial collection to 2 or 4 h post-collection (one-way RM-MANOVA: $F_{110,2016}$=0.33, p=1.00, Table 2). Stearic acid did not change significantly over the period of 4 h (Friedmann test statistic=1.37, p=0.50). For each female, individual FA relative percentages were averaged over initial collection and 2 and 4 h post-collection for analyses of maternal states.

Interannual Comparison

No significant differences were found in relative percentages of individual milk FAs between 2010 and 2011 perinatal SSLs (Mann-Whitney U tests, p>0.05). Thus, milk FA composition of perinatal females from both sampling years were combined and used in analyses between maternal states and within each maternal state. Significant differences were found, however, in relative percentages for five of the 56 identified milk FAs (lauric acid (12:0): p=0.01, *cis*-5-octadecenoic acid (18:1n-13): p=0.45, stearidonic acid: p=0.02, paullinic acid (20:1n-7): p=0.03, erucic acid (22:1n-9): p=0.02, Mann-Whitney U tests), between 2010 and 2011 foraging SSLs. Biosynthesized FAs, lauric acid and *cis*-5-octadecenoic acid (Cook 1991, Iverson et al. 2004), were found at <0.5% of total FAs. Dietary FAs stearidonic acid, paullinic acid, and erucic acid (Cook 1991, Iverson et al. 2004) were significantly different between years, but only stearidonic acid was found at a relative percentage considered to have precise determination (i.e., >0.5% of total FAs, Dahl et al. 2003, Falk-Petersen et al. 2004). Thus, although three out of a total of 27 identified dietary FAs in milk were significantly different between 2010 and 2011 in milk of foraging SSLs, likely only the difference in stearidonic acid resembles a change in diet, if any, between years. Consequently, it is unlikely that collection year biologically impacted the milk FA composition between years to the extent that pooling data would invalidate results. Thus, milk FA

Table 2: Dominant Fatty Acids Over Time. Relative percentages of dominant fatty acids (FA, >0.5% of total FAs) and classes of FAs (total %, in bold) in milk collected from the stomachs of four Steller sea lion pups (P: with perinatal mother, F: with foraging mother) at initial collection (0h) and 2 and 4 h post-collection. SAFAs: saturated FAs, MUFAs: monounsaturated FAs, PUFAs: polyunsaturated FAs.

	Pup P2 (2010)			**Pup P6 (2011)**			**Pup F1 (2010)**			**Pup F10 (2011)**		
	time post-collection (h)			**time post-collection (h)**			**time post-collection (h)**			**time post-collection (h)**		
FA	**0**	**2**	**4**	**0**	**2**	**4**	**0**	**2**	**4**	**0**	**2**	**4**
14:0	5.52	5.51	5.38	5.30	5.23	5.36	5.42	5.38	5.34	4.93	4.87	4.96
16:0	17.17	17.11	17.01	16.68	16.37	16.74	15.03	14.73	14.70	13.86	13.61	13.67
18:0	1.59	1.57	1.52	1.76	1.68	1.73	1.82	1.78	1.76	1.69	1.65	1.66
SAFAs	**25.70**	**25.62**	**25.46**	**23.73**	**23.28**	**23.82**	**22.28**	**21.89**	**21.80**	**22.02**	**21.78**	**21.87**
16:1n-11	0.51	0.51	0.51	0.65	0.66	0.66	0.61	0.61	0.61	0.70	0.69	0.71
16:1n-7	7.07	7.12	7.03	6.28	6.29	6.32	5.68	5.70	5.72	4.55	4.52	4.60
18:1n-11	2.66	2.46	2.43	2.70	2.73	2.72	2.54	2.54	2.56	2.87	2.88	2.92
18:1n-9*cis*	32.28	32.37	32.04	29.04	29.41	28.95	25.49	25.19	25.33	18.32	18.35	18.55
18:1n-7	6.54	6.57	6.50	4.69	4.75	4.72	4.35	4.33	4.36	2.91	2.92	2.96
20:1n-11	2.86	2.85	2.79	3.11	3.13	3.06	4.15	4.07	4.05	4.77	4.79	4.81
20:1n-9	1.27	1.27	1.25	1.23	1.24	1.22	1.53	1.50	1.50	1.86	1.86	1.88
22:1n-11	0.86	0.89	0.86	0.79	0.72	0.76	1.20	1.20	1.21	2.01	2.01	2.30
MUFAs	**56.66**	**56.63**	**56.30**	**48.49**	**48.93**	**48.40**	**45.56**	**45.13**	**45.32**	**40.93**	**43.06**	**45.63**
18:2n-6*cis*	1.52	1.53	1.50	1.78	1.79	1.80	1.51	1.52	1.52	1.57	1.57	1.58
18:3n-3	0.45	0.46	0.45	0.84	0.84	0.84	0.88	0.89	0.87	1.07	1.07	1.07
18:4n-3	0.36	0.39	0.47	0.82	0.82	0.83	1.03	1.05	1.02	1.79	1.79	1.78
20:4n-6	0.75	0.76	0.93	0.85	0.85	0.86	0.60	0.61	0.67	0.71	0.71	0.71
20:4n-3	0.61	0.61	0.79	1.26	1.24	1.26	1.47	1.48	1.44	2.09	2.10	2.09
20:5n-3	4.46	4.48	4.40	5.95	5.95	6.01	6.34	6.43	6.29	7.89	7.88	7.86
22:5n-3	1.96	1.99	1.92	2.51	2.52	2.53	3.24	3.30	3.24	4.14	4.15	4.15
22:6n-3	5.87	5.92	5.78	7.77	7.78	7.84	11.01	11.17	10.94	15.66	15.58	15.55
PUFAs	**17.63**	**17.76**	**18.25**	**21.79**	**21.79**	**21.98**	**26.06**	**26.44**	**26.00**	**37.05**	**39.16**	**40.50**
Dominant FA total %	**94.30**	**94.35**	**93.57**	**94.01**	**94.00**	**94.20**	**93.90**	**93.47**	**93.12**	**93.41**	**92.99**	**93.82**

composition of foraging females from both sampling years were combined and used in analyses comparing milk FA composition between maternal states and within foraging females.

Spatial Separation of Perinatal and Foraging Females

Milk FA composition of both perinatal and foraging SSLs contained the same suite of FAs (Table 3). Out of 56 identified FAs in milk, the three most abundant FAs for both maternal states were oleic acid, palmitic acid, and DHA. Saturated fatty acids accounted for 21.77% to 27.17% of total FAs, MUFAs for 38.90% to 60.59% of total FAs, and PUFAs for 17.63% to 38.13% of total FAs in milk when milk from both perinatal and foraging SSLs was examined.

Foraging SSLs were distinguished from perinatal SSLs based on MDS ordination of milk samples using the 18 dominant FAs (stearic acid not included, Fig. 2). Although some perinatal and foraging females overlap in their respective clusters, there is a visible separation between the two maternal states. The perinatal female from 2010 (letter B) is within the cluster of 2011 perinatal females (letters A, C - I) (Fig. 2). Foraging females from 2010 (letters J, M - Q) are not visually separated from 2011 foraging females (letters K, L, R - T) (Fig. 2).

Principal component analysis of the 18 dominant FAs (stearic acid not included) in milk of perinatal and foraging SSLs produced four PCs with eigenvalues >1.0, and in combination these PCs explained 90.53% of the total variance (PC1: 41.07%, PC2: 33.54%, PC3: 8.89%, PC4: 7.03%). Clustering of sampling groups is illustrated via a plot of the first two PCs (Fig. 3a). Fatty acids mainly causing separation of individuals were revealed through the first four PCs sorted by magnitude. Based on the loadings of FAs along PC1, foraging SSLs were separated from perinatal SSLs primarily due to higher positive loadings of *cis*-8,11,14,17-eicsoatetraenoic acid (20:4n-3), alpha-linolenic acid, stearidonic acid, and DHA (Fig. 3b). Separation of maternal states based on FA loadings along PC2 was mostly caused by higher positive loadings of linoleic acid, arachidonic acid, palmitic acid, and palmitoleic acid (16:1n-7) (Fig. 3b).

Table 3: M**ilk Fatty Acids of Perinatal and Foraging Steller Sea Lions.** Relative percentages of identified fatty acids (FA), FA classes (italicized), and ratios (italicized) in milk of perinatal and foraging Steller sea lions. Values are expressed as the mean % of total FAs ± 1 standard deviation. Dominant FAs (>0.5% of total FAs) are indicated in bold. SAFAs: saturated FAs, MUFAs: monounsaturated FAs, PUFAs: polyunsaturated FAs. Asterisk indicates a significant difference (*: p <0.05) between perinatal and foraging females based on univariate test statistics.

	Perinatal	**Foraging**
FA	n = 9	n = 11
12:0*	0.11 ± 0.01	0.16 ± 0.06
13:0*	0.02 ± 0.01	0.03 ± 0.01
iso 14:0*	0.02 ± 0.00	0.02 ± 0.00
14:0	**5.19 ± 0.84**	**5.93 ± 0.75**
iso 15:0*	0.12 ± 0.03	0.17 ± 0.02
ante iso 15:0*	0.04 ± 0.01	0.05 ± 0.01
15:0*	0.35 ± 0.05	0.43 ± 0.06
iso 16:0	0.05 ± 0.01	0.06 ± 0.01
16:0	**15.35 ± 1.04**	**15.05 ± 0.91**
iso 17:0	0.13 ± 0.02	0.16 ± 0.04
17:0	0.61 ± 0.18	0.49 ± 0.06
18:0	**1.79 ± 0.24**	**1.85 ± 0.25**
20:0	0.06 ± 0.02	0.08 ± 0.01
total % SAFAs	*23.86 ±1.38*	*24.50 ±1.50*
14:1n-9*	0.11 ± 0.03	0.08 ± 0.02
14:1n-7*	0.03 ± 0.01	0.02 ± 0.00
14:1n-5*	0.29 ± 0.03	0.21 ± 0.03
16:1n-11	**0.71 ± 0.13**	**0.66 ± 0.05**
16:1n-9*	0.57 ± 0.10	0.42 ± 0.06
16:1n-7*	**6.07 ± 0.50**	**4.99 ± 0.53**
16:1n-5	0.22 ± 0.03	0.24 ± 0.03
17:1n-9*	0.37 ± 0.07	0.30 ± 0.04
18:1n-13*	0.35 ± 0.08	0.26 ± 0.03
18:1n-11*	**3.68 ± 0.76**	**2.45 ± 0.32**
18:1n-9*cis**	**28.74 ± 4.87**	**20.42 ± 2.89**
18:1n-7*	**4.89 ± 0.95**	**3.44 ± 0.59**
18:1n-5	0.42 ± 0.05	0.47 ± 0.03
20:1n-11	**4.50 ± 1.49**	**4.54 ± 0.61**
20:1n-9	**1.42 ± 0.26**	**1.61 ± 0.20**
20:1n-7*	0.20 ± 0.02	0.38 ± 0.12
22:1n-11*	**1.00 ± 0.64**	**1.78 ± 0.57**
22:1n-9*	0.16 ± 0.04	0.47 ± 0.25
22:1n-7*	0.02 ± 0.01	0.06 ± 0.02
24:1n-9*	0.08 ± 0.03	0.17 ± 0.05
*total % MUFAs**	*53.83 ±4.00*	*42.96 ±3.51*

Table 3 continued:

	Perinatal	**Foraging**
FA	n = 9	n = 11
16:2n-4	0.26 ± 0.05	0.28 ± 0.06
16:3n-4	0.08 ± 0.04	0.11 ± 0.04
16:4n-1*	0.09 ± 0.08	0.18 ± 0.10
18:2n-7*	0.06 ± 0.01	0.05 ± 0.01
18:2n-6*cis**	**1.80 ± 0.16**	**1.51 ± 0.15**
18:2n-4	0.10 ± 0.02	0.10 ± 0.02
18:3n-6*	0.04 ± 0.02	0.05 ± 0.01
18:3n-4	0.12 ± 0.05	0.10 ± 0.03
18:3n-3*	**0.69 ± 0.17**	**1.01 ± 0.12**
18:4n-3*	**0.60 ± 0.32**	**1.68 ± 0.60**
18:4n-1	0.22 ± 0.08	0.22 ± 0.05
20:2n-6 and/or 20:2n-9*	0.15 ± 0.02	0.23 ± 0.05
20:3n-6	0.08 ± 0.01	0.09 ± 0.02
20:4n-6*	**0.78 ± 0.09**	**0.64 ± 0.10**
20:3n-3*	0.10 ± 0.06	0.15 ± 0.04
20:4n-3*	**0.97 ± 0.29**	**1.73 ± 0.34**
20:5n-3*	**4.57 ± 1.35**	**7.83 ± 1.00**
21:5n-3*	0.25 ± 0.06	0.38 ± 0.10
22:4n-6	0.07 ± 0.01	0.06 ± 0.01
22:5n-6*	0.09 ± 0.02	0.12 ± 0.03
22:4n-3*	0.04 ± 0.01	0.06 ± 0.02
22:5n-3	**2.73 ± 0.46**	**3.18 ± 0.61**
22:6n-3*	**8.43 ± 1.35**	**12.78 ± 1.88**
*total % PUFAs**	*22.31 ± 3.34*	*32.54 ± 3.59*
*total % PUFAs : total % SAFAs**	*0.94 ± 0.13*	*1.34 ± 0.20*
*total % PUFAs : total % MUFAs**	*0.42 ± 0.09*	*0.77 ± 0.14*
*total % n-3 FAs : total % n-6 FAs**	*6.09 ± 0.70*	*10.71 ± 1.10*

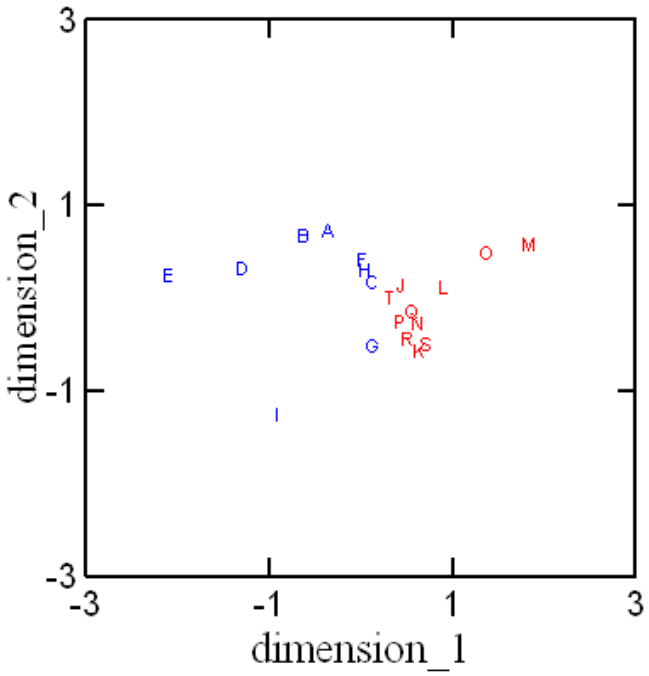

Figure 2: Multidimensional Scaling Ordination of Perinatal and Foraging Steller Sea Lion Milk Fatty Acids. Multidimensional scaling (MDS) ordination using 18 dominant milk fatty acids (FA, >0.5% of total FAs) from perinatal (blue, A – I) and foraging (red, J – T) Steller sea lions who gave birth on Chiswell Island in 2010 or 2011. Blue letters represent milk samples from perinatal females of both sampling years (B: 2010, A, C - I: 2011). Red letters represent milk samples from foraging females of both sampling years (J, M - Q: 2010, K, L, R - T: 2011). Axes of MDS plot ordination represent new variables (i.e., dimension_1 and dimension_2) which summarize the original variables (i.e., dominant FAs). Stress of final configuration: 0.094. Data on milk FAs of these perinatal and foraging females can be found in Tables 4 and 5, respectively. Dominant FAs consisted of myristic acid (14:0), palmitic acid (16:0), *cis*-5-hexadecenoic acid (16:1n-11), palmitoleic acid (16:1n-7), *cis*-7-octadecenoic acid (18:1n-11), oleic acid (18:1n-9*cis*), vaccenic acid (18:1n-7), linoleic acid (18:2n-6*cis*), α-linolenic acid (α-18:3n-3), stearidonic acid (18:4n-3), gadoleic acid (20:1n-11), gondoic acid (20:1n-9), arachidonic acid (20:4n-6), *cis*-8,11,14,17-eicsoatetraenoic acid (20:4n-3), eicosapentaenoic acid (20:5n-3), cetoleic acid (22:1n-11), docosapentaenoic acid (22:5n-3), and docosahexaenoic acid (22:6n-3).

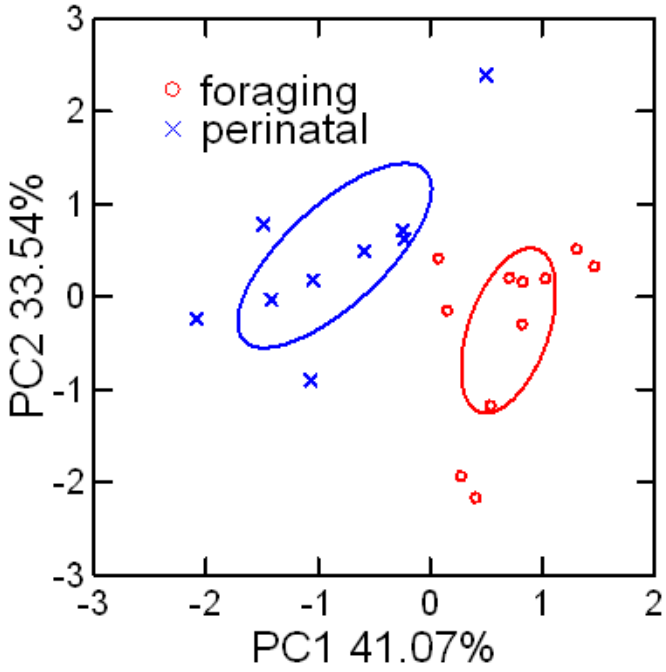

Figure 3a: Principal Component Analysis of Perinatal and Foraging Steller Sea Lion Milk Fatty Acids. Principal component analysis based on 18 dominant milk fatty acids (FA, >0.5% of total FAs) of perinatal (blue x) and foraging (red open circle) Steller sea lions that gave birth on Chiswell Island in 2010 or 2011. Milk samples from perinatal and foraging females for both 2010 and 2011 were included in analysis. Plot of principal component 2 (PC2) versus principal component 1 (PC1) with 95% confidence ellipses. PC 1 and PC2 collectively explain 74.61% of the observed variation. Dominant FAs consisted of myristic acid (14:0), palmitic acid (16:0), *cis*-5-hexadecenoic acid (16:1n-11), palmitoleic acid (16:1n-7), *cis*-7-octadecenoic acid (18:1n-11), oleic acid (18:1n-9*cis*), vaccenic acid (18:1n-7), linoleic acid (18:2n-6*cis*), α-linolenic acid (α-18:3n-3), stearidonic acid (18:4n-3), gadoleic acid (20:1n-11), gondoic acid (20:1n-9), arachidonic acid (20:4n-6), *cis*-8,11,14,17-eicsoatetraenoic acid (20:4n-3), eicosapentaenoic acid (20:5n-3), cetoleic acid (22:1n-11), docosapentaenoic acid (22:5n-3), and docosahexaenoic acid (22:6n-3).

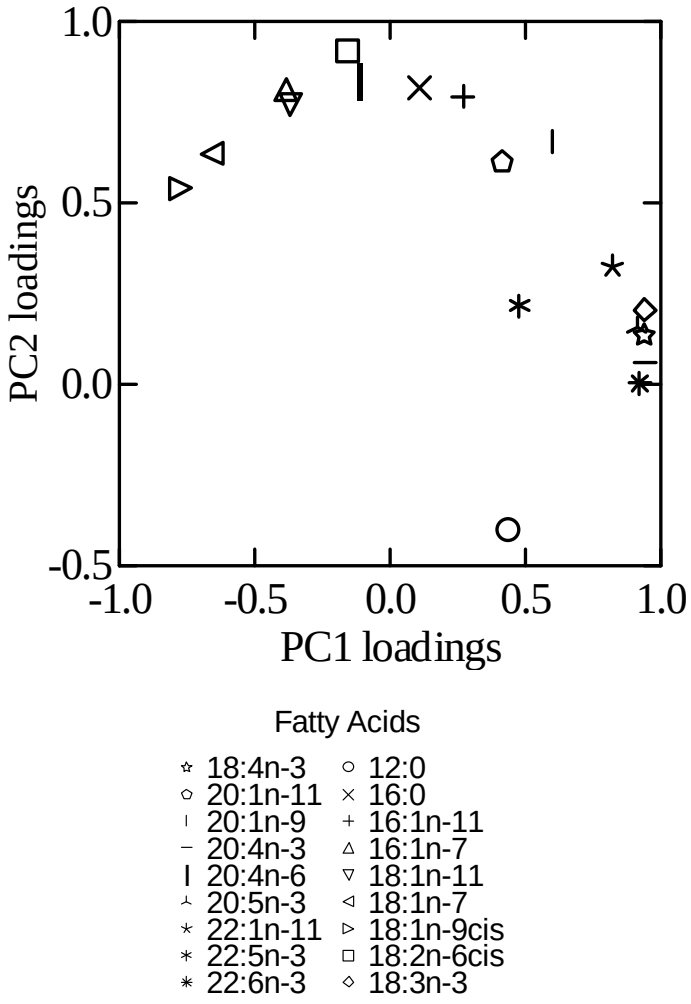

Figure 3b: Principal Component Loadings Plot of Dominant Fatty Acids. Plot of first (PC1) and second principal component (PC2) loadings of 18 dominant fatty acids (FA, >0.5% of total FAs) used in principal component analysis of Steller sea lion milk.

Milk Fatty Acid Composition Between Perinatal and Foraging Females

Composition of dominant milk FAs of foraging SSLs differed significantly from perinatal females. Milk FA composition between maternal states was significantly different based on the eight most abundant dietary FAs (MANOVA: $F_{8, 11}$=16.87, p<0.01, Fig. 4) and the six dominant biosynthesized/dietary FAs (stearic acid not included, MANOVA: $F_{6,13}$=6.76, p<0.01, Fig. 5).

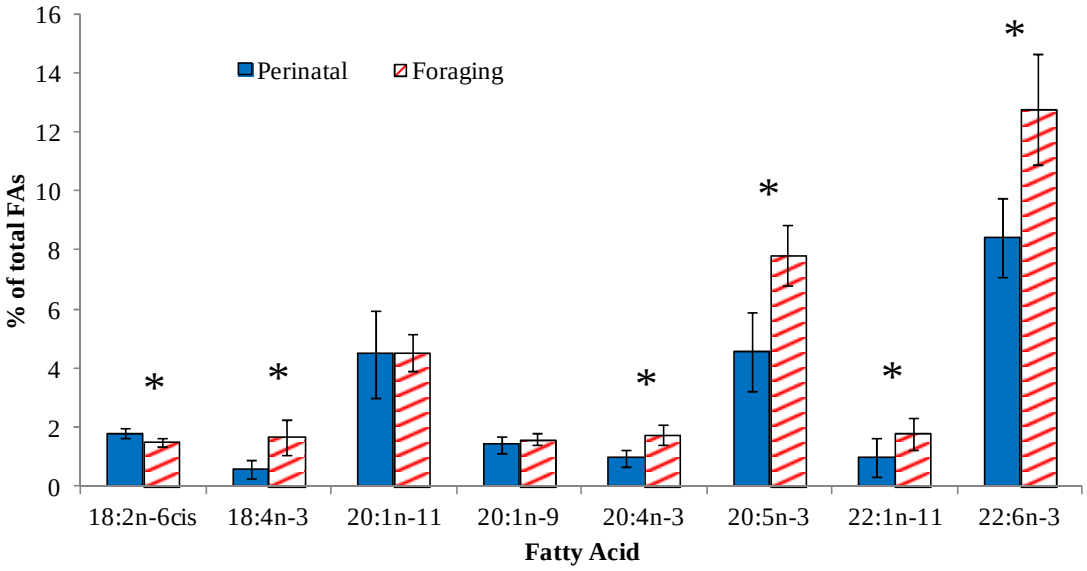

Figure 4: Abundant Dietary Fatty Acids in Steller Sea Lion Milk. Dietary fatty acids (FA, based on Iverson et al. 2004) of highest abundance (% of total FAs) in milk of perinatal (n=9, blue solid) and foraging (n=11, red diagonal lines) Steller sea lions. Mean values are expressed as percent of total FAs (i.e., % of all 56 FAs). Error bars represent one standard deviation. Asterisk indicates a significant difference between perinatal and foraging females using a Mann-Whitney U test (p<0.05). 18:2n-6*cis*: linoleic acid, 18:4n-3: stearidonic acid, 20:1n-11: gadoleic, 20:1n-9: gondoic acid, 20:4n-3: *cis*-8,11,14,17-eicosatetraenoic acid, 20:5n-3: eicosapentaenoic acid, 22:1n-11: cetoleic acid, 22:6n-3: docosahexaenoic acid.

Relative percentages of individual FAs between maternal states were significantly different for 12 out of the 19 dominant FAs (stearic acid included) and for 36 out of all 56 identified FAs (Mann-Whitney U tests, Table 3). Most notable differences among dominant dietary FAs were an increase of 181.10% in stearidonic acid and of 78.76% in *cis*-8,11,14,17-eicsoatetraenoic acid, and a decrease of 16.30% in

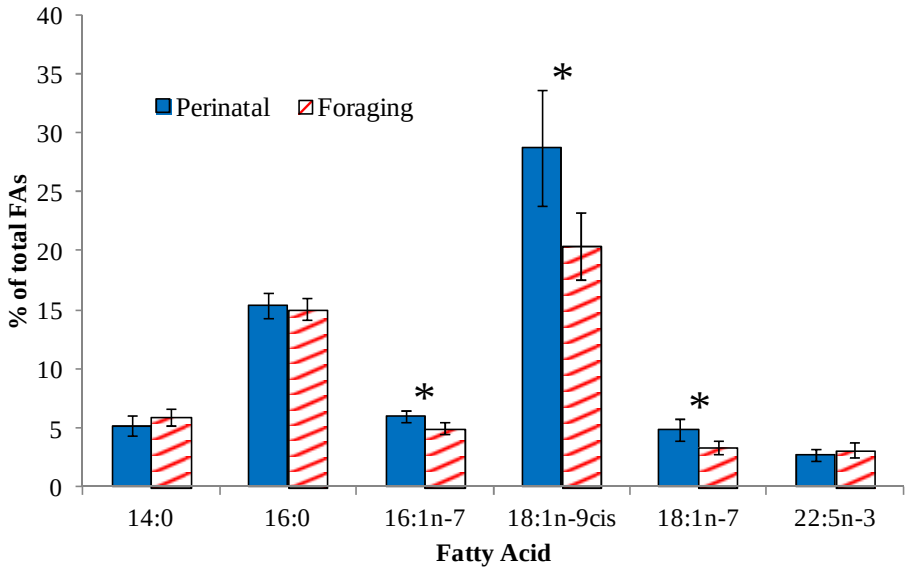

Figure 5: Abundant Biosynthesized and/or Dietary Fatty Acids in Steller Sea Lion Milk. Biosynthesized and/or dietary milk fatty acids (FA, based on Iverson et al. 2004) of highest abundance (% of total FAs) of perinatal (*n*=9, blue solid) and foraging (*n*=11, red diagonal lines) Steller sea lions. Mean values are expressed as percent of total FAs (i.e., % of all 56 FAs). Error bars represent one standard deviation. Asterisk indicates a significant difference between perinatal and foraging females using a Mann-Whitney U test ($p<0.05$). 14:0: myristic acid, 16:0: palmitic acid, 16:1n-7: palmitoleic acid, 18:1n-9*cis*: oleic acid, 18:1n-7: vaccenic acid, 22:5n-3: docosapentaenoic acid.

linoleic acid and of 17.87% in arachidonic acid, from perinatal to foraging state. The relative percentage of DHA was significantly different between maternal states (Mann-Whitney U test statistic=98.00, $p<0.01$), ranging from 5.86% to 10.53% of total FAs in perinatal females and from 10.21% to 15.60% of total FAs in foraging females. Relative percent of EPA, found as low as 2.37% of total FAs in a perinatal female and as high as 9.59% of total FAs in a foraging female, was also significantly different (Mann-Whitney U test statistic=97.00, $p<0.01$). Percentages of gadoleic acid (20:1n-11, Mann-Whitney U test statistic=57.00, $p=0.57$) and gondoic acid (20:1n-9, Mann-Whitney U test statistic=74.00, $p=0.06$) were not significantly different. Biosynthesized/dietary FAs showing the greatest change on a relative percent basis between maternal states were palmitoleic acid and oleic acid which decreased, and docosapentaenoic acid (22:5n-3) which increased from perinatal to foraging maternal state.

Based on the overall milk FA composition, total percent of both PUFAs (Student's t-test, $p<0.01$) and MUFAs (Student's t-test, $p<0.01$) were significantly different between perinatal and foraging SSLs, with MUFAs being higher in perinatal females and PUFAs being higher in foraging females (Table 3, Fig. 6). Ratio comparisons between maternal states of total percent PUFAs to total percent SAFAs (Student's t-test, $p<0.01$) and total percent PUFAs to total percent MUFAs (Student's t-test, $p<0.01$, Fig. 7) were also significantly different (Table 3). All milk samples were enriched in n-3 FAs compared with n-6 FAs, but foraging females had a significantly higher ratio of total percent n-3 FAs to total percent n-6 FAs than perinatal females (Mann-Whitney U test statistic=77.00, $p<0.01$, Table 3, Fig. 8).

Milk FA Composition Within Perinatal and Foraging Females

Within perinatal females, there was no significant difference in any individual FA among days in perinatal period (Kruskal-Wallis tests, $p>0.05$, Fig. 9). Similarly, total percent EPA plus DHA (Kruskal-Wallis test statistic=2.67, $p=0.45$, Table 4), ratio of total percent PUFAs to total percent MUFAs (Kruskal-Wallis test statistic=2.67, $p=0.45$, Table 4), and ratio of total percent n-3 FAs to total percent n-6 FAs (Kruskal-Wallis test statistic=3.01, $p=0.39$, Table 4) were not significantly different among days in perinatal period. Within foraging females, there was no significant difference in any individual FA among the minimum number of foraging trips observed (Kruskal-Wallis tests, $p>0.05$, Fig. 10). Total percent EPA plus DHA (Kruskal-Wallis test statistic=3.48, $p=0.48$, Table 5), ratio of total percent PUFAs to total percent MUFAs (Kruskal-Wallis test statistic=3.07, $p=0.54$, Table 5), and ratio of total percent n-3 FAs to total percent n-6 FAs (Kruskal-Wallis test statistic=5.08, $p=0.28$, Table 5) were not significantly different among the minimum number of foraging trips observed.

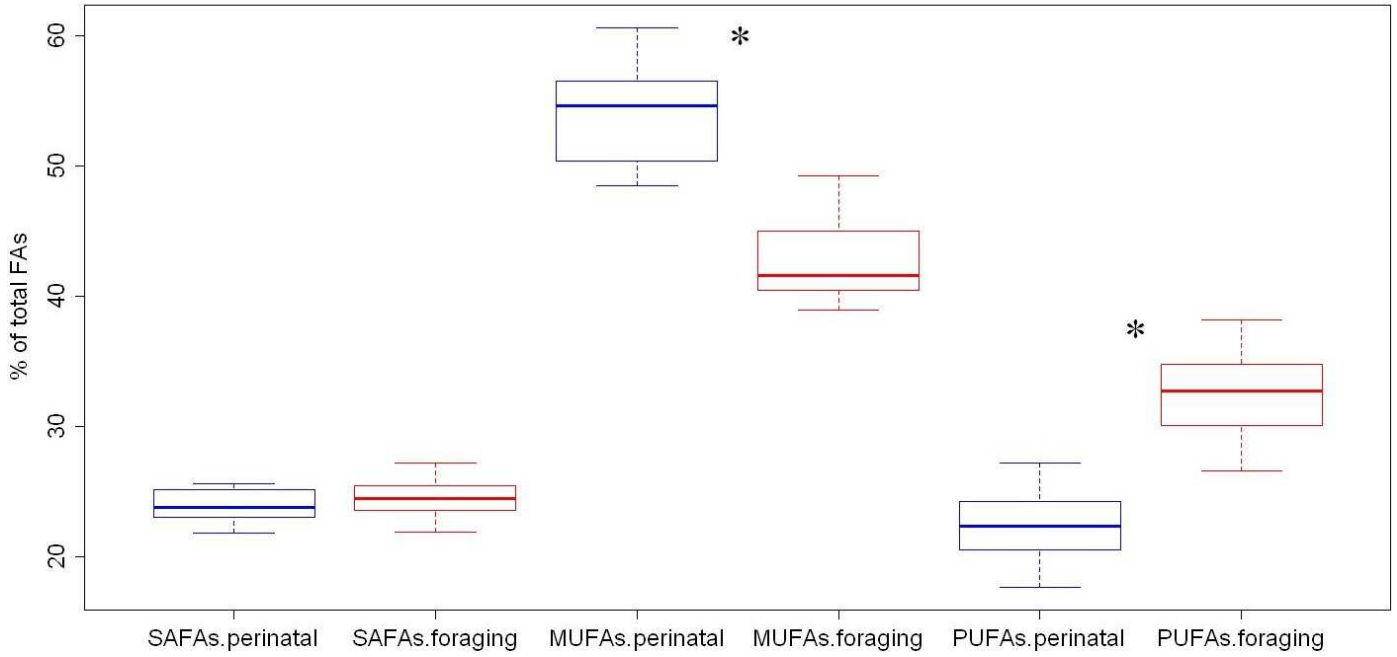

Figure 6: Fatty Acid Classes in Perinatal and Foraging Steller Sea Lion Milk. Total percent (% of total FAs) of saturated fatty acids (SAFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) in milk of perinatal (blue, n=9) and foraging (red, n=11) Steller sea lions. Asterisk indicates a significant difference between perinatal and foraging Steller sea lions using a Student's t-test, p<0.01). The median value for each maternal state's SAFAs, MUFAs, and PUFAs is represented by a bold line within each box. Boxes represent values between the lower quartile (i.e., 25% of data are less than this value) and upper quartile (i.e., 25% of data are greater than this value). Whiskers represent the maximum and minimum value**.**

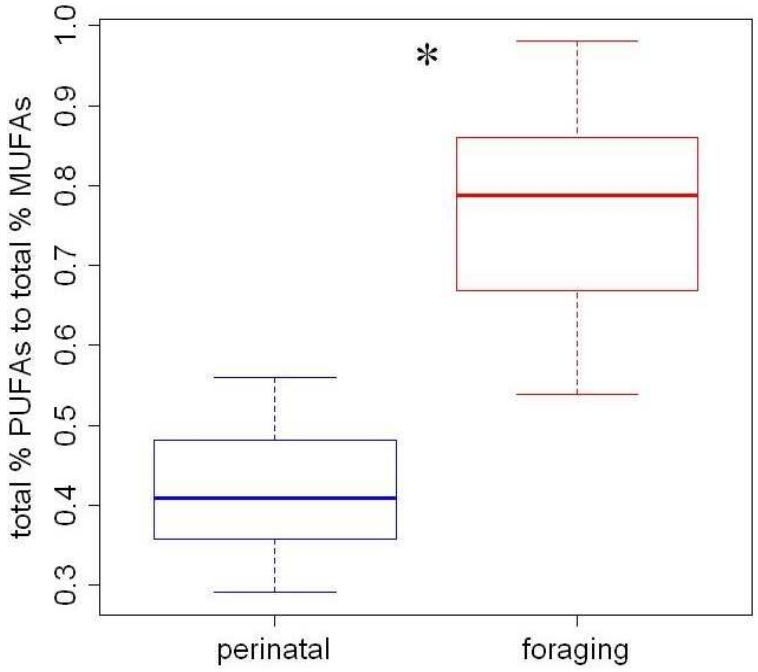

Figure 7: Ratio of Polyunsaturated to Monounsaturated Fatty Acids in Perinatal and Foraging Steller Sea Lion Milk. Visual comparison of the ratio of total percent polyunsaturated fatty acids (PUFAs) to total percent monounsaturated fatty acids (MUFAs) in milk of perinatal (n=9) and foraging (n=11) Steller sea lions. Asterisk indicates a significant difference between perinatal and foraging Steller sea lions (Student's t-test, p<0.01). The median value for each maternal state's ratio of total percent PUFAs to total percent MUFAs is represented by a bold line within each box. Boxes represent values between the lower quartile (i.e., 25% of data are less than this value) and upper quartile (i.e., 25% of data are greater than this value). Whiskers represent the maximum and minimum values.

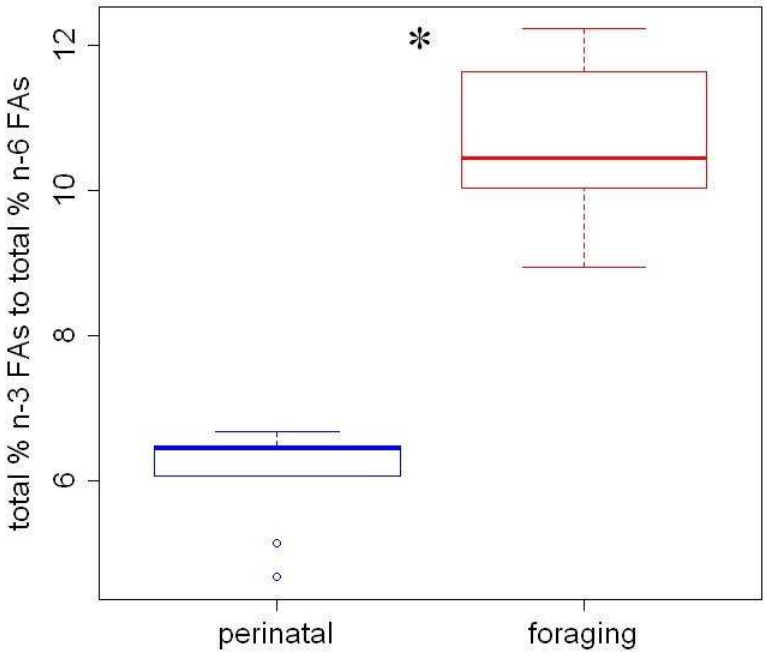

Figure 8: Ratio of n-3 Fatty Acids to n-6 Fatty Acids in Perinatal and Foraging Steller Sea Lion Milk. Visual comparison of the ratio of total percent n-3 fatty acids (FA) to total percent n-6 fatty acids in milk of perinatal (n=9) and foraging (n=11) Steller sea lions. Asterisk indicates a significant difference between perinatal and foraging Steller sea lions (Mann-Whitney U test, p<0.01). The median value for each maternal state's ratio of total percent n-3 FAs to total percent n-6 FAs is represented by a bold line within each box. Boxes represent values between the lower quartile (25% of data are less than this value) and upper quartile (25% of data are greater than this value). Whiskers represent the maximum and minimum values, excluding outliers. Outliers, i.e., values smaller than the lower quartile or larger than the upper quartile by at least 1.5 times the interquartile range (difference between upper and lower quartiles), are indicated by small, open blue circles.

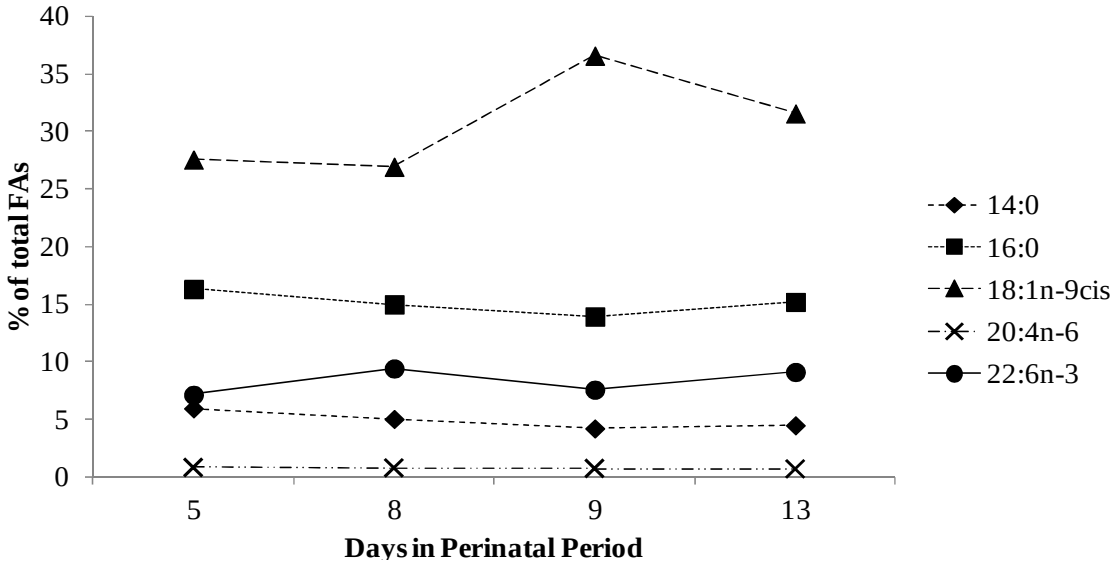

Figure 9: Milk Fatty Acid Relative Percentages Among Days in Perinatal Period. Relative percentages of five abundant fatty acids (FA, >0.5% of total FAs) across days in the perinatal period for perinatal females. No significant difference was found in any individual FA among days in the perinatal period (Kruskal-Wallis one-way analysis of variance test, $p>0.05$).

Table 4: Milk Fatty Acid Groupings of Perinatal Steller Sea Lions. Milk fatty acid groupings of perinatal Steller sea lions based on length of the perinatal period. 'MDS letter' corresponds to individuals in multidimensional scaling (MDS) ordination. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, MUFAs: monounsaturated FAs, PUFAs: polyunsaturated FAs.

Perinatal period (days)	Female	MDS letter	Total % DHA+EPA	Ratio total % PUFAs : total % MUFAs	Ratio total % n-3 FAs : total % n-6 FAs
5	P2	B	10.30	0.32	5.14
5	P6	F	13.77	0.45	6.49
5	P7	G	14.07	0.48	6.38
8	P3	C	15.35	0.53	6.67
8	P4	D	11.95	0.36	6.07
8	P8	H	15.79	0.56	6.45
8	P9	I	12.80	0.39	6.44
9	P5	E	9.96	0.29	4.67
13	P1	A	13.03	0.41	6.48

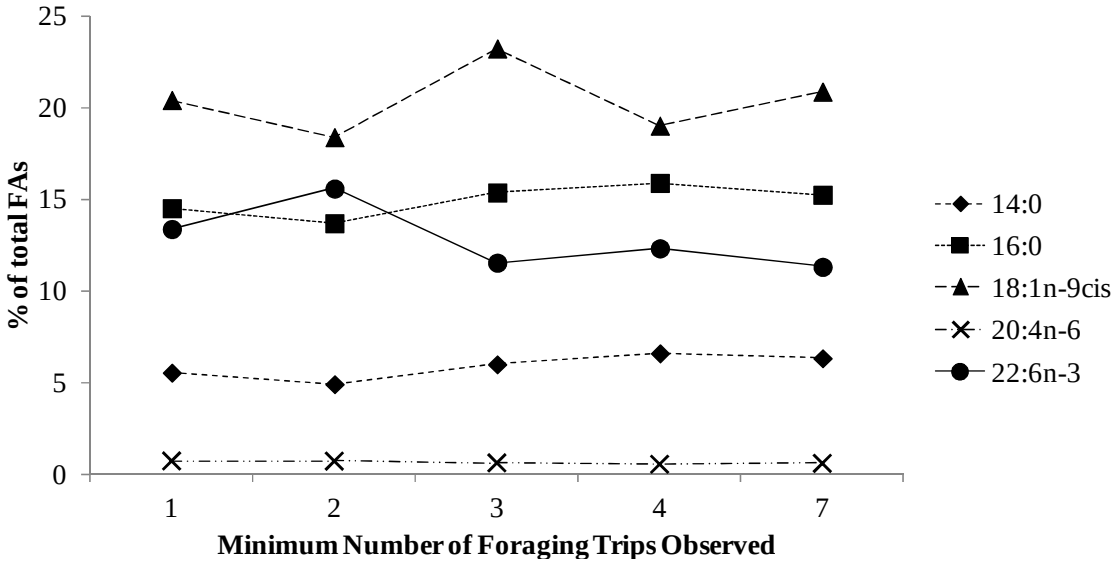

Figure 10: Milk Fatty Acid Relative Percentages Among the Minimum Number of Foraging Trips Observed. Relative percentages of five abundant fatty acids (FA, >0.5% of total FAs) across the minimum number of foraging trips observed for foraging females. No significant difference was found in any individual FA among the minimum number of foraging trips observed (Kruskal-Wallis one-way analysis of variance test, p>0.05).

Table 5: Milk Fatty Acid Groupings of Foraging Steller Sea Lions. Milk fatty acid groupings of foraging Steller sea lions based on the minimum number of foraging trips. 'MDS letter' corresponds to individuals in multidimensional scaling (MDS) ordination. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, MUFAs: monounsaturated FAs, PUFAs: polyunsaturated FAs.

Minimum # trips	Female	MDS letter	Perinatal period (days)	Total % DHA+EPA	Ratio total % PUFAs : total % MUFAs	Ratio total % n-3 FAs : total % n-6 FAs
1	F1	J	11	17.39	0.58	9.24
1	F4	M	11	21.07	0.76	10.29
1	F9	R	11	21.81	0.84	9.76
1	F11	T	10	23.46	0.98	10.44
2	F10	S	10	23.48	0.90	11.64
3	F3	L	9	16.11	0.54	8.93
3	F7	P	15	21.48	0.79	11.72
4	F2	K	11	20.76	0.84	12.22
4	F5	N	12	22.58	0.88	11.44
4	F6	O	15	19.24	0.66	11.63
7	F8	Q	13	19.35	0.68	10.44

DISCUSSION

This study examined the correlation between milk FA composition and maternal states in SSLs birthing and breeding on Chiswell Island, a small rookery in the Gulf of Alaska. Milk FA composition was determined using milk collected from SSL pup stomachs. Fatty acid composition of milk samples, collected from SSL pup stomachs, was inspected over a period of four hours post-collection to verify milk FA composition remains unchanged over time, and thus validate that milk samples taken from SSL pups at different times post-suckling can be compared without biases. Milk FA composition was compared between perinatal and foraging SSLs to determine if milk FA composition can be used to distinguish between maternal states. Investigations of physiological differences between maternal states are important to conduct to understand the demands of the perinatal period and replenishing the body while feeding. Milk FA composition within perinatal SSLs was examined to determine if composition changes over the perinatal period, and within foraging SSLs to investigate if composition changes during early lactation foraging trips. Relative percentages of FAs that influence offspring energy reserves, thermoregulation, growth, and development were compared in milk between perinatal and foraging SSLs to investigate if offspring are receiving differing levels of these FAs in milk depending on their mother's maternal state, and the impact that this may have on offspring.

The suite of 56 FAs identified in milk collected from SSL pup stomachs is also found in other pinniped milks (Iverson 1988, Iverson 1993, Lea et al. 2002, Baylis et al. 2009). Milk FA composition of otariids and phocids is characterized by FAs with 14 to 22 carbons and high proportions of long-chain PUFAs (e.g., n-3 FAs) (Jangaard & Ke 1968, Iverson 1988, Brown et al. 1999, Staniland & Pond 2004). Fatty acid composition of milk collected from SSL pup stomachs, which was used as a proxy of maternal milk FA composition (Iverson 1988), is consistent with this general pattern including high relative percentages of myristic acid, palmitic acid, palmitoleic acid, oleic acid, vaccenic acid (18:1n-7), gondoic acid, EPA, and DHA. Similar to other pinniped milk, total percent MUFAs in SSL milk was higher than total percent SAFAs or PUFAs (Iverson 1988, Adams 2000, Baylis & Nichols 2009), which likely

indicates the importance of MUFAs for offspring (Best et al. 2003, Maillet & Weber 2006, Wheatley et al. 2008).

Milk Fatty Acid Stability Over Time

Fatty acid composition of milk samples, collected from SSL pup stomachs, did not change over a period of four hours post-collection, which agrees with FA composition of milk collected over time directly from the stomachs of two California sea lion pups remaining unchanged over a period of eight hours post-suckling (Iverson 1988). Thus, FAs within milk were not modified over time and any differences in FA composition of milk samples, taken from SSL pups at different times post-suckling, are likely not a result of FA modification. The result of this study lends further support that gastric hydrolysis of milk fat triacylglycerols occurs due to gastric lipases in the stomach, and released FAs stay within the milk globule until reaching the small intestine (Patton et al. 1982, Iverson 1988).

Distinguishing Between Perinatal and Foraging Females

Milk FA composition was significantly different between perinatal and foraging SSLs and therefore can be utilized to distinguish between maternal states of lactating SSLs of the same geographic region. Individuals from each maternal state formed discrete groups in MDS and PCA plots illustrating that although individual variation in milk FA composition may occur (Staniland & Pond 2004), differences due to maternal state outweigh individual variation. To distinguish the maternal state of a lactating female when milk FA composition from several females is analyzed, individual FAs or classes of milk FAs can be examined. Relative percentages of dominant (>0.5% of total FAs) dietary FAs and biosynthesized/dietary FAs in milk can be utilized to make a distinction between perinatal and foraging females, as well as the ratios of total percent PUFAs to total percent MUFAs and total percent n-3 to total percent n-6 FAs. A difference in milk FA composition between maternal states, as was seen for lactating SSLs, is probable for other lactating otariids due to a similar lactation strategy (i.e., perinatal fast before subsequent foraging during lactation, Oftedal et al. 1987a, Iverson 1993) and foraging behavior (e.g.,

foraging close to birthing rookery, Merrick & Loughlin 1997). If researchers collect milk from lactating females in addition to other tissues of interest (e.g., blubber, blood), they can use milk FA composition to determine if individuals of both maternal states are in the sampling group. This could then lead to investigations on how blubber or blood, for example, differs between perinatal and foraging females. The ability to determine maternal state via milk FA composition provides a means to not only investigate physiological differences between lactating perinatal and foraging females when other methods (e.g., direct observation) are not possible, but also to investigate differences in resources (i.e., milk FAs) provided to offspring.

Differences in milk FA composition between perinatal and foraging SSLs are likely due to dissimilar diets between maternal states (i.e., prey species consumed before a female arrives on the rookery to give birth, with dietary FAs being sequestered in blubber, versus prey species consumed while foraging after birth, with dietary FAs being shuttled to milk). Changes in milk FA composition between maternal states in other pinniped species are caused by differences in overall diet pre- and post-birth (Iverson et al. 1997a, Smith et al. 1997). SSL prey species near the Chiswell Island rookery fluctuate seasonally (Adams et al. 2008), and therefore differences in milk FA composition between perinatal and foraging SSLs due to a change in diet are likely. Hard parts of fish prey in SSL feces indicate seasonal diet differences in Resurrection Bay with walleye pollock (*Theragra chalcogramma*) and Pacific herring (*Clupea pallasii*) dominating in spring, and walleye pollock and Pacific salmon (*Oncorhynchus* spp.) present during summer (ASLC unpublished data). Given the proximity of Chiswell Island and Resurrection Bay, there is likely similar seasonal access to fish prey around Chiswell Island as found in Resurrection Bay. Seasonal fluctuations in diet based on seasonal abundance of prey are found in other pinnipeds (Prime & Hammond 1990, Pierce et al. 1991, Beck et al. 1993), and for SSLs in Alaska (Merrick et al. 1997, Sinclair & Zeppelin 2002, Womble & Sigler 2006). Therefore, in this study it is likely that milk FAs of perinatal SSLs are influenced by feeding on pollock and herring, which are dominant prey species nearby the Chiswell rookery during spring (Adams et al. 2008), while milk FAs of

foraging SSLs primarily arise from feeding on pollock and salmon, which are dominant prey species nearby the rookery during summer.

Examination of how milk FA compositions of Chiswell SSLs compares with FA compositions of probable prey species is challenging because FA compositions of SSL prey species around Chiswell Island are not known. However, FA composition of many SSL prey species have been investigated in Prince William Sound (PWS), Alaska (Iverson et al. 1997b, 2002), which is northeast of Chiswell Island. Although FA composition of fishes vary within species based on age, maturity, body size, and geographic location (Iverson et al. 1997b, Budge et al. 2002), comparing FAs of SSL prey species from PWS to milk FAs of perinatal and foraging SSLs may shed light on prominent prey species of each maternal state.

Given that walleye pollock are prominent prey to both maternal states, comparing relative percentages of FAs in herring and salmon of PWS to milk FAs of perinatal and foraging SSLs, respectively, may verify a change in diet. The relative percentage of oleic acid is higher in herring compared with salmon, comprising of 11.29% to 14.81% and 6.63% to 11.98% of total FAs, in herring and salmon, respectively (Iverson et al. 1997b, 2002). The ratio of total percent DHA to EPA is lower in herring compared with salmon, ranging from 1.34 to 1.63 and 1.58 to 4.11, in herring and salmon, respectively (Iverson et al. 1997b, 2002). When comparing relative percentage of oleic acid and total percent DHA to EPA between perinatal SSLs and herring, and between foraging SSLs and salmon, percentages did not stand out that indicated a diet of herring in perinatal females or a diet of salmon in foraging females. The relative percentage of oleic acid was higher in milk of perinatal females (28.74% of total FAs) compared with herring and was also higher in milk of foraging females (20.74% of total FAs) compared with salmon. The ratio of total percent DHA to EPA was higher in milk of perinatal females (1.84 DHA:EPA) compared with herring, but lower in milk of foraging females (1.63 DHA:EPA) compared with salmon. The relative percent of oleic acid and total percent DHA to EPA may not have been similar in milk of perinatal SSLs compared with herring, and in milk of foraging SSLs compared with salmon, because 1) SSLs are opportunistic foragers consuming a wide variety of prey species (Merrick & Loughlin 1997, Sinclair & Zeppelin 2002), and thus FAs of other prey species are likely

influencing milk FA composition, and 2) FA modification (i.e., elongation, desaturation, oxidization) and maternal use of FAs occurs from consumed prey to milk, and thus relative percentages of milk FAs do not exactly match relative percentages of prey FAs (Iverson et al. 2004, Staniland & Pond 2004, 2005). In addition, FA composition of fishes vary within species based on geographic location (Iverson et al. 1997b, Budge et al. 2002), and therefore FA composition of fishes in PWS may not be similar to FA composition of fishes in the vicinity of Chiswell Island.

Beyond diet, differences in milk FA composition between SSL maternal states could be attributed to selective mobilization and/or mammary gland activity. During their perinatal period, lactating SSLs rely entirely on their blubber stores to produce high fat milk for their offspring (Oftedal et al. 1987a). Comparisons of FA composition in paired blubber and milk samples from pinnipeds showed certain FAs, including palmitic acid, stearic acid, arachidonic acid, and DHA are higher in milk relative to blubber, which may be due to selective mobilization of FAs from blubber to milk, mammary gland de novo FA synthesis, or selective uptake via the mammary gland (Iverson et al. 1995a, Grahl-Nielsen et al. 2000, Wheatley et al. 2008). These physiological processes are probably occurring to enrich milk in important FAs, such as DHA, that are required for pup growth and development (Wheatley et al. 2008). Furthermore, blubber is a limited resource for lactating SSLs (Adams 2000, Pitcher et al. 2000, Davis et al. 2002), and thus lactating perinatal SSLs, who are not replenishing their blubber FAs through dietary consumption, may be mobilizing their blubber FAs in a way that allows for adequate nourishment of pups throughout the perinatal period (Wheatley et al. 2008). If percentages of dietary FAs in milk of perinatal SSLs were altered due to selective mobilization and/or mammary gland activity in this study, differences in milk FA composition between maternal states may be partially due to female metabolism rather than diet alone.

Milk Fatty Acid Composition Within Perinatal and Foraging Females

Milk FA composition was similar within perinatal SSLs at Chiswell Island with different lengths of perinatal periods. Relatively constant milk FA composition over the course of the perinatal period has

also been found for black bears (Iverson & Oftedal 1992) and throughout lactation for harp (*Pagophilus groenlandicus*) and hooded seals (*Cystophora cristata*, Debier et al. 1999), northern elephant seals (*Mirounga angustirostri*, Riedman & Ortiz 1979), and beluga whales (*Delphinapterus leucas*, Birkeland et al. 2005) in which females are fasting throughout lactation. Milk FA composition of perinatal SSLs may not have changed over the perinatal period due to similar rates of mobilization and mammary gland synthesis/uptake of FAs throughout the perinatal period. The lower relative percentages of milk FA groupings for perinatal females P2 and P5 (Table 4), when compared with other perinatal females, are not likely due to length of perinatal period, which might have been the case if relative percentages of milk FA groupings sequentially lowered with increasing perinatal period length. Females P2 and P5 correspond to letters B and E, respectively, on the MDS ordination (Fig. 2) and do not appear to be spatially separated from other perinatal females.

Within foraging SSLs at Chiswell Island with different numbers of foraging trips, milk FA composition was similar. Milk FA compositions of individuals who had taken one foraging trip resembled composition represented by females who had taken multiple trips, which suggests that after only one foraging trip, assuming it is successful, milk dietary FAs of a foraging female come directly from dietary intake (Robinson 1963, Hamosh et al. 1970, Iverson 1993). After several foraging trips, dietary FAs, including n-3 FAs, are not enriched on a relative percentage basis. This may result if changes in lipoprotein lipase activity of the mammary gland and other tissues occur after feeding, causing shuttling of dietary FAs to other tissues besides the mammary gland, such as blubber or the liver, for maternal metabolism (Wheatley et al. 2007, 2008, Fowler et al. 2014).

Relative percentages of milk FA groupings were lower for foraging females F1 and F3 (Table 5) compared with other foraging females, and are likely not a result of low food availability or length of perinatal period. Although lower relative percentages in females F1 and F3 may be explained if foraging was unsuccessful (e.g., no influx of n-3 FAs from foraging), this was not possible to confirm. Through remote-monitoring at Chiswell Island, female F1's single foraging trip was approximately 47 h and female F3's three foraging trips were approximately 8, 6, and 16 h. Female F1's lower relative

percentages of milk FA groupings may be due to low food availability given her long foraging trip, as long foraging trips are linked with low food availability in California sea lions (Ono et al. 1987). However, female F3 exhibited lower relative percentages of milk FA groupings as well, and her foraging trip lengths were shorter and similar to average foraging trip length for Chiswell females (16.5 h) who are not food-limited (Maniscalco et al. 2006). If relative percentages of milk PUFAs were found to change over the perinatal period in SSLs, then it may have been possible that relative percentages of milk FA groupings of females F1 and F3 were slightly different from other foraging females due to the length of their perinatal periods. However, milk FA composition was similar over the course of the perinatal period in SSLs and perinatal periods of females F1 and F3 are similar to other foraging females (Table 5). Females F1 and F3 correspond to letters J and L, respectively, on the MDS ordination (Fig. 2), and they are spatially in close proximity to other foraging females, showing similarity in milk FA composition to other foraging females.

Milk FA composition was similar within foraging SSLs at Chiswell Island, likely because these females foraged in the vicinity of the rookery and consumed the same prey species and age-class of prey during early lactation foraging trips. Although there are seasonal fluctuations in SSL prey species around Chiswell Island (Adams et al. 2008), consumption of prey for all foraging females in this study was over the course of 12 days during summer, so it is likely that all foraging females were feeding on similar prey taxa. This study examined milk FA composition of foraging females only in the early stages of foraging post-parturition (i.e., up to seven foraging trips), and therefore it cannot be determined if and how milk FA composition changes over the remainder of lactation. Milk FA composition changes from early to mid and late foraging in Antarctic fur seals (Iverson et al. 1997a) and harbor seals (Smith et al. 1997), but milk FA composition of foraging females at any stage of lactation differed from perinatal females in both species (Iverson et al. 1997a, Smith et al. 1997).

Differences in Fatty Acids Important for Pups Between Maternal States

Saturated FAs, MUFAs, and PUFAs have different chemical properties and are important for offspring in different ways. Saturated FAs contain more chemical energy per unit mass than either MUFAs or PUFAs (Maillet & Weber 2006), and therefore are likely utilized by pups for energy to fuel metabolism (Wheatley et al. 2008). Similar relative percentages of myristic acid and palmitic acid, as well as total percent SAFAs, between maternal states likely serve to provide SSL pups with a continual supply of energy-dense FAs (Bryden & Stokes 1969, Wheatley et al. 2008) throughout the perinatal period and during early foraging trips. Total percent MUFAs is different when comparing inner and outer blubber layers of pinnipeds, with long-chain MUFAs (e.g., 20:1, 22:1) being more abundant in inner blubber and medium-chain MUFAs (e.g., 14:1, 16:1, 18:1) being more abundant in outer blubber (Best et al. 2003, Olsen & Grahl-Nielsen 2003, Wheatley et al. 2007, Fowler et al. 2014). Medium-chain MUFAs remain fluid at low temperatures (Sinensky 1974), and therefore are thought to be important for thermoregulation (Fowler et al. 2014). Medium-chain MUFAs were higher, but long-chain MUFAs (e.g., 20:1, 22:1) were lower, in milk of perinatal SSLs when compared with foraging SSLs. This may suggest that milk MUFAs received by pups of perinatal SSLs are geared toward building an outer blubber layer that is able to maintain fluidity at low temperatures (Best et al. 2003, Strandberg et al. 2008), whereas milk MUFAs received by pups of foraging SSLs are aimed toward building an inner blubber layer. Polyunsaturated FAs, on a total percent basis, were significantly higher in milk of foraging SSLs compared with perinatal SSLs. This may be in part due to the influx of n-3 FAs, which caused total percent PUFAs to increase from perinatal to foraging maternal state.

Milk of both perinatal and foraging SSLs contained the same FAs known to be important for offspring growth and development, but several n-6 and all n-3 FAs increased in relative percent from perinatal to foraging maternal state. The only two n-6 FAs that decreased in relative abundance from perinatal to foraging maternal state were linoleic acid and arachidonic acid. Linoleic and arachidonic acids are the two primary FAs for offspring growth (Needleman et al. 1986, Innis 1991, Piomelli 1994), and therefore higher relative percentages of these FAs in milk of perinatal SSLs compared with foraging

SSLs may indicate higher growth rate in pups of perinatal SSLs as opposed to pups of foraging SSLs. Higher relative percentages of EPA and DHA, as well as all other n-3 FAs, in milk of foraging SSLs compared with perinatal SSLs may suggest a higher neural development rate in pups of foraging SSLs as opposed to pups of perinatal SSLs.

The significant differences between maternal states in the ratio of total percent PUFAs to total percent SAFAs and total percent PUFAs to total percent MUFAs demonstrate differences in milk FA classes when they are collectively compared between perinatal and foraging SSLs. A higher ratio in milk of total percent PUFAs to total percent SAFAs for SSL pups with foraging mothers may mean less fat deposition when compared with SSL pups with perinatal mothers, as has been shown for infant Syrian hamsters (*Mesocricetus auratus*, Liao et al. 1982). Differing plasma and liver cholesterol levels have been found in rats when fed varying ratios of PUFAs to SAFAs (Chang & Huang 1999), and therefore different ratios may indicate differences in tissue cholesterol levels, including steroid hormones, between pups of perinatal and foraging SSLs.

The significant increase in the ratio of n-3 to n-6 milk FAs given to pups of foraging SSLs compared with pups of perinatal SSLs raises the question of how this change influences growth, development, and biochemistry of phospholipids of pups. The ratio of n-3 to n-6 PUFAs in milk impacts growth and neurodevelopment of offspring (Saste et al. 1998, Uauy et al. 1999). Varying ratios of n-3 to n-6 FAs within dietary intake impact biochemistry of tissue phospholipids in various mammals (rats (spontaneously hypertensive and normotensive Wistak/Kyoto): Yamamoto et al. 1987, humans (*Homo sapiens*): Clark et al. 1992, mice ($B6D2F_1$ hybrid): Huang et al. 1992) as well as eicosanoid production (humans (*Homo sapiens*): Ferretti et al. 1998, rats (C57BL/6): Broughton & Wade 2002). The ratio of n-3 to n-6 FAs in milk significantly increased, on a total percent basis, in lactating SSLs from perinatal to foraging maternal state likely due to the influx of n-3 FAs in milk of foraging females from dietary consumption. For pups of foraging females, a higher ratio of n-3 to n-6 milk FAs may promote more DHA incorporation into tissue phospholipids, and n-3 FA eicosanoid production, than pups of perinatal females (Innis 2003). In addition, it may suppress arachidonic acid production and arachidonic acid

derived eicosanoids, due to n-3 FAs out-competing n-6 FAs for use of enzymes in metabolic pathways (Innis 2003). While in some species a high ratio of dietary n-3 to n-6 FAs leads to reduced arachidonic acid levels and impaired growth (humans (*Homo sapiens*): Carlson et al. 1993, rats (Sprague-Dawley): Amusquivar et al. 2000), for sea lions and other marine mammals the high ratio of n-3 to n-6 FAs in milk delivers adequate FAs for growth (Oftedal et al. 1987b, Iverson et al. 1993, Mellish et al. 1999).

Importance of Food Resources

Availability of food sources near rookeries is crucial for lactating SSLs and their pups. Lactation pattern of SSLs are likely collectively explained by maternal metabolic requirements and physiological adaptations (Trillmich & Weissing 2006). Otariid milk output decreases when food availability is low (Ono et al. 1987, Iverson 1993), and milk output influences rate of pup growth (Iverson et al. 1993), which impacts pup survival (Iverson et al. 1993, Festa-Bianchet et al. 2000). If SSL pups do not obtain sufficient amounts of milk, including essential milk FAs needed for growth and development, such as when their mothers are experiencing a low plane of nutrition, pup survival may be adversely impacted. Therefore, it is important that n-6 and n-3 FAs are replenished in the milk so pups continue to have an adequate supply of these FAs for growth and development (Connor & Neuringer 1988, Innis 1991, 2005). In neonatal mammals, dietary deficiencies in n-3 FAs during gestation and postnatal development result in depletion of DHA in retina and cerebral cortex phospholipids (rhesus monkeys (*Macaca mulatta)*: Neuringer et al. 1984, 1986, rats (Wistar): Bourre et al. 1989, mice (OF1): Carrié et al. 2000). Neonatal humans given milk with deficient levels of DHA develop decreased sensitivity of rod photoreceptors in the retina (Birch et al. 1992). Steller sea lion mothers nurse their offspring for up to 12 months, but sometimes as long as two years or more (Gentry 1970, Sandegren 1970, Pitcher & Calkins 1981, Trites et al. 2006), and therefore have a long time to deliver FAs to offspring through milk. However, lack of n-6 and n-3 FAs in SSL milk for the first three months of lactation, possibly due to lack of prey, could have long-term adverse effects on offspring retinal and brain biochemistry, which have been noted in infant rhesus monkeys (Connor et al. 1990, Anderson et al. 2005).

Through remote-monitoring at Chiswell and neighboring islands post-sampling, all sampled pups were observed with their mothers throughout the summer of their sampling year. Furthermore, all sampled pups, except one, were observed with their mothers during autumn (i.e., after August 15, Maniscalco et al. 2006) of the year in which they were born. Observations of sampled pups suggest that milk FA composition provided to pups during the 2010 and 2011 summers adequately nourished pups throughout the year they were born, and thus females were not food limited during sampling years. This agrees with previous research of lactating SSLs birthing on Chiswell Island concluding lactating females were able to find sufficient food in the vicinity of the rookery during 2001-2004 summers (Maniscalco et al. 2006), and of lactating SSLs in the western distinct population segment suggesting females have not had difficulties obtaining prey (Milette & Trites 2003, Trites et al. 2006). To directly assess if adequate food is available for lactating SSLs in the vicinity of the Chiswell rookery, dietary information of these females needs to be investigated. Information on diet of lactating SSLs birthing on Chiswell Island, as well as of lactating SSLs birthing on other rookeries, would provide a useful tool for management decisions regarding fisheries near rookeries to assure sufficient food supply for lactating females.

CONCLUSION

Fatty acid composition of milk samples, collected from SSL pup stomachs, did not change over a period of four hours post-collection, verifying FAs within milk are not modified over time. Milk collected from the stomach of a pup, even if obtained up to four hours post-suckling, can therefore be a proxy to define maternal state through milk FA composition. Milk FA composition between perinatal and foraging SSLs was significantly different when individual FAs or FA classes were considered. Maternal state of individual lactating SSLs, within a geographic region and within the early part of lactation (i.e., up to 7 foraging trips), can therefore be determined through FA analysis of milk extracted from the stomach of pups. Milk FA composition was similar within each maternal state in lactating SSLs. Milk FA composition remained relatively constant within perinatal SSLs with varying lengths of perinatal periods, suggesting that selective mobilization of FAs and mammary gland activity over the course of the perinatal period remains relatively steady. Within foraging SSLs, with varying numbers of foraging trips, milk FA composition did not change, which may indicate these females are foraging in the vicinity of the rookery and consuming similar prey species during early foraging trips. Maintaining healthy populations of prey species for lactating SSLs is therefore essential for pup survival and conservation of SSLs.

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APPENDIX A

Detailed Methods of Sample Collection, Lipid Extraction, Fatty Acid Esterification, and Fatty Acid Analysis

Sample Collection

Milk samples were collected from the stomachs of 20 SSL pups on July 1, 2010 (with milk, *n*=7) and June 30, 2011 (with milk, *n*=13) at the Chiswell Island rookery (59°36.13'N, 149°34.05'W, Fig. 1). Chiswell Island is located in the northern Gulf of Alaska within the range of the endangered western distinct population segment of SSLs and is within the United States Fish and Wildlife Service Alaska Maritime National Wildlife Refuge. The rookery was raided in a strategic manner leading to separation of pups from their mothers. Steller sea lion pups were randomly captured using 62-cm diameter hoop nets and then physically restrained while a milk sample was withdrawn from the stomach via gastric intubation with a 10-mm veterinary stomach tube. During restraint, a pup's mouth was held open for insertion of the tube and once proper insertion in the stomach occurred, suction was applied to the tube, milk was extracted, and the tube was removed.

In 2010, 28 pups were intubated over a period of 6 hours, and 7 milk samples were collected. In 2011, 28 pups were intubated over a period of 5 hours and 13 milk samples were collected. In this study, all milk samples from pup stomachs were collected at least five days post-parturition, past the timeframe in which colostrum is produced (Boersma et al. 1991, Csapó et al. 1995, Macias & Schweigert 2001, Pavlíková et al. 2010). In addition, all milk samples had a coloration of white or off-white rather than yellow as is observed in colostrum (Macias & Schweigert 2001). Thus, all milk samples collected were considered mature milk, not colostrum. Milk samples, ranging in volume between 2 to 52.5 ml, were collected from each pup and put into separate 50-ml polypropylene conical centrifuge falcon tubes (VWR International, LLC, Radnor, PA, U.S.A.). Milk samples were kept at ambient temperature, approximately 10.5ºC, in both sampling years. For 19 of 20 pups, 1 ml aliquots (i.e., subsamples) of the original milk sample were taken at 0, 2, and 4 hours (h) post-collection. For the remaining pup, a 1 ml aliquot was

taken at initial collection only (i.e., 0 h) due to low milk volume collected from its stomach. Each subsample was immediately placed into a 4-ml glass vial, with a teflon-lined cap, containing 2 ml chloroform ($CHCl_3$, OmniSolv grade, EMD Millipore, Billerica, MA, U.S.A.) with 0.01% butylated hydroxytoluene (v/w) (BHT, 2,6-bis(1,1-dimethylethyl)-4-methylphenol, Sigma-Aldrich Co. LLC, St. Louis, MO, U.S.A.) and Duraseal (Diversified Biotech, Dedham, MA, U.S.A.) applied to the vial cap. All milk subsamples preserved in $CHCl_3$ with 0.01% BHT were stored at -20°C until processed. Pups were either permanently or temporarily marked using hot-iron branding (2010) or hair shaving (2011), respectively, to identify sampled pups via remotely-operated cameras once mothers and pups reunited following collections. Collections for this project were performed under National Marine Fisheries Service permit number 14324 and under the ASLC Institutional Animal Care and Use permit number R10-03-01.

Lipid Extraction

Sample processing was conducted at the University of Alaska Fairbanks Kodiak Seafood and Marine Science Center. All glassware was rinsed with methylene chloride (CH_2Cl_2, HPLC grade, Honeywell Burdick & Jackson, Morristown, NJ, U.S.A.) prior to use. Each milk subsample in $CHCl_3$ with 0.01% BHT was thawed and homogenized using a vortexer (VWR Mini Vortexer, VWR International, LLC) until milk and $CHCl_3$ with 0.01% BHT were visually indistinguishable, and 50% of the homogenate, by weight, was transferred to a 10-ml conical glass tube (Fisherbrand, Thermo Fisher Scientific Inc., Waltham, WA, U.S.A.) fitted with a Teflon-lined screw cap for lipid extraction. Each subsample was weighed, to the ten thousandths decimal place, on a digital analytical scale (Mettler AE163, Mettler-Toledo, Columbus, OH, U.S.A.). The remaining milk subsample in solvent was immediately stored at -20°C.

Lipids were extracted using the Folch method (Folch et al. 1957) as modified by Budge et al. (2006). A quantity of 0.5 ml of methanol (MeOH, HPLC grade, Avantor Performance Materials, Center Valley, PA, U.S.A.) with 0.01% BHT (v/w, Sigma-Aldrich Co. LLC) was added to the 1.5 ml aliquot of

milk subsample followed by the addition of 7.5 ml 2:1 $CHCl_3$:MeOH ($CHCl_3$: OmniSolv grade, EMD Millipore, MeOH: HPLC grade, Avantor Performance Materials) with 0.01% BHT (v/v/w, Sigma-Aldrich Co. LLC). The vial with the subsample and reagents was capped and the subsample/reagents were mixed using a vortexer (VWR Mini Vortexer, VWR International, LLC) for approximately 30 sec. Next, 2 ml 0.9% sodium chloride (NaCl, ACS grade, VWR International, LLC) was added, and the subsample/reagents were mixed using a vortexer (VWR Mini Vortexer, VWR International, LLC) for approximately 10 sec. Samples were centrifuged (Thermo IEC Centra CL2, Thermo Fisher Scientific Inc.) at 2,600 rpm for 18 min to separate aqueous from organic layers. The bottom layer, containing lipids, was separated using a disposable glass Pasteur pipet (VWR International, LLC) and filtered through a scoop of anhydrous sodium sulfate ($NaSO_4$, ACS grade, Avantor Performance Materials) on Whatman #1 filter paper (Whatman International Ltd, Maidstone, England, U.K.) into a clean pre-weighed 10-ml conical glass tube (Fisherbrand, Thermo Fisher Scientific Inc.). Filter paper and $NaSO_4$ were rinsed with a portion of 5 ml of $CHCl_3$ (OmniSolv grade, EMD Millipore), and added to the lipid extract. Chloroform was evaporated under a nitrogen (Airgas USA, LLC, Nor Pac region, Vancouver, WA, U.S.A.) stream using a Reacti-vap III evaporator (Pierce, Rockford, IL, U.S.A.). Percent lipid was determined gravimetrically. Lipids were dissolved in hexane (practical grade, EMD Millipore) at a concentration of 100 mg of lipid per ml hexane and tubes were flushed with nitrogen (Airgas USA, LLC, Nor Pac region), capped, and stored at 4°C.

Fatty Acid Esterification

Hexane was evaporated from the sample under a nitrogen (Airgas USA, LLC, Nor Pac region) stream using a Reacti-vap III evaporator (Pierce). Fatty acids in the extracted lipids were converted to their methyl ester counterparts using acid catalyzed esterification as described in Budge et al. (2006). Briefly, 1.5 ml of CH_2Cl_2 (HPLC grade, Honeywell Burdick & Jackson) with 0.01% BHT (Sigma-Aldrich Co. LLC) and 3.0 ml of Hilditch reagent (0.5 N sulfuric acid in methanol, sulfuric acid: ACS grade, VWR International, LLC) were added to all extracted lipids in each subsample. Nitrogen (Airgas

USA, LLC, Nor Pac region) was added to the headspace of the sample and reagents in the conical glass tube, the tube was capped, and the tube was placed in a heating block (COD reactor, Hach Company, Loveland, CO, U.S.A.) operated at 100ºC for 1 h (Budge et al. 2006). Samples were cooled to room temperature and 3 ml hexane (practical grade, EMD Millipore) and 1 ml distilled water were added to the reaction media and mixed using a vortexer (VWR Mini Vortexer, VWR International, LLC). The mixture was centrifuged for 3 min using a Thermo IEC Centra CL2 centrifuge (Thermo Fisher Scientific Inc.) at 2,600 rpm. After centrifugation, the top layer containing hexane and fatty acid methyl esters (FAME) was transferred to a 10-ml conical glass tube (Fisherbrand, Thermo Fisher Scientific Inc.). The mixture was extracted two more times with hexane (practical grade, EMD Millipore), following the procedure described above, and the extracts were combined. A quantity of 2 ml of distilled water was added to the FAMEs in hexane and the mixture was vortexed (VWR Mini Vortexer, VWR International, LLC) and then centrifuged (Thermo IEC Centra CL2, Thermo Fisher Scientific Inc.) for 2 min at 2,600 rpm. The top layer, containing hexane and FAMEs, was transferred to a 10-ml conical glass tube (Fisherbrand, Thermo Fisher Scientific Inc.) and a scoop of anhydrous $NaSO_4$ (ACS grade, Avantor Performance Materials) was added to draw out any remnants of water. All liquid was transferred to a pre-weighed 10-ml conical glass tube (Fisherbrand, Thermo Fisher Scientific Inc.). Hexane was evaporated under a nitrogen (Airgas USA, LLC, Nor Pac region) stream using a Reacti-vap III evaporator (Pierce). The reaction yield was determined gravimetrically and FAMEs were dissolved in hexane (practical grade, EMD Millipore) to a concentration of 50 mg FAME per ml hexane. Fatty acid methyl esters in hexane were transferred to a 2-ml crimp-top amber gas chromatography vial (Agilent Technologies, Wilmington, DE, U.S.A.) and nitrogen (Airgas USA, LLC, Nor Pac region) was added to the headspace of the sample.

Fatty Acid Analysis

Fatty acid methyl esters were analyzed by gas chromatography using an Agilent model 6850N Series II (Agilent Technologies) coupled to a flame ionization detector (Agilent Technologies) and fitted with a DB-23 (60 m × 0.25 mm i.d., 0.25 µm film) capillary column (Agilent Technologies). Data were

collected and analyzed using the GC ChemStation software (Rev.A.08.03, Agilent Technologies). Hydrogen (Airgas USA, LLC, Nor Pac region) was used as carrier gas at linear constant flow of 1 ml/min and average velocity of 30 ml/sec. The temperature of the inlet was 250ºC and the split ratio 100:1. Initial oven temperature was 140ºC and increased at a rate of 2ºC per min up to 180ºC, followed by a rate of 0.5ºC per min to 200ºC, and a rate of 1ºC per min to 203 ºC. The final oven temperature was 220ºC using a temperature rate of 20ºC per min and final hold time of 1.25 min. Total run time was 65.10 min. Detector temperature was 275ºC, and hydrogen (Airgas USA, LLC, Nor Pac region), air, and nitrogen (Airgas USA, LLC, Nor Pac region) flows were maintained at 40, 450, and 35 ml/min, respectively. Sample injection was performed by an autosampler model 6850 (Agilent Technologies), and injection volume was 1µl. Fatty acid methyl ester peaks were identified based on retention times of known standard mixtures 189-19 FAME (Sigma-Aldrich Co. LLC), PUFA 1 (marine source, Sigma-Aldrich Co. LLC), PUFA 2 (animal source, Sigma-Aldrich Co. LLC), PUFA 3 (Menhaden oil, Sigma-Aldrich Co. LLC), Bacterial Acid Methyl Esters (Sigma-Aldrich Co. LLC), and 189-13 FAME (Sigma-Aldrich Co. LLC). Authentic standards of docosatrienoic acid (22:3n-3, Nu-Check-Prep, Inc., Elysian, MN, U.S.A.), adrenic acid (22:4n-6, Nu-Check-Prep, Inc.), and osbond acid (22:5n-6, Nu-Check-Prep, Inc.) were also used.

During examination of multiple chromatograms at 50 mg FAME per ml hexane, it was apparent that the sample load was too high causing misshaped chromatogram peaks with poor symmetry, which resulted in poor performance for peak area integration. Therefore, each sample was analyzed twice using two concentrations, one at full concentration and one at a 1:10 diluted concentration. For the latter, each sample was diluted using hexane (practical grade, EMD Millipore, i.e., 10 µl subsample and 90 µl hexane to equal a diluted concentration of 5 mg FAME per ml hexane) and reanalyzed to accurately measure the chromatogram peak areas of most abundant FAs. Chromatogram peak areas of all other FAs were determined using samples with a concentration of 50 mg of FAME per ml of hexane and their chromatogram peak areas divided by 10 to yield a final chromatogram peak area. Chromatogram peak areas for three FAs (22:4n-6, 22:4n-3, and 22:5n-6) were manual integrated for all samples.

To ensure accurate identification of FAs, six subsamples were analyzed using a gas chromatography model 6890 interfaced with a mass spectrometer detector model 5973N (Agilent Technologies) fitted with a DB-23 (60 m × 0.25 mm i.d., 0.25 µm film) capillary column (Agilent Technologies). Helium (Airgas USA, LLC, Nor Pac region) was used as carrier gas at linear flow of 1 ml/min and average velocity of 27 ml/sec. The temperature of the inlet was 250ºC and the split ratio 100:1. Initial oven temperature was 140ºC and increased at a rate of 2ºC per min up to 180ºC, followed by a rate of 0.5ºC per min to 200ºC, and a rate of 1ºC per min to 203ºC. The final oven temperature was 220ºC using a temperature rate of 20ºC per min and final hold time of 1.25 min. Total run time was 65.30 min. The mass spectrometer was operated in electron impact mode at 70eV and the mass ranged scanned was 15-435 amu at a rate of 3.42 scans/sec. Acquisition started after a 1.45 min of solvent delay. Transfer line, source, and quadrupole temperatures were 280, 230, and 150ºC, respectively. Sample injection was performed by an autosampler model 7683 (Agilent Technologies), and injection volume was 1µl. Data were collected and analyzed using the MSD ChemStation (Rev. E.02.02.1431, Agilent Technologies). Mass spectra of FAME in standards, listed above, were compared to spectra available in the NIST/EPA/NIH Mass Spectral Library (NIST 05 v.2.0, National Institute of Standards and Technology, Gaithersburg, MD, U.S.A.). In parallel, mass spectra of FAME in samples were also compared to library spectra available and/or spectra of FAME standards. Fifty-six FAs were identified in SSL milk (Table 1).

APPENDIX B

Lipid Recovery of Cow Milk Enriched with Salmon Oil and Steller Sea Lion Milk Using a Modified Folch Method

A preliminary study was conducted to investigate lipid recoveries of the Folch procedure as modified by Budge et al. (2006). One pint of commercial half & half (Lucerne, Lucerne Foods, Inc., Pleasanton, CA, U.S.A.), purchased at a local grocery store contained 10% fat and was used as proxy for Steller sea lion milk samples. Known quantities of Alaska salmon oil (Alaska Protein Recovery, Juneau, AK, U.S.A.) were added to the milk product to determine lipid recoveries.

Commercial half & half (H&H) was spiked with 7.7, 14.3, and 25.0% of salmon oil (SO) to yield three groups of samples that contained 16.9%, 22.9%, and 32.5% of lipids, respectively. The fourth group used for comparison, control, consisted of H&H without SO (10% cow lipids). Three independent lipid extractions were conducted for each sample group using a modified Folch method (Budge et al. 2006). The range of lipid content selected in this study encompassed values that were lower and higher than the 21.6% lipid content in Steller sea lion milk previously reported by Adams (2000). Samples containing 16.9% lipids were composed of a mixture of 45.0 ml H&H and 3.75 ml of SO. The two ingredients were homogenized in a 150 ml Pyrex beaker (Corning Incorporated, Tewksbury, MA, U.S.A.) using a Ultra-Turrax model T10 (IKA Works, Inc., Wilmington, NC, U.S.A.). Similarly, the samples containing 22.9% and 32.5% lipids were composed of 45.0 ml H&H and 7.5 ml of SO and 45.0 ml H&H and 15 ml of SO, respectively. Lipids were extracted as described in Appendix A.

Expected lipid amounts and percentages, extracted lipid amounts and percentages in samples, and lipid recoveries of H&H samples and mixtures of H&H supplemented with SO are listed in Table B-1. Steller sea lion milk, collected from the stomachs of pups (n=20), averaged 19.8% lipid. Extracted lipid amounts (mg) and percentages for SSL milk samples (n=20) are listed in Table B-2.

Table B-1: Expected and Extracted Lipid Amounts and Percent Lipid, and Recovered Percent Lipid, for Commercially Available Half & Half and Half & Half Supplemented with Salmon Oil. Half & half (H&H) samples (*n*=3) and H&H supplemented with salmon oil (H&H w/SO) mixtures (*n*=3 for each mixture) containing a range of lipids (mg, %), extracted lipids (mg, %), and lipid recoveries (%) using a sample size of 0.5 g (i.e., 500 mg). Values are expressed as the mean ± 1 SD.

Sample type	Expected lipid (%)	Sample volume (mg)	Expected lipid (mg)	Extracted lipid (mg)	Extracted lipid (%)	Recovered lipid (%)
H&H	10.00	500	50.00	38.53 ± 4.93	7.71 ± 0.01	77.07 ± 0.10
H&H w/oil (7.7% oil)	16.90	500	84.50	60.33 ± 7.81	12.07 ± 0.02	71.40 ± 0.09
H&H w/oil (14.3% oil)	22.90	500	114.50	77.63 ± 13.99	15.53 ± 0.03	67.80 ± 0.12
H&H w/oil (25.0% oil)	32.50	500	162.50	87.67 ± 6.09	17.53 ± 0.01	53.95 ± 0.04

Table B-2: Extracted Lipid Amounts, Total Sample Amounts, and Extracted Percent Lipid for Steller Sea Lion Milk. Extracted lipid amounts (mg), total sample amounts (mg), and calculated extracted lipid (%) for Steller sea lion milk. P: perinatal female, F: foraging female. For all females except F3, three subsamples (*n*=3) were used to calculate values. For female F3, only one milk subsample was obtained (*n*=1). Values are expressed as the mean ± 1 SD.

Female ID	Extracted lipid (mg)	Sample amount (mg)	Extracted lipid (%)
P1	65.63 ± 6.60	396.13 ± 130.28	17.67 ± 5.05
P2	97.77 ± 8.44	529.53 ± 153.91	20.13 ± 8.71
P3	80.00 ± 29.26	474.93 ± 152.05	16.98 ± 4.14
P4	74.53 ± 5.20	508.07 ± 47.72	14.70 ± 0.49
P5	68.47 ± 12.09	376.93 ± 61.02	18.39 ± 3.66
P6	93.77 ± 10.59	419.64 ± 70.79	22.51 ± 1.95
P7	97.40 ± 7.82	344.67 ± 82.10	29.33 ± 6.89
P8	102.10 ± 8.09	408.57 ± 34.61	25.20 ± 3.81
P9	92.53 ± 4.82	374.37 ± 29.99	24.83 ± 2.44
F1	132.63 ± 11.49	586.03 ± 96.24	22.86 ± 2.19
F2	111.53 ± 23.26	418.55 ± 128.95	27.15 ± 2.46
F3	84.10	500.00	16.82
F4	81.41 ± 25.57	337.63 ± 137.31	25.30 ± 5.54
F5	52.70 ± 23.08	505.80 ± 10.00	10.36 ± 4.32
F6	112.6 ± 21.04	546.80 ± 85.92	20.53 ± 0.99
F7	65.03 ± 10.83	626.40 ± 165.95	10.61 ± 1.30
F8	114.07 ± 13.83	617.77 ± 66.94	18.45 ± 0.63
F9	59.07 ± 13.97	576.80 ± 195.45	10.54 ± 1.35
F10	75.57 ± 8.37	487.67 ± 79.56	15.66 ± 2.20
F11	98.90 ± 12.39	366.83 ± 75.33	27.24 ± 2.07

Lipid contents determined for H&H and H&H supplemented with SO were lower than expected, reflecting low lipid recoveries. Expected lipid recovery using the Folch method (Folch et al. 1957), based on a diverse range of samples each containing 1 g, is approximately 95% (Folch et al. 1957). Lipid recoveries for H&H and H&H supplemented with SO using the Folch method (Folch et al. 1957) as modified by Budge et al. (2006) were lower (77% or less, Table B-1) than published lipid recovery data (i.e., 95.72%, Folch et al. 1957). Lipid recoveries may have been lower due to a reduction in sample size (i.e., 0.5 g sample used), as lipid losses during the various extraction steps are magnified in small sample sizes. Lipid recoveries may also have been lower because a sample to solvent ratio of 0.5 g to 9 ml (1:18) was used (Budge et al. 2006), rather than a ratio of 1 g to 20 ml (1:20), which is recommended by Folch et al. (1957) as the minimum sample to solvent ratio for lipid extraction. The ratio of sample to solvent used could also explain why samples with higher lipid content showed the lowest recovery. Determination of lipid content through the Folch method (Folch et al. 1957) as modified by Budge et al. (2006) did not reliably estimate lipid content. The result of this small study agrees with Budge et al. (2006) that the Folch procedure as modified by Budge et al. (2006) should not be used to determine lipid content of milk.