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**MILK COMPOSITION OF THE
NEW ZEALAND SEA LION
AND
FACTORS THAT INFLUENCE IT**

**A thesis presented in partial fulfilment of
the requirements for the degree of
Doctor of Philosophy
in
Zoology**

**at Massey University, Palmerston North,
New Zealand**

Federico Germán Riet Sapriza

2007

ABSTRACT

The objectives of the present study were to: 1) describe the gross chemical milk composition of the New Zealand sea lion (NZSLs), *Phocarctos hookeri*, in early lactation; 2) validate an analytical method for sea lion milk composition; 3) investigate a series of temporal, individual and dietary factors that influence the milk composition of the NZSL and; 4) investigate the temporal and spatial differences in the fatty acids signatures of sea lion milk.

A comprehensive literature review revealed that data on milk composition in otariid species is either missing or limited, that to be able to fully describe their milk composition extensive sampling was required and that the temporal, maternal and offspring factors that influence milk composition in pinnipeds are poorly understood. The review identified that considerable work has been conducted to infer diet via the application of fatty acids signature analysis of milk and blubber. There are many factors (i.e. metabolism, *de novo* synthesis and endogenous sources) that contribute to the differences in fatty acid composition between the diet and milk or blubber.

Milk samples from NZSL were used to test whether a new method would give similar results as the standard methods of milk analysis. Agreement between analytical methods for milk components was assessed using different measures of statistical fitness and the results indicated that the new method was comparable to the standard methods and applicable to the milk of sea lions, pinnipeds and to ecological studies of lactation. Milk from NZSLs was collected over a period of seven years (1997, 1999 to 2003, and 2005) in early lactation to describe the composition of milk of NZSL and to test for differences between years. The results indicated that: i) the milk protein concentration was comparable to other species of pinnipeds; ii) the milk fat concentration and the milk energy content of NZSL is the lowest reported for otariids in early lactation; however iii) the milk fat concentration was significantly different between years. These results suggested that the milk composition of NZSLs was influenced by annual changes in the environment; however, there may be other unidentified factors. Month, maternal body

condition, age, body weight and length, offspring sex and age, and attendance pattern were compared with milk components. The results identified that month, maternal body condition and age significantly affected milk fat concentration. These results and the fact that maternal body condition varied significantly between years and mothers nursing male pups had lower body condition and produced milk lower in energy content suggested that local food resources along with other unidentified factors have an effect on the reproductive success of NZSLs. To test whether the fatty acid signature analysis (FASA) of lipid rich tissues (milk, blubber and serum) of otariids could be used to infer diet a mixture of vegetable oil (with distinctive fatty acid signature) was fed to 24 lactating NZSL and tissue samples were collected at different time intervals. Significant increases in the concentration of specific fatty acids in serum and milk were observed with peaks within 12hrs and 24hrs respectively of ingestion. Concentrations in milk remained elevated for up to 72hrs and there were differential rates of incorporation into milk. These findings confirm the potential of FASA to infer the composition of the diet. The variation in milk fatty acid signatures from lactating NZSL from four years (1997, 2003, 2004 and 2005) of sampling were measured in order to test whether differences occurred between years. Fatty acids signatures from five potential prey species including the commercially important arrow squid were incorporated into the analysis to associate the changes in milk fatty acids with a shift in prey choice. The results indicated that milk fatty acid signatures were different in 1997 and 2003; however, it was not possible to relate these differences to the five prey species. The variability in the annual arrow squid catch data suggested that local food resources around the Auckland Islands may also be variable.

In conclusion, the milk produced by the NZSL has the lowest concentration of fat and energy in early lactation reported for any otariid species. The main factors that contributed to changes in milk quality were stage of lactation, year and maternal body condition. The yearly variation in the quality of milk appears to be a result of their lactation strategy or to variable local food conditions that also affect maternal body condition. Therefore monitoring the annual milk quality may be a means to monitor the health of a pinniped

population and potential management tool for pinniped species. This thesis has shown that annual changes in the diet of NZSL can be assessed with milk fatty acid signatures.

PREFACE

A lack of understanding of the lactation strategies adopted by New Zealand sea lions (NZSL) was the keystone that initiated the work presented in this thesis. *A priori* there were some questions that were proposed such as what's the gross chemical milk composition of the milk?; Does it vary in relation to environmental conditions?; What are the factors that are determining the milk quality produced by NZSLs?; Do these factors have detrimental effects on the quality of the milk of NZSLs and thus on their reproductive success as measured by pup survival?

The interaction with the commercial squid fisheries is evident and thus the question that comes to mind is whether both fisheries and NZSLs are targeting the same food source, and if so would the competition adversely impact on the lactating NZSL? Would this interaction reduce the quality and quantity of milk produced by the NZSL?

I started this project with the idea of analysing the gross chemical composition of milk of NZSL and relating its composition to a number of maternal and offspring characteristics and temporal factors. Although there has been some work in this area but not necessarily on sea lions, I realize that there were many bias incorporated in these studies, for instance, methodologies were not standardized for the analysis of milk composition.

The first step in this project was to validate for the milk of NZSL an analytical method based on infra-red technology that is usually applied in the analysis of milk of dairy animals. Next it was evident that the factors such as maternal characteristics that influence the milk composition needed to be investigated and eventually the long and short term effects of diet. It soon became obvious that the complex mechanisms of milk fat synthesis/secretion related to the physiology of sea lions and the factors governing this mechanism would make it difficult to fully understand the relation between milk and diet and to draw objective conclusions. I found that there was little known about the mechanism of transfer of dietary lipids to milk lipids, in particular in pinnipeds.

Finally, my study focused on the milk composition and the factors that affect it because of its importance in the dynamics and recovery of the population of the species. Information on the quality of the milk can be used as an indirect index of the reproductive success and as a measure of the health of the population.

DEDICATION

"I dedicate this thesis to both my grandmother, Pochola (Jorgelina Bustamante de Riet), and my grandfather Hector (Hector Maria Sapriza) that passed away while doing this thesis, and of course to my parents. They more than anyone, have been the best support and encouragement I could have hoped for. Any accomplishment of mine is due in no small part to their support."

"Quiero dedicar esta tesis de doctorado a mi abuela Pochola (Jorgelina Bustamante de Riet) y a mi abuelo Héctor (Héctor Maria Sapriza), que fallecieron en el periodo de esta tesis, y por supuesto a mis padres. Ellos, más que nadie, han sido el mejor apoyo y ánimo que yo haya podido esperar. Cualquiera de mis logros, son en parte, debido a su apoyo."

ACKNOWLEDGMENTS

First of all, I am deeply indebted to my supervisor Dr. Padraig J. Duignan. He motivated me to take this as a Master's project and at early stages of my studies encouraged me to develop it towards a PhD project. I am very grateful for his support, vision, trust, and encouragement towards any aspect of project and my future career in marine mammalogy.

I would like make an honourable mention of Dr. Nicolas Lopez Villalobos that as a co-supervisor was brave enough to take the responsibility to be my chief supervisor in the last stages of this project. He has been throughout this project an important key that has greatly contributed to the mathematical aspect of this project. With his support, experience and advice I was able to enhance in many ways this thesis. Mil gracias, estoy eternamente agradecido!

I would like to express my sincere appreciation and gratitude to my other supervisors, Prof. Duncan D. S. Mackenzie, Dr. Alastair McGibbon, Dr. Louise B. Chilvers, and Prof. Brian Springett.

Duncan's M. understanding of the physiology of lactation in dairy animals has made enormous contribution to this thesis. During this project he had make me think further and challenge me by making many valuable suggestion and constructive criticisms.

Louise C. understanding of the ecology of marine mammals and great experience working with these animals has enriched this research in many ways. Throughout this project she has been a source of support and encouragement and I have great appreciation for her willingness to help and listen.

My sincere thanks go to Alastair for providing me his valuable insights on many aspect related to milk analysis, milk lipids and fatty acid.

I would like to record my gratitude to Ian W. for his supervision at early stages of this project. He has made great contribution to this thesis with his knowledge of pinniped biology and ecology.

I convey special acknowledgement to Ms Allain Scott for her indispensable help dealing with administrative and bureaucratic matters during my stay in New Zealand and while in Uruguay that facilitated me to carry out my research.

The financial support provided by The Swedish National Board of Student Aid (CSN) allowed me to undertake my studies for which I am grateful.

Words fail me to express my appreciation to IVABS for its financial support that allowed me to finish my postgraduate studies.

This project was supported by grants from Massey University Research Fund, Lewis Fitch and McGeorge Fund, Whale and Dolphin Adoption Project, Department of Conservation and Fonterra Research Centre, New Zealand.

The research was conducted under permit from the Animal Ethics Committee-Approval AEC86 (1 July 1999) of the Department of Conservation approved the handling and capture of NZSL and collection of milk samples. Massey University Ethics committee approved the handling and experiments conducted in Chapter 6 of this thesis.

Many people participated in the field that assisted in the capture of sea lions, collection, handling and care of the samples; and in the logistics of this research. For this reason I would to thank the veterinarians Aurelie Castinel and Mana Stratton and, Jacinda Amey, Rod Hood, Maurice Brown, Malcolm Wylie, Pete McClelland, Wally Hockley for their assistance in the field

I am grateful to Pete McClelland, Greg Lind and Sharon Trainer (Department of Conservation, Southland) for the significant logistic support provided throughout the field trips. And also to the crew members aboard the Marine Countess who provided valuable logistical assistance.

There are a number of people that I would like to show my gratitude because their participation in the laboratory work was crucial to the success of this research. I am grateful to Errol Conaghan, Mark Reynolds, Russell Richardson, Yvonne van der Does, D. Kreegher (Mineral analysis) (Fonterra Research Centre); and to Maggie Zou, Whiba Desai and Fliss Jackson (Institute of Food Nutrition and Human Health, Massey University, Palmerston

North), L. Meynier (IVABS) for their invaluable help and their technical support with laboratory analysis.

Special thanks to Mark Reynolds for his enormous assistance, help and advice during the fatty acids analyses. I am very thankful to Russell Richardson for his assistance and advice in the application of the ASE.

I cannot thank enough Yvonne van der Does for her willingness to help me in any matter during my laboratory work at Fonterra.

I would like to thank Maggie Zou (Institute of Food Nutrition and Human Health) for her help in showing me how to extract the lipid from the serum samples.

I am in debt with Laureline Meynier for her helps and assistance in the blubber lipid extraction procedures.

I am much obliged to Sara Iverson (Dalhousie University, Canada) for providing the laboratory protocol for the extraction of milk and blubber lipids.

I would like to thanks Dan Costa and Nick Gales for their collaboration in this project since they provided the milk samples and data on the females from summer season 1997. Milk samples and NZSL female's data from 1997 were used in Chapters, 3, 4 and 6.

I would like to show my appreciation to Laureline Meynier for her collaboration in this project. She provided the fish prey fatty acids data that was used in Chapter 6.

I acknowledge my gratitude to Simon Childerhouse for collaborating with this project by sharing females age data and some data on the sex of pups

I am deeply and forever indebted to my parents for their love, support and encouragement throughout my entire life. I am grateful to my brothers Gustavo and Jeronimo. I owe a special gratitude to Alicia, Licha, Mark, Mara and Luis, Lorena Errico, my families Riet and Sapriza, and the family Antunez for their continuous and unconditional support of all my undertakings.

My friends in Uruguay deserve special mention for their inseparable support.

I am also thankful to Santiago Marquez and Sebastian Aliaga for their unconditional friendship and support during all these years. I would like to

express my gratitude to Rosario, my girlfriend, for her love, support and caring.

Throughout my studies in New Zealand there were many friends that were by my side during the good and difficult times. Without their support and friendship this project could not have been possible. I owe a big debt of gratitude to: Nacha Holmes and Erich Seydewitz, Gerardo Canales and Carolina Arenas, Gonzalo Tuñon, Javier and flia Baudracco, Patric Dulin and Rebeca Maseda, Sabina Jaeger, Megan McGregor, Alfredo Ordóñez, Marcos and Gabriela Fernandez, Juan Pellegrino and Virginia Serra, Gonzalo Carracelas and Virginia Porcile, Mateo, Alejandra Yanegui, Natalia Benquet y Alexis Pérez.

Finally, I would like to thank everybody who was been important to the successful realization of the thesis, as well as expressing my apology that I could not mention personally one by one

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LIST OF ABBREVIATIONS

| | |
|---------|---|
| • AMF | Anhydrous milk fat |
| • AOAC | Association of Official Analytical Chemists |
| • BCF | Bias Correction Factor |
| • BCI | Body condition index |
| • CART | Classification and regression tree |
| • CCC | Concordance correlation coefficient |
| • CDA | Canonical Discriminant analysis |
| • CLO | Cod liver oil |
| • CoNVO | Cocktail of Natural Vegetable Oils |
| • DFA | Discriminant Function Analysis |
| • ENSO | El Niño Southern Oscillation |
| • FA | Fatty acid |
| • FAME | Fatty acid methyl esters |
| • FASA | Fatty Acid Signature Analysis |
| • ICC | Intraclass correlation coefficient |
| • MUFA | Monounsaturated fatty acids |
| • NEFA | Non-esterified fatty acids |
| • NPN | Non-protein nitrogen |
| • NZSL | New Zealand sea lion |
| • PUFA | Polyunsaturated fatty acids |
| • QFASA | Quantitative Fatty Acid Signature Analysis |
| • r | Pearson correlation coefficient |
| • r^2 | Coefficient of determination |
| • RPE | Relative prediction error |
| • R-G | Roese Gottlieb method for fat determination |
| • SAFA | Saturated fatty acids |
| • SDA | Stepwise Discriminant analysis |
| • TAG | Triacylglycerol |

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

FOREWORD

The objective of this chapter is to introduce the thesis in a broader context and describe briefly the biology and ecology of New Zealand sea lions (*Phocarctos hookeri*). The pertinent literature to this research is found in this Chapter. The rationale of the research and the outline and main aims of this thesis are presented at the end of this Chapter.

GENERAL INTRODUCTION

Maternal investment during lactation is higher than during gestation, and is the most energetically expensive period in a mammal's life cycle (Higgins and Gass, 1993; Gales *et al.*, 1996). In early post natal life the neonate is unable to feed itself, hence it has to rely on the mother for its food supply in the form of milk, and this process of milk production is known as lactation (Arnould and Boyd, 1995a). During the period of full dependence on the female, parental investment will directly influence the growth rate and survival of the young. In general, reproductive success for mammals will be affected by several factors such as litter size, sibling competition, food availability, parental foraging patterns, time of weaning, and parental age and experience (Lunn *et al.*, 1993).

Pinnipeds are the principal group of aquatic mammals that are adapted to reside on land and at sea (Oftedal *et al.*, 1987a; Boness and Bowen, 1996). They rely on land or ice to give birth and nurse their pups, and as a consequence, foraging at sea and nursing of the young on land are separated by space and time (Bonner, 1984; Boness and Bowen, 1996). While staying on the terrestrial environment, for some species the mother and the pup are vulnerable to potential terrestrial predators, therefore pinnipeds have evolved strategies to diminish the risk of predation (Bonner, 1984; Ferguson, 2006). Other issues that concern the survival of the pup are: 1) the build up of an insulation layer against heat loss, and 2) the supply of enough energy to

enable the pup to sustain itself during periods of fasting. These problems are tackled by secreting and rapidly transferring lipid-rich and energy-dense milk to the pup (Goldsworthy and Crowley, 1999; Georges *et al.*, 2001). Lactation strategies in pinnipeds have evolved to meet particular environmental conditions and because of their worldwide distribution they have evolved a diversity of lactation strategies (Bonner, 1984; Schulz and Bowen, 2005; Ferguson, 2006). There are three taxonomic groups of pinnipeds: *Otariidae* the sea lions and fur seals; *Odobenidae* the walrus; and *Phocidae* the true seals, and they have adopted distinctive lactation strategies (Bonner, 1984; Boness and Bowen, 1996).

This research describes the gross chemical milk composition of NZSL over a period of seven years during early lactation at Enderby Island, Auckland Islands. Because milk was collected over seven years it allowed me to study the inter-annual and intra-annual (in early lactation) changes in milk composition. In addition, the relation between changes in milk composition and maternal and offspring factors are tested. Hypotheses about temporal and spatial changes in milk fatty acid composition in relation to diet are also investigated.

LACTATION STRATEGIES IN PINNIPEDS

Otariidae: foraging lactation strategy

The period of lactation in otariids extends from four months in northern fur seals (*Callorhinus ursinus*) to up to three years in Galapagos fur seals (*Actocephalus galapagoensis*) (Table 1) (Bonner, 1984). The otariid lactation strategy is characterized by an alternation between nursing the pup and foraging at sea and therefore is known as the “foraging lactation strategy” or as “income breeders” (Figure 1) (Boyd, 2000). Before giving birth otariid mothers return to shore a short time and then after giving birth they stay with the pups for around one week in which the bond between mother and newborn is established. This period is known as the perinatal period.

It has been proposed that the duration of lactation among otariids may have evolved in relation to environmental predictability associated with latitude (Schulz and Bowen, 2005). The productivity of the marine environment and its predictability has shaped the maternal strategies of pinnipeds. In higher latitudes seals are exposed to radical seasonal productivity variation but this is very predictable and the duration of lactation is usually short. Whilst in lower latitudes seals are generally exposed to a more constant seasonal pattern of productivity throughout the year and therefore their lactation is usually longer. However, every few years due to El Niño/La Niña (El Niño Southern Oscillation or ENSO) conditions, productivity becomes very unpredictable (Gentry *et al.*, 1986).

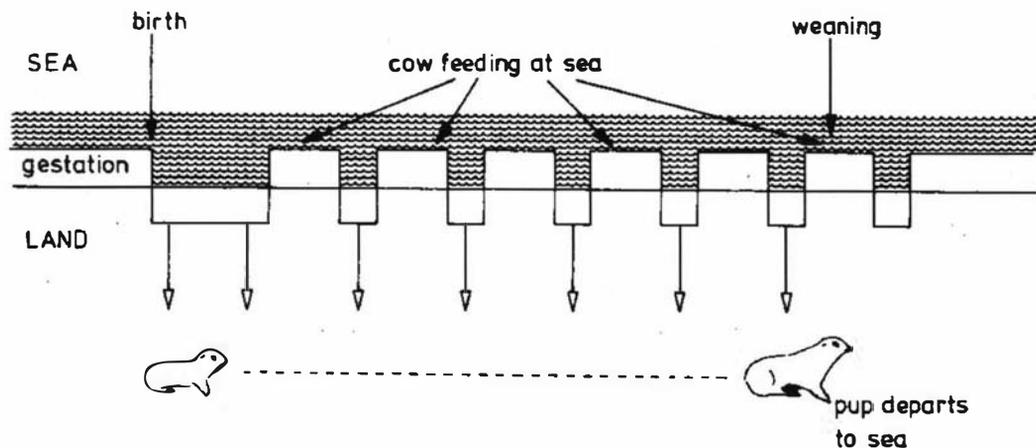


Figure 1. Maternal foraging strategy of otariid seals (Bonner, 1984).

ENSO events have a profound effect on climate and ocean ecosystems (Cane, 1983). Upwelling zones in the eastern Pacific undergo a negative transition from normal highly rich productivity to profoundly decreased productivity (Barber and Chavez, 1983). Pinnipeds which feed at the top of the food chain, are severely affected by low food availability, which in turn disrupts normal maternal foraging and attendance patterns, suckling patterns, pup growth and pup behaviour (Trillmich *et al.*, 1986b; Oftedal *et al.*, 1987b).

Trillmich *et al.* (1986b) investigated attendance and diving behaviour of South American fur seals (*Arctocephalus australis*) during El Niño conditions for a two-month period in 1983. Their study revealed that low foraging success extended the stay at sea searching for food at high-energy cost and led to the inability of females to replenish their energy reserves. Moreover, during the same event, California sea lions (*Zalophus californianus*) extended significantly their foraging trips (Melin *et al.*, 2000) and pup milk intake was lower than in non El Niño years (Oftedal *et al.*, 1987b). During years of shortage of krill near South Georgia, lactating Antarctic fur seal (*Arctocephalus gazella*) females made fewer and longer trips that resulted in decreased mass and growth of pups (Lunn *et al.*, 1993). The prolonged foraging trips doubled in time and as a consequence, the mortality of pups increased to 32%; 68% which died from malnutrition (Costa *et al.*, 1989).

A characteristic of otariid breeding and lactation strategies is that they are similar among the different species (see Figure 1) (Bonner, 1984). Galapagos fur seals during pregnancy spend extended periods at sea fattening and arrive at the colony between two and three days before giving birth. The newborn pup is fed for five to ten days during the perinatal period, and then the mother's attendance pattern consists of feeding trips at night and then she returns in the morning (Trillmich and Lechner, 1986). Foraging trips lasted around two days (Trillmich and Lechner, 1986), whereas suckling attendance periods lasted from half a day to one and a half days, the length of which is related to the age of the pup (Kooyman and Trillmich, 1986). Their conspecific, the Galapagos sea lions (*Zalophus californianus wollebaeki*), attended their pups almost every day and foraged during the day and returned at night (Trillmich and Lechner, 1986). The tropical Galapagos fur seals and sea lions have a lactation period that lasts from one to three years and one year, respectively.

In comparison, otariid species with high latitude distribution such as Antarctic fur seals (polar/temperate), northern fur seals (*Callorhinus ursinus*) (temperate), and subantarctic fur seals (*Arctocephalus tropicalis*) (temperate) have short lactations. The first two species wean their pups at four months while the third species weans their pups at ten months of age (Table 1) (Oftedal *et al.*, 1987a). Pregnant Antarctic fur seals arrive ashore two days before parturition and feed their pups for about five to seven days and then alternate foraging trips lasting for three to five days with maternal attendance periods lasting between three to ten days (Doidge *et al.*, 1986; Boyd, 1999). Northern fur seals have similar breeding patterns, females arrive ashore half a day to two and a half days before giving birth and nurse the newborn for six to seven days followed by a first maternal postpartum foraging trip of between four and seven days (Doidge *et al.*, 1986; Donohue *et al.*, 2002). The mean duration of foraging trips were longer than in Antarctic fur seals, lasting for six to eight days with constant periods of maternal attendance that lasted from one and a half to two and a half days. Subantarctic fur seals have one of the longest attendance cycles known in fur seals. The females arrive ashore one

to two days prepartum and then spend eight days nursing the newborn; thereafter alternating long foraging trips of 11 to 23 days with long maternal attendance periods ashore of up to four days (Goldsworthy, 1999; Georges and Guinet, 2000b). This attendance pattern is constant during the whole period of lactation and until the pup is weaned at 10 months of age (Table 1) (Goldsworthy, 1999).

Conclusion-Otariidae: foraging lactation strategy

Otariids living in low latitudes live in an environment where food productivity is low and very unpredictable, while otariids nursing their pups at high latitudes have the benefits of a more predictable environment with high seasonal productivity. Therefore, we may expect to see shorter lactation periods in subpolar species due to the short seasonal period of high productivity. But this is not always the case. Thus, it appears that the length of lactation in otariids might be influenced by the seasonal availability and predictability of food sources, whilst foraging trip length and rate of energy transfer is determined by the distance from breeding site to food source (Boness and Bowen, 1996). The question that arises is whether milk composition is directly or indirectly influenced by interspecific differences in attendance patterns, or in other words, the length of time spent at sea foraging. This question has been under the spotlight and has been investigated in several species including subantarctic fur seals (Goldsworthy and Crowley, 1999; Georges *et al.*, 2001), Galapagos fur seals and sea lions (Trillmich and Lechner, 1986), Australian sea lions, *Neophoca cinerea* (Kretzmann *et al.*, 1991), Juan Fernandez fur seals, *Arctocephalus philippii* (Ochoa-Acuna *et al.*, 1999); Australian fur seals, *Arctocephalus pusillus doriferus* (Arnould and Hindell, 1999), and Antarctic fur seals (Arnould and Boyd, 1995b; Goldsworthy and Crowley, 1999).

***Phocidae*: fasting lactation strategy**

The two main characteristics that separate maternal strategies adopted by phocids from that of the otariids is that phocids have shorter lactation periods and they generally fast during the whole lactation period (Figure 2 and Table 2). Phocids are capital breeders and their lactation strategy is known as the fasting lactation strategy (Boyd, 2000) however; as discussed later in detail, not all phocids species are embedded into this strategy. Pregnant phocid females arrive at haul out sites a few days before pupping and when nursing is completed the pup is abruptly weaned (Figure 2) (Oftedal *et al.*, 1987a). Phocid seals that breed on pack or fast ice are known as pagophilic seals while seals that breed on dry land are known as land whelping seals (Bonner, 1984).

Ice breeding seals or pagophilic seals, have adopted remarkable breeding and lactation strategies to decrease predation pressure. Firstly they have shortened their lactation period and secondly they have exploited higher latitudes where terrestrial predators are virtually absent (Bonner, 1984). However, ringed seals (*Phoca hispida*), a fast-ice phocid, that are threatened by predators, such as polar bear and arctic fox, have diminished predation pressure by giving birth and nursing their pups in snow and ice dens (Bowen, 1991; Hammill and Smith, 1991). If predation is non existent or basically avoided by choice of breeding site, then any variations in the maternal strategies must be related to other ecological factors such as stability of breeding substrate.

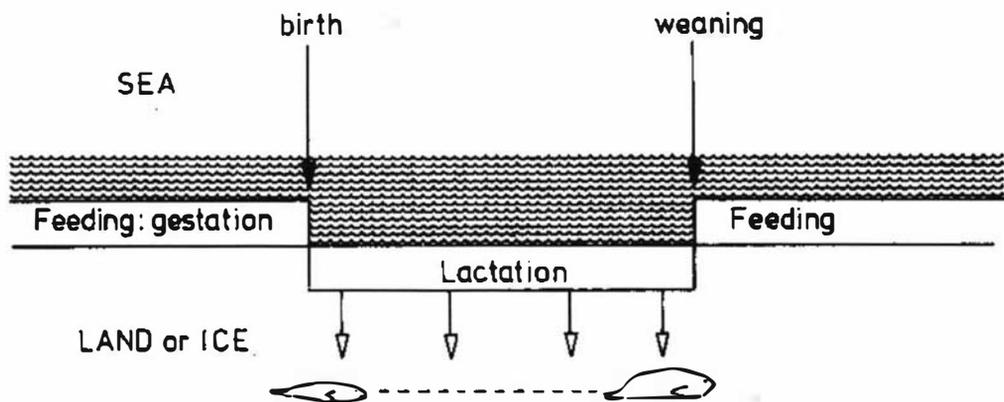


Figure 2. Maternal foraging strategy of phocid seals (Bonner, 1984).

Breeding on ice packs (ice floating on the sea surface) or fast ice allows seals a rapid access to deep waters. However, breeding on ice packs has several pitfalls. Ice packs provide little shelter and are an unstable habitat at the mercy of wind and water currents with the potential to drift away separating mother and pup. In a more stable environment, seals can extend the lactation period. Thus, pack ice breeding phocids have the shortest nursing period (4 to 30 days), while land and fast-ice breeding phocids have the longest (36 to 75 days) (Table 2). The very short lactation period seen in pagophilic seals has been suggested to be related to the unstable pack ice surface (Bonner, 1984; Bowen *et al.*, 1985; Oftedal *et al.*, 1987a).

The shortest lactation period in pack ice breeding species is seen in the hooded seal, *Cystophora cristata*, that nurse pups for only four days (Bowen *et al.*, 1985) and the harp seal, *Phoca groenlandica*, for 12 to 13 days (Kovacs *et al.*, 1991) whilst the longest are found in seals that pup on fast-ice and land, such as the Baikal seal (*Phoca sibirica*) and Mediterranean monk seal (*Monachus monachus*), respectively (Table 2). The Baikal seal lactates for 60 to 75 days, whereas Mediterranean monk seals lactate for between 42 and 49 days (Table 2). In comparison with Baikal seals and Mediterranean monk seals, southern (*Mirounga leonina*) and northern elephant seals (*Mirounga angustirostris*) lactate for significantly shorter periods of 21 and 28 days, respectively (Table 2). The relatively short lactation seen in phocid pinnipeds

can be expected to affect milk composition and the dynamics of energy transfer from mother to pup since most phocids fast during the nursing period. Indeed the high fat content found in phocid milk can be attributed by the short lactation period in which a large amount of energy must be transferred to the pup in a limited time.

Two maternal strategies have been identified within ice breeding phocids. One is carried out by species such as harp seals, hooded seals and grey seals, *Halichoerus grypus*. This strategy involves a very short lactation period with the transfer of energy rich milk to the pup (Table 2 and 3). Their pups are very inactive and in most cases do not enter the water for many weeks after they have been abruptly weaned and face a long post-weaning period without feeding (Lydersen and Kovacs, 1999).

The second strategy is found in bearded seals (*Erignathus barbatus*) and ringed seals (*Phoca hispida*). These species have among the longest lactation periods within the ice breeding phocids: females do feed during lactation, milk has lower energy content, and pups are more active. It was believed that only the income breeder, the otariid group, had evolved a maternal foraging cycle in which there are periods of pup attendance interrupted by periods of foraging at sea. Recent studies on energetics and diving behaviour in harbor seals (*Phoca vitulina*) have proven the exception to the rule, and have shown that maternal body mass has important consequences for lactation strategies in phocid species (Bowen *et al.*, 2001). Compared with other phocids the bearded seals' pups have lower weight gain during lactation and this is a consequence of the more active lifestyle of the pup, the lower energy content of the milk, and the larger birth size of the pup (Lydersen *et al.*, 1996). In the study on bearded seals the sample size was only three pups, and therefore the conclusions should be interpreted with caution. Nevertheless, it is apparent that ice breeding seals such as bearded and ringed seals, with longer nursing periods and lower energy rich milk, are unable to sustain a long nursing period without foraging. There is some evidence to suggest that these seals have adopted an "otariid-like" maternal foraging cycle (Hammill *et al.*, 1991; Kelley and Wartzok, 1996; Lydersen *et*

al., 1996). Adopting an otariid-like maternal foraging cycle during lactation has also been suggested for other species of phocids.

Foraging cycle behaviour may have developed in small phocids such as the harbour seal as a response to nutrient depletion during the lactation period (Boness and Bowen, 1996). Harbor seals are only slightly larger than most otariids and smaller than some, thus it is very likely that maternal size limits the amount of energy that the female can store. The combined demands on the female harbour seal's reserves for her own maintenance and for milk production is likely to be too great for the limited energy she can store in the form of blubber (Bowen *et al.*, 1992; Boness *et al.*, 1994; Boness and Bowen, 1996; Oftedal, 2000). Harbor seals are among the smallest of the phocid seals, but there are other phocid species with small female body mass which strongly suggests that perhaps half of the phocids species forage during lactation and hence have adopted an "intermediate strategy" between that of income breeders otariids and the larger income breeder phocids (Boness *et al.*, 1994). Boness and his co-workers (1994) proposed that harbour seals begin to forage when the gain of energy, to replenish the energy stores, was highest and the risk of losing the pup was the lowest.

Lactation strategies in other small phocids warrant further investigation, and special consideration should be given to what extent and under which conditions females begin to forage. Body size have been identified as one of the major factors influencing the lactation strategies in pinnipeds (Ferguson, 2006). Therefore smaller body size phocid species cannot store sufficient energy in the form of blubber and thus cannot withstand the cost of producing fat-rich milk. It appears that there are physiological restraints that are most likely to interact and influence their lactation strategy (Boness and Bowen, 1996; Bowen *et al.*, 2001).

Within the group of land-whelping seals, elephant seals have one of the shortest lactation periods. Northern and Southern elephant seals have a similar breeding pattern. A wide variety of social behaviour traits according to

age, sex and season are a result of well-defined seasonal cycles and formation of large colonies. As in all phocids, lactation is short lasting 23 days in southern elephant seals, and 28 days in northern elephant seals during which the pup has a rapid growth rate (Ortiz, 1984; Hindell *et al.*, 1994) followed by a long post-weaning fasting period of two to three months (Ortiz *et al.*, 1978). During the post weaning period, male pups suckle from other lactating females with the benefits of growing bigger. Male pups will benefit from stealing milk since elephant seals have marked sexual dimorphism and hence there is a selective advantage in increased size in males (Figure 3). Phocids have to some extent adopted different maternal strategies within their group and are not as conservative as believed. Therefore the question arises what are the factor/s or selective pressures that are influencing the maternal strategies in phocids and are they the same as those governing otariids.

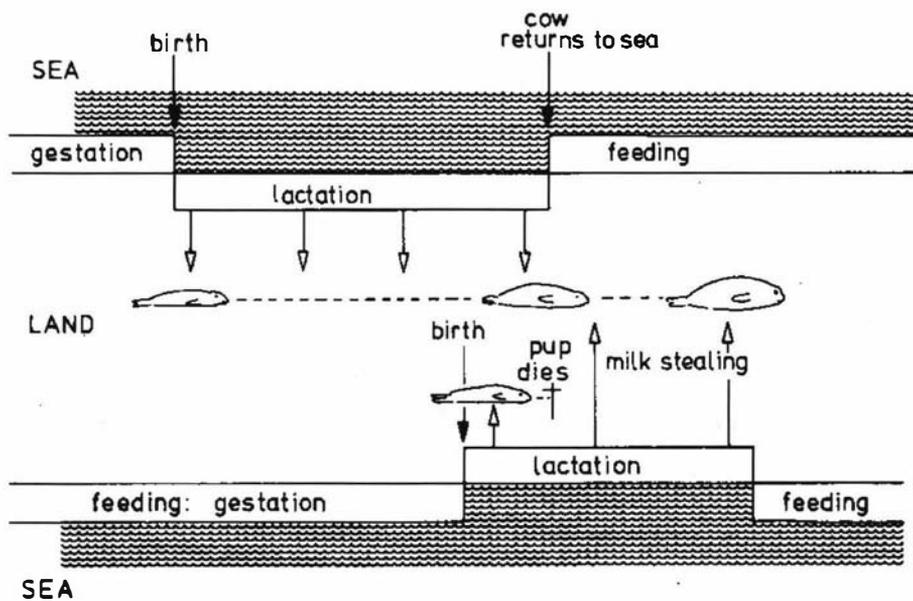


Figure 3. Lactation strategy in northern elephant seals (Bonner, 1984).

Variation in lactation strategies with latitude, as noted in otariids, has not been proposed for phocids. However there are environmental factors associated with latitude that have apparently influenced the evolution of lactation strategies in phocids and that recently have been tested using phylogenetic analysis (Schulz and Bowen, 2005). Lactation length in phocids

has evolved driven by the selective pressure of the breeding substrate and the cost of milk production, and to some extent predation (Schulz and Bowen, 2005). Predation does not apply for most land breeding phocids because they breed on predator-free islands, or for species such as monk seals and elephant seals that have short lactation periods. For this reason there may be selective pressures other than breeding substrate that drove the shortening of the lactation length in phocids (Schulz and Bowen, 2005).

Conclusion- Phocidae: fasting lactation strategy

The maternal strategy adopted by most phocids is quite distinctive. They usually lactate for a short period compared to their counterparts the otariids, and they fast during the lactation period and therefore they have to rely entirely on body reserves to produce milks that are amongst the most nutrient rich and energy dense of any species.

Compared to otariids, phocids usually have large maternal body size which enables them to withstand the cost of lactation while fasting; however, it appears that harbour seals, a small body mass phocid species, cannot support the cost of lactation due to nutrient depletion and has adopted an alternative foraging strategy similar to otariids. Further investigation in energetic and maternal investment in phocids is needed to assess the extent of the “otariid-like foraging strategy” in this group of pinnipeds.

Odobeniidae (walrus): aquatic lactation strategy

Walrus live in the higher northern latitudes and two subspecies have been recognized, the Atlantic walrus, *Odobenus rosmarus rosmarus* and the Pacific walrus *O. r. divergens* (Brenton, 1979b). They are the most highly social of pinnipeds, and always occur in groups. Walrus seems to prefer ice floes for haul out, resting, moulting, and whelping (Fay, 1981). Moreover the migration pattern of females and pups are associated with ice movement (Fay, 1982).

Most, if not all, pinnipeds rely on land or ice to nurse their pups and sometimes the mother and/or pup do not make their way into the water until the weaning period is commenced. Walruses have adopted a different strategy to those seen in otariids and phocids in that they whelp their young on ice floes and after a few days both mother and pup return to the sea. This particular strategy is known as “aquatic nursing”. It involves the pup being nursed in the sea, on ice or on land and at sea the mother forages while the pup remains at the surface (Kovacs and Lavigne, 1992).

The lactation period lasts for two years, and at the age of five months the pup starts to consume solid food, mainly benthic invertebrates (Fisher and Stewart, 1997). Fisher and Stewart (1997) suggested that the long duration of lactation might be associated with the specific mode of feeding of walruses. The main prey are bivalves that belong to the benthic fauna (Fisher and Stewart, 1997), thus walruses must be able to dive and stay at the bottom searching for prey. The whelping must learn to find its food, and this may explain the long lactation period that presumably increases the survival of the pup and increases its weaned mass (Kovacs and Lavigne, 1992). Walrus milk contains the lowest mean fat and protein concentrations recorded so far in pinnipeds (Table 4). The low content of energy in the milk may be explained in part by their long lactation period and hence there is not the need for a rapid transfer of energy rich milk, with its high cost for the mother in terms of thermoregulatory demands (blubber depletion). Since walruses dwell in the same environment as their pagophilic phocid counterparts, they must face the same high thermoregulatory demands and predation pressure.

Conclusion- Odobeniidae: aquatic lactation strategy

Walrus have adopted a unique lactation strategy in which nursing of the pup and maternal feeding at sea are not spatially or temporally disassociated as with other pinnipeds. As a result they are able to extend their lactation period with low cost for the female and increased survival for the pup.

General Conclusion- Lactation Strategies in pinnipeds

Two distinctive maternal strategies have been presented, that of the phocids, fasting strategy although not entirely true, and that of the otariids, foraging strategy. Both strategies rely on energy reserves for the production of energy dense and nutrient rich milk. Nevertheless, it has been demonstrated that some smaller phocids have adopted an otariid-like foraging cycle in that they start to feed at mid lactation probably because they are unable to withstand the cost of prolonged fasting. Walrus have adopted an aquatic nursing strategy which allows them to nurse and feed in the same space and time.

MILK COMPOSITION: A COMPARATIVE REVIEW

Milk is secreted by the mammary glands, and it is a complex fluid that contains five main components, water, lipids, proteins, sugars and minerals (Ling *et al.*, 1961; Jenness, 1974; Johnson, 1974). Several of these can be divided further into more specific components. The concentrations of all the components in milk may vary both between species (see Table 22, Appendix) and within species at different stages of lactation and under different nutritional and environmental condition.

In Table 22 (Appendix) the gross composition of milk of some species has been presented for comparative purpose. Examples are given of the milk from at least one species within every order of the class mammalia to give a representative overview of the milk composition in comparison with pinniped milk. More extensive reviews of the comparative composition of milk across species can be found elsewhere (Ben Shaul, 1962; Jenness and Sloan, 1970; Linzell, 1972; Jenness, 1974). The more limited data on the mineral composition of milk of various species is presented in Table 5.

Methods of milk analysis

In order to study lactation the concentration of at least the main milk components needs to be determined. The methods for milk analyzed are standardized and known as reference methods and although they have been developed to analysis the milk of domestic mammals they are also applicable to mik of non domestic mammals. The methods that have been used for the analysis of milk in pinnipeds have been adapted from the official reference methods approved by the Association of Official Analytical Chemists (AOAC) for the analysis of cow milk.

The Roesse-Gottlieb (R-G) method, which is the official method for the determination of the total concentration of lipid in milk, has been used to analyse the milk of pinnipeds (Ofstedal and Iverson, 1995). The R-G uses ammonium hydroxide and alcohol to disrupt the fat globule membrane followed by extraction of the lipid with petroleum ether and the gravimetric determination of the lipid after evaporation of the solvent (International Dairy Federation, 1987). However, this method is not well suited for a) samples collected from stomachs of pups or for b) samples that have suffered extensive hydrolysis or oxidation during storage (Iverson and Ofstedal, 1995). The total lipid concentration in such samples may be underestimated (Iverson and Ofstedal, 1995).

Two alternative methods that have been used for the determination of total milk lipid in pinnipeds are the Folch method (Folch *et al.*, 1957) and the Bligh and Dyer method (Bligh and Dyer, 1959). For both methods the extraction of lipid is based on a mixture of methanol and chloroform. However, the later method was developed for the extraction of lipid from lean fish samples and is not recommended for samples high in fat such that of pinnipeds' milk (Iverson *et al.*, 2001a; Budge *et al.*, 2006).

A stoichiometric CHN method that measures elemental (C, H, N) composition and determines the proximate composition based on the

stoichiometric relationship for representative proteins, lipids, and carbohydrates in milk was developed for pinniped milk (Arnould *et al.*, 1995) by adapting a method for analysing tissue composition (Gnaiger and Bitterlich, 1984). However, the results of Arnould *et al.* (1995) should be taken with caution since they were similar to those determined by the Bligh and Dyer method which has been shown to underestimate lipid concentration in pinniped milk.

The reference method and the one most commonly used to determine total milk protein is the Kjeldahl method (International Dairy Federation, 2002) which measures the nitrogen concentration in the sample. Milk protein concentration is usually reported as total nitrogen less the non protein nitrogen (NPN) times 6.38. The factor 6.38 was derived for cow milk proteins, which may differ for pinniped milk. A factor for the protein of pinniped milk has not been determined, and therefore total protein concentration may be over or under estimated slightly. Variation in the NPN concentration in pinniped milk is potentially more serious if it is not measured directly. However, NPN only accounts for approximately 6% and 3-7% of the total nitrogen in cow milk and in pinniped milk, respectively (Ashworth *et al.*, 1966; Jenness, 1974; Oftedal, 1984b; Trillmich and Lechner, 1986; Carlini *et al.*, 1994; Arnould *et al.*, 1995). Thus the Kjeldahl method is accepted for the estimation of the protein concentration of pinniped milk (Oftedal and Iverson, 1995).

Milk lipids

Milk lipid concentration

A comprehensive and comparative review of the milk of 100 species of mammal is available in Jenness (1974) and more recently in Oftedal (1984b) and Oftedal and Iverson (1995). Pinnipeds are the group of mammals that produces milk with highest milk fat concentration and thus milk fat is the major contributor to the energy content of milk (Table 22, Appendix). Among pinnipeds, milk fat concentration varies considerably between species and

within species. Overall phocids, produce milk with a higher concentration of fat than otariids although some otariids produce milk with high fat concentrations (Table 3 and 4). In most species the milk fat concentration increases as the mammary gland is being emptied, pinnipeds are not an exception. As in other mammals, milk fat is influenced by stage of lactation and by the nutritional status (Ling *et al.*, 1961) although it is not clear how the latter is mediated in pinnipeds. This and other factors that affect milk composition in pinnipeds and in particular milk fat concentration are discussed below (see section FACTORS THAT INFLUENCE MILK COMPOSITION).

Milk fatty acid composition

Milk lipid composition is influenced by environmental and physiological factors, including, age, stage of lactation, gestation length and diet. Some intra-species differences reflect inherited variation (Jenness, 1974). Milk lipids belong to various lipid classes, but triacylglycerides account for almost 97-98% of lipid in pinniped milk (Iverson and Oftedal, 1995). Other lipids classes include di- and mono-acylglycerols, phospholipids, free cholesterol, cholesterol esters and free fatty acids (Ling *et al.*, 1961; Garton, 1963)

The distribution of chain lengths differ among taxa. The reason for this is not clear but it is likely that the differences in milk lipid composition among mammals can be explained by fatty acid synthetase of the mammary glands (Iverson and Oftedal, 1995). Despite this, little attention has been given to this topic, especially in carnivores including pinnipeds (Iverson and Oftedal, 1995). Milk FA composition reflects the origins of the FAs, since some FAs (dietary) pass directly from the gut, others are transferred from body tissues and some are synthesized by the mammary glands. For instance, ruminants secrete milk lipids with a high proportion of short chain and medium chain FA synthesised in the mammary glands, whereas carnivores such as pinnipeds secrete milk lipids high in long chain fatty acids (Iverson and Oftedal, 1995). These long chain fatty acids may originate from either *de novo* synthesis or from circulating blood lipid that the mammary gland uptakes. The milk fatty acid composition of pinnipeds is characterized by a high proportion of long chain

FA, which is a reflection of their diet. This and other topics are discussed later in this review (see section FATTY ACIDS A SOURCE OF INFORMATION).

Milk proteins

Proteins found in milk are either caseins or whey proteins, and the kind and number of protein varies significantly between species (Jenness, 1979). Proteins that occur in the milk of most species studied to date are caseins, blood serum albumin, immunoglobulins, and alpha-lactalbumin; whereas there is some evidence to indicate that the beta-lactoglobulin family only occurs in the milk of ruminants and some species of artiodactyls (Jenness, 1979). The primary function of casein is nutritional and serves as a source of amino acids.

Little is known about proteins and their function in the milk of terrestrial mammals hence few conclusions can be made in relation to homologous proteins in the milk of pinnipeds. For instance, the whey protein alpha-lactalbumin has not been detected in otariid milk and is virtually absent in phocid milk (Pilson and Kelly, 1962; Peaker and Goode, 1978; Stewart *et al.*, 1983). Alpha-lactalbumin is crucial for biosynthesis of lactose in milk and therefore the absence of lactose in pinniped milk has been associated with the lack of this protein (Dosako *et al.*, 1983). By comparison with bovine milk, casein micelles found in northern fur seals milk were significantly larger, but the reason for this has not been addressed (Dosako *et al.*, 1983). Caseins have been reported to account for 44 to 72% of the total protein in phocid milk (Jenness, 1974; Shaughnessy, 1974), whereas in otariids, such as northern fur seals and Galapagos fur seals, casein accounted for 52% and 75%, respectively (Ashworth *et al.*, 1966; Dosako *et al.*, 1983; Trillmich, 1988).

The amino acid concentration in the milk of pinnipeds was slightly higher than in that of terrestrial mammals. The proportion of total essential amino acids, total branched-chain amino acids, total sulphur amino acids, and most individual amino acids in relation to the total amino acids in pinniped milk was within or near the range of mean values for that of other species

(Trillmich, 1988; Davis *et al.*, 1995). Furthermore, the amino acid pattern and total amino acid concentration of milk was affected by stage of lactation in terrestrial mammals but not in pinnipeds (Trillmich, 1988; Davis *et al.*, 1994; Davis *et al.*, 1995). This contradicts the view of Davis *et al.* (1994) that changes in amino acid pattern and total amino acid concentration during lactation appears to be unrelated to phylogenetic order.

Milk carbohydrates

Lactose (disaccharide) is synthesized in the mammary gland and is the dominant sugar in most milk (Table 22, Appendix). In addition to lactose there are a great variety of saccharides in milk (Jenness *et al.*, 1964; Urashima *et al.*, 2001b). However, the milks of marine mammals contain only traces or no lactose at all (Table 22, Appendix). For instance, in human milk there has been detected more than 100 oligosaccharides, or saccharides that contain three or more monosaccharide residues. The chemical structure of around 80 have been reported (Newburg and Neubauer, 1995).

In comparison with measurements of the concentrations of milk fat and protein, carbohydrates have been given little attention in pinnipeds but data have been reported for Australian fur seals and Hooded seal (Urashima *et al.*, 2001a), harp seal (Stewart *et al.*, 1983), Crabeater seal (*Lobodon carcinophagus*) (Messer *et al.*, 1988; Urashima *et al.*, 1997), Arctic harbour seal (*Phoca vitulina vitulina*) (Urashima *et al.*, 2003), sirenians (Pervaiz and Brew, 1986) and cetaceans (Table 22, Appendix) (Urashima *et al.*, 2000; Urashima *et al.*, 2002). It has been reported that phocid milk contains several oligosaccharides of unknown structure, low concentrations of free lactose and traces of glucose and galactose (Messer *et al.*, 1988; Urashima *et al.*, 1997; Urashima *et al.*, 2001a). In the milk of most mammals apart from pinnipeds and cetaceans, lactose is the predominant carbohydrate (Ofstedal, 1984b). As a consequence, pinnipeds have among the lowest milk carbohydrate concentration of any mammal. The chemical characterization of carbohydrates in hooded seal, crabeater seal and Australian fur seals,

California sea lions and northern fur seals has revealed that, unlike phocids, otariid milk does not contain free reducing saccharides or lactose (Pilson and Kelly, 1962; Pilson, 1965; Dosako *et al.*, 1983; Stewart *et al.*, 1983; Messer *et al.*, 1988; Urashima *et al.*, 2001a)

The biological function of milk oligosaccharides in phocids may be similar to that in terrestrial mammals, but this does not apply to otariids since they produce milk without free saccharides (Urashima *et al.*, 2001a). The concentration of carbohydrates in Antarctic fur seal milk decreases significantly throughout lactation (Arnould and Boyd, 1995a) but comparable data are lacking on the specific carbohydrates present at various stages of lactation in otariids species.

A carbohydrate, lactose, that it is usually found in mammalian milk it been reported to be lacking or virtually absent in pinniped milk. A lack of the protein α -lactalbumin, which is an essential component of the lactose synthetase complex, would explain the lack of lactose in otariid milk. However, it is present at low activity in the milk of northern fur seals (Schmidt *et al.*, 1971) which suggests an altered α -lactalbumin molecule with low biological activity rather than its complete absence in the milk of otariids (Johnson *et al.*, 1972). The lack (otariids) or traces of lactose (phocids) in milk have been associated with the need for water conservation in pinnipeds (Peaker and Goode, 1978) and thus related to the evolutionary history of otariids and phocids and their adaptations for water conservation. Secretion of lactose into milk requires an obligatory movement of water to maintain isotonicity with other body fluids (Peaker, 1977) and a consequent loss of water from the mother.

An alternative explanation for the virtual absence of lactose in otariid milk could be attributed to their inability to digest this sugar. Intestinal lactase activity is common in mammalian species however, the activities of intestinal disaccharidases in some pinnipeds have been reported to be minimal (Kretchmer and Sunshine, 1967; Johnson *et al.*, 1972; Crisp *et al.*, 1988). For

instance, lactose intolerance in California sea lions pups and adults has been demonstrated (Sunshine and Kretchmer, 1964) although intestinal lactase activity has been shown in crabeater seal pups (Crisp *et al.*, 1988). However, it is perhaps more likely that the ability to digest lactose was lost as an adaptation to the primary lack of the sugar in the milk of pinnipeds. The secretion of lactose and the virtual absence of lactose in milk of some species of pinniped (Pilson, 1965; Peaker, 1977; Dosako *et al.*, 1983; Arnould and Boyd, 1995a; Urashima *et al.*, 2001a) demands further attention. Without doubt further investigation is needed to identify the specific carbohydrates and understand their role in pinniped milk secretion and as a source of energy.

Milk minerals

There are a great variety of minerals in milk present in a variety of chemical forms (Table 5). The major cations are sodium (Na), potassium (K), calcium (Ca) and magnesium (Mg) and the major anions are phosphorus (P) as phosphate, chloride (Cl) and citrate (Jenness, 1974; Peaker, 1977). Some of these and their concentrations in the milk of different species are shown in Table 5. A peculiarity of pinniped milk is that the ratio of Ca:P (0.5-0.9:1) is the inverse of the ratio in terrestrial mammals (1.6:1) (Peaker, 1977) (Table 5). The reason for the inverse Ca/P ratios warrants investigation.

The main ions in the aqueous phase of milk of terrestrial animals are Na^+ , K^+ and Cl^- (Peaker, 1977) and they have a central role in determining milk volume. The volume of milk secreted is determined by the amount of lactose secreted and a mechanism that maintains the concentration of the ions relatively constant. These solutes draw water into the alveolar lumina to maintain the isosmotic conditions between milk and blood (Peaker, 1977). What remains unclear is how pinnipeds in the virtual absence of lactose are able to control the secretion of the aqueous phase. It appears that in the absence of lactose that Na^+ , K^+ and Cl^- concentrations in pinnipeds are higher than in other mammals and the ratio of $\text{Na}^+:\text{K}^+$ is 1:1 for pinniped milk while in many other mammals it is 1:3 (Peaker, 1977). These data strongly suggest

that the mechanism controlling the secretion of water in pinnipeds is quite different from that in terrestrial mammals and that further investigation in this area is warranted.

General conclusion – Milk composition

It is evident that the milk composition of pinnipeds differs markedly from that of other species of mammal in two main areas, a) in the concentration of milk fat and b) in the virtual absence of lactose. These two main differences are a reflection of the lactating strategies of pinnipeds and their lactation physiology in which rapid transfer of milk energy to the pup and the conservation of water are crucial.

FACTORS THAT INFLUENCE MILK COMPOSITION

Lactation strategies and milk composition are such important aspects of the reproduction in pinnipeds that they have been the subject of many investigations (Bonner, 1984; Oftedal *et al.*, 1987a; Costa, 1991b; Oftedal, 1992; Boness and Bowen, 1996; Trillmich, 1996). The chemical composition of milk from most pinniped species has been analyzed however, the number of samples has often been very limited and how the composition varies in relation to factors such as stage of lactation, attendance pattern or maternal body condition, have not been fully elucidated. More attention has been given to the milk composition and the factors that influence the milk produced by phocids (Riedman and Ortiz, 1979; Lavigne *et al.*, 1982; Oftedal *et al.*, 1988; Baker, 1990). By contrast, although the composition of otariid milk has been studied for most species, there are greater uncertainties and controversies about the factors that are affecting its composition.

There are methodological factors in the data collection that obscure the understanding of the lactation strategies in mammals. Compilations of data on milk composition of several mammalian species have been published (Ben

Shaul, 1962; Jenness and Sloan, 1970; Linzell, 1972). However, little attention was given in these earlier reviews to critically evaluate the information presented (Oftedal, 1984b). The data in the literature on milk composition of pinnipeds, are often difficult to evaluate and must be interpreted with caution (Oftedal *et al.*, 1983; Oftedal, 1984b; Oftedal and Iverson, 1995). Many if not most of these studies include only a small number of samples, collected at unrecorded stages of lactation and suffer from bias due to the sampling regime. Errors due to incorrect analytical procedures and methodological difficulties may further complicate any interpretation of the data and interspecific comparisons (Oftedal, 1984b).

Milk composition and maternal characteristics

The lactation strategy in otariids involves periods of nursing ashore while fasting, which are alternated with foraging trips to sea to replenish energy reserves. Animals that fast for an extended period during the lactation period must face serious metabolic adjustments. These animals must minimize glucose use to reduce the catabolism of amino acids and thus spare them and tissue proteins, for other vital functions (Oftedal *et al.*, 1987a). Otariids produce milk with a low concentration of carbohydrates and with the primary and secondary source of energy being fat and protein, respectively (Table 4).

Pup growth will be promoted by good maternal body condition (with substantial reserves especially lipid in the form of blubber) prior to the initiation of lactation and with good nutrient intake during foraging trips at the initiation and during the duration of lactation (Arnould *et al.*, 1996b). Availability of food and maternal foraging success may play an important role in transferring energy to the pup while fasting, and even regaining energy while foraging (Arnould and Hindell, 2002). Consequently it is important to understand the relative contributions of maternal body mass, body condition, age and foraging success to changes in milk composition and yield in lactating otariids.

Body condition indexes have been widely used in pinnipeds for many reasons: as indicators of nutritional state, to measure the response to environmental perturbations, during moulting stage, to relate to reproductive success and growth (Costa *et al.*, 1989; Oftedal *et al.*, 1993; Arnould, 1995; Guinet *et al.*, 1998; Pitcher *et al.*, 1998; Georges and Guinet, 2000a; Georges and Guinet, 2001). Methods of estimating body condition have not been standardized, making interspecies comparisons difficult. One index of body condition is body mass divided by body length (Arnould, 1995), whereas a second method calculates the individual residual value of the linear regression between the body mass and body length (Guinet *et al.*, 1998). The latter index has proven to be a better predictor of body condition in otariids (Trites, 1992; Boltnev *et al.*, 1998; Trites and Jonker, 2000; Winship *et al.*, 2001; Arnould and Warneke, 2002)

In South American fur seals, drastic environmental perturbations, such as ENSO, changed the attendance and foraging patterns in the lactating females and their foraging success (Trillmich *et al.*, 1986a). The authors concluded that a scarcity of food prolonged the foraging trips and that most likely had a negative effect on milk quality and volume. The reduction in maternal foraging success may have decreased body weight and body condition as well as decreasing the benefit to cost ratio of foraging (Trillmich *et al.*, 1986a). Furthermore, failure in reproductive performance has been also reported in pinnipeds due to changes in body condition. Body condition in Cape fur seal (*Arctocephalus pusillus pusillus*) females influenced their ability to become pregnant or maintain pregnancy (Guinet *et al.*, 1998). Females of this species with better condition during pregnancy were more likely to be pregnant than females with low body condition. It was also found that even though nearly all sexually mature Steller's sea lion females were pregnant, poor body condition, as a result of nutritional stress, caused lower pup production in the subsequent season (Pitcher *et al.*, 1998). This indicates that food resources were not sufficient to support the energy demands of the reproductive strategy in this species. Similarly, when food resources were

scarce for Cape fur seals resulting in low body condition, pregnancy was likely to fail through abortion (Guinet *et al.*, 1998). In Antarctic waters, variation in food availability in any year has also been associated with low pup production in the following year for Antarctic fur seals around South Georgia (Lunn and Boyd, 1993).

In contrast, in years when food was plentiful pup production increased in the current breeding season because lactating females secreted more milk, which supported high pup growth and they also accumulated larger energy reserves and were in better body condition for the following season (Lunn *et al.*, 1993; Georges and Guinet, 2000b). An increased number of pups was most likely the result of an increase in the number of females in which embryos implanted and which carried a foetus to term (Lunn and Boyd, 1993). Lactating otariid females with low foraging success may spend more time at sea and therefore increase the chances of pup mortality due to malnutrition, hypothermia, trauma, or infection, and therefore reduce their reproductive success (Trillmich and Limberger, 1985; Ono *et al.*, 1987).

Since in other species variation in milk composition indicate the effects of environmental and physiological factors (Oftedal and Iverson, 1995; Martin *et al.*, 1998; Purushottam and Kiran, 2003), it is important to determine the relationships between these factors and their effects on milk composition in pinnipeds. Undoubtedly, body mass and body condition are influenced by individual foraging success and may be a proxy for the availability of local food resources. The concentration of lipid in milk has been correlated with maternal body mass in Australian fur seals (Arnould and Hindell, 1999) and Antarctic fur seals (Arnould and Boyd, 1995a), whereas no relationship was found in Australian sea lions (Kretzmann *et al.*, 1991; Gales *et al.*, 1996). However, body mass is to some degree determined by body length, and may not reflect the quantity of body reserves (Guinet *et al.*, 1998) and therefore body mass may not be an appropriate predictor of milk quality. This is further supported by the fact that in terrestrial mammals such as dogs (Oftedal, 1984a) and dairy cows, which vary in body size within the species, milk composition does not vary with body size (Wilson *et al.*, 1969; Agenäs *et al.*,

2003). Milk fat concentration has exhibited great variability in individuals, within species, and between species of pinnipeds however, body size is unlikely to account for this variability but rather physiological factors or other factors linked to ecological constraints. Either way, the answer is very likely to have a physiological basis (Iverson, 1993).

In humans and in dairy animals such as the cow and goat, body condition (e.g. cow body condition is scored) affects the lipid content of the milk (Brown *et al.*, 1986; Garnsworthy and Huggett, 1992; Cabiddu *et al.*, 1999). Similarly, lactating subantarctic fur seals in good body condition (body mass/body length) produced milk with a greater concentration of lipid (Georges *et al.*, 2001). Thus, indicating that variation in individual foraging success likely affects body condition and consequently milk quality in this species.

It is proposed that during nursing the pup, while fasting on land, milk is synthesised initially from the nutrients released from the newly digested food but as the flow of nutrients from the intestine diminishes there is an increasing dependence on nutrients mobilized from maternal body stores (Iverson, 1993). Therefore, one would expect that females with better body condition (measured from the relationship between body mass and body length) would produce milk with higher lipid content than females with lower body condition, as observed for subantarctic fur seals (Georges *et al.*, 2001). However this has not been tested in other otariids.

It could be suggested that female age may also influence foraging and reproductive success. Older females may be more experienced in finding food in years of poor food availability. They may also be better able to provide for the pup because they have reached maturity and the drive to divert nutrients towards their own growth is reduced. Thus older Antarctic fur seal and northern fur seal females had better reproductive performance, as indicated by greater natality rates, pups with heavier birth weights, births earlier in the season, and better chances of giving birth the following season, than younger females (Lunn *et al.*, 1994; Boltnev and York, 2001). In Antarctic fur seals

there was no apparent affect of maternal age on the time budget for foraging attendance (Boyd *et al.*, 1991); however, in years of poor food resources, the foraging time budget was adjusted (Boyd, 1999) which increased the cost of foraging in that year by 30-50% (Boyd *et al.*, 1994). This is consistent with the hypothesis that mothers adjust their behaviour to maximize energy delivery to the pup.

Body length has been used as an indirect measurement of age (Rosas *et al.*, 1993; Lunn *et al.*, 1994; Trites and Bigg, 1996; Georges and Guinet, 2001; Winship *et al.*, 2001). Maternal age estimated from body length, did not increase the concentration of fat in the milk of subantarctic fur seals (Georges *et al.*, 2001) however, it did in Australian sea lions (Kretzmann *et al.*, 1991) and Australian fur seals (Arnould and Hindell, 1999). Nevertheless, age estimation from body length has limitations since growth occurs at a progressively decelerating rate with age and the variation within a year class and the overlap between year classes is great. Therefore body length is not reliable to assign a pinniped to a particular age (Bryden, 1972; Bengston and Siniff, 1981; Rosas *et al.*, 1993; Trites and Bigg, 1996; Winship *et al.*, 2001) and the relationship between age of a lactating pinniped and the composition of her milk remains unclear.

Maternal age is very likely to influence body condition. Young lactating otariids females must store enough energy reserves and regain energy at sufficient rate, in a compensatory manner, to withstand the cost of lactation, the cost of their metabolic needs, and their body growth. Obviously, their smaller body size may limit the amount of energy stored in their body. The relationship between maternal age-body condition and maternal investment and milk composition needs urgent attention but is difficult to achieve in most free-living pinniped populations. For a species such as the NZSL for which age structure data are available for at least one breeding colony, it may be possible to measure the reproductive success of lactating females in relation to their age and consequently the effect on milk composition.

Conclusion – Maternal characteristics

The influence of body condition on milk composition in otariids has not been fully elucidated. Inter-annual changes in food availability are likely to affect body condition and as a result, influence the female's reproductive success and lactation performance. Attempts have been made to investigate the effect of maternal age on milk composition. However, for most species, age has only been estimated from body length of the female and it is now known that length is not a good estimator of age. Further work is required on this using samples collected from known age animals. It is possible that age acts indirectly on milk composition through body condition, but this hypothesis also needs to be tested further in a species for which there are data on animals of known age representing all age classes that are reproductively active.

Milk composition and attendance pattern

Interspecies differences in otariid milk composition might be explained by the hypothesis that females of a species making longer trips to sea will secrete milk with higher fat than those species that make shorter foraging trips (Trillmich and Lechner, 1986; Arnould and Boyd, 1995b). According to this hypothesis, the longer the foraging trip, the greater the energy content in the milk (Costa, 1991b). The latter is in concordance with the central place foraging theory that states that parents that have feeding grounds away from a central place (nest or breeding site) should make fewer trips, and return with a greater amount of energy per trip. While parents feeding close to a central place would make many short feeding trips and return with comparatively lower energy return per trip (Stephens and Krebs, 1986). This theory has been tested in birds and otariids (Costa, 1991a; Staniland *et al.*, 2007).

The high concentration of nutrients in the milk is sufficient to sustain the pup while fasting during its mother's absence (Gentry *et al.*, 1986; Arnould and Boyd, 1995b). This is true for subpolar species such as the subantarctic

fur seals, and temperate species such as the Juan Fernandez fur seals and the Guadalupe fur seals, all of which have amongst the longest foraging trips reported for any otariids (Figure 4 and Table 6). There must be physiological and reproductive advantages in producing milk with a high concentration of solids when absent for long periods at sea (Francis *et al.*, 1998; Ochoa-Acuna *et al.*, 1999; Georges and Guinet, 2000b). Firstly, the need for water is reduced, which puts less pressure on the female's own water balance, and secondly the mammary gland capacity is less likely to be a limiting factor for the production of a concentrate milk. The holding capacity of the mammary gland of any otariid is not known. A further mystery surrounds the ability of the mammary gland of the otariids to resume lactation after foraging trips of up to 12 days, which is without precedence for terrestrial mammals. It is not known what is regulating the secretion of milk in the absence of the stimulus of the suckling pup and milk removal. Experiments investigating the control of milk secretion in terrestrial mammals, in particular in dairy animals, indicate that autocrine factors and cell stretching may be important (Peaker and Wilde, 1987; Knight *et al.*, 1998) but they are yet to be investigated in pinnipeds.

In respect to lactation species that make long maternal foraging trips at sea face many obstacles. They may be limited by the amount of milk that the mammary gland is able to store, and they may be at risk of involution of the mammary gland as the suckling stimulus and milk removal are crucial for the maintenance of mammary gland function in other species (Ofstedal *et al.*, 1987a; Ochoa-Acuna *et al.*, 1999). The mechanisms by which involution of the mammary gland are constrained in the absence of the suckling stimulus, remains to be elucidated. Ofstedal *et al.* (1987a) suggested that mammary glands might have a large storage capacity coupled with low secretion of milk with a high solids content while at sea. This is consistent with Antarctic fur seals that had a weak negative relationship between foraging trip duration and rate of milk production while at sea, whilst a positive correlation was found between rate of milk production and duration of visit on land (Arnould and Boyd, 1995a).

Mammary glands size in pinnipeds is estimated based on the mammary glands weight relative to body weight, have indicated that most otariids have large mammary glands in comparison with terrestrial mammals (Ofstedal *et al.*, 1987a; Ochoa-Acuna *et al.*, 1999). Arnould and Boyd (1995b) measured the mammary glands capacity of Antarctic fur seals by complete manual evacuation and demonstrated that mammary glands were not necessarily full when the mother arrived ashore. There is a lack of information on mammary gland size and capacity in pinnipeds that needs to be addressed. The limited data available suggests the capacity of the mammary gland is not likely to limit foraging trip duration, rather it is limited by the mother reaching a set point of nutritional satiation (Gentry *et al.*, 1986).

The relationship between trip duration and fat content in milk applies between and within otariids species. The within species relationship between milk fat concentration and trip duration has been demonstrated in a few species. Arnould and Hindell (1999) found a significant relationship between milk lipid content and duration of the preceding foraging trip for Australian fur seals and a similar relationship was demonstrated for Antarctic fur seals (Arnould and Boyd, 1995b) By contrast trip duration and milk fat content were not related in studies carried out on Australian sea lions (Kretzmann *et al.*, 1991) and subantarctic fur seals (Georges *et al.*, 2001). The poor relationship found in subantarctic fur seals was thought to be a consequence of individual maternal foraging skills, and thus, the quality of the milk would have been determined by this factor (Georges *et al.*, 2001).

Australian sea lions conform with the hypothesis that species that make short foraging trips secrete relatively low fat milk (Trillmich and Lechner, 1986; Costa, 1991a) however, Cape fur seals do not. Australian sea lions inhabit a low energy marine environment, therefore, as an adaptive response they secrete low energy milk that provides a relatively low energy intake for their pups and necessitates a relatively lengthy lactation period (Kooyman and Trillmich, 1986). On the other hand, Cape fur seals have moderate foraging trips averaging 5.23 days and were expected to produce a milk higher in lipid

than 23.16% observed by Gamel *et al.* (2005). However, the milk was collected only in early lactation and it is not necessarily representative of the entire lactation period. A similar pattern to that seen in Australian sea lions is also seen in the Galapagos fur seals which produce low energy milk. The later species had mean absence duration of 1.3 days and a prolonged lactation period (Table 1 and 6). Duration of the foraging trips of lactating Galapagos fur seals seemed to adjust according to short term fluctuations of food abundance. Even though the absolute level of primary productivity of this water is unknown, it is lowest in summer due to reduced upwelling (Kooyman and Trillmich, 1986). Both temperate and tropical species, Australian sea lions and Galapagos fur seals respectively, have adopted a different strategy to polar species in that they are obligated to extend their lactation period. As otariid females depend upon their dietary intake to sustain lactation (Costa, 1991b), by adjusting their foraging trips to food availability they are able to sustain a long lactation period. This strategy is not necessary at higher latitudes where the marine environment has a burst of high primary productivity during the short summer season allowing otariids to forage successfully and complete lactation in a short period.

Subantarctic and Juan Fernandez fur seals breed at lower latitudes but do not follow the pattern seen in other otariids at similar latitudes in that they have markedly extended foraging trips with a mean of 15.9 and 12.3 days respectively (Figure 4, Table 6). However, they do conform to the relationship seen in other otariids between trip length and milk fat concentration in that they secrete milk with fat contents of 38.6 % and 41.4 %, respectively. The Guadalupe fur seal is the only species that has an attendance pattern, foraging cycles of 11.5 days at sea and 5 days on land (Table 6), similar to that of Juan Fernandez fur seals. In addition the two species secrete milk with the same percentage of milk fat and their lactation duration is similar (Francis *et al.*, 1998).

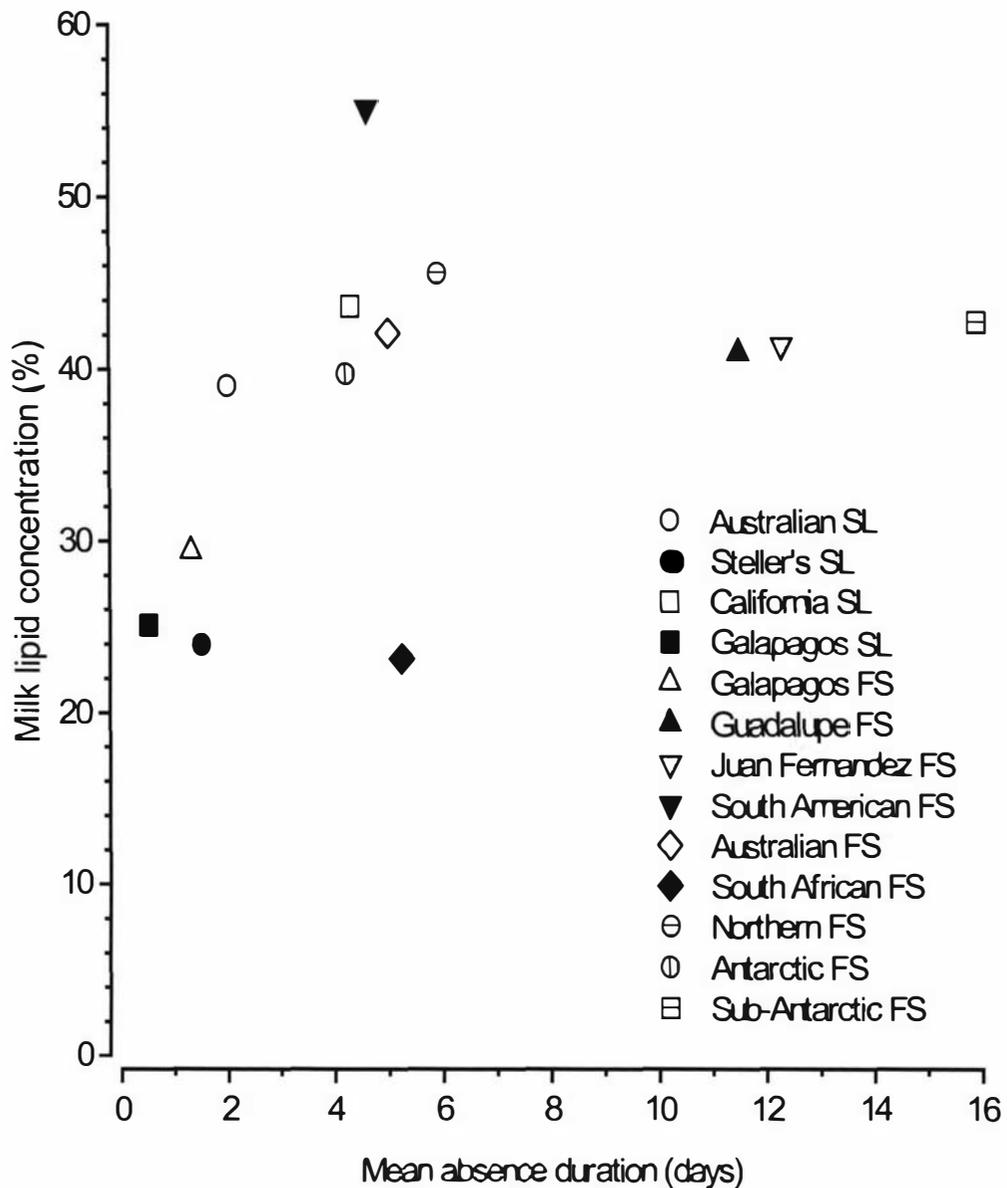


Figure 4. The relationship between foraging trip length and milk fat content in 13 species of otariids (Data from sources in Table 4 and 6). SL= sea lions, FS= fur seals.

Juan Fernandez and subantarctic fur seals have the longest inter-suckling intervals and highest milk fat content during the first month postpartum that have been reported for any otariids (Ochoa-Acuna *et al.*, 1999; Georges *et al.*, 2001). Both species leave their local low productive waters and travel long distances to waters of higher productivity (Francis *et al.*, 1998; Georges *et al.*, 2001). The similarity of the attendance patterns of Antarctic and subantarctic fur seals breeding at Macquarie island indicated

that prey availability might be playing a major role influencing pattern of foraging and attendance cycles (Goldsworthy and Crowley, 1999). This was also shown to be true for Juan Fernandez fur seals that had a correlation between foraging trip, visit duration ashore and primary productivity, indicating that food source location and availability were determining the foraging pattern (Francis *et al.*, 1998). Consequently, the lactation strategy adopted with long foraging trips is very likely to have consequences for milk production and composition.

It appears that long foraging trips are followed by long nursing bouts ashore. Some of the milk lipid secreted when nursing (while fasting) ashore could be obtained from body stores; hence the rate of milk production and consequently energy transfer to the pup must be largely influenced by maternal body lipid storage capacity. Furthermore, 42-79% of the milk energy transferred to the pups of Antarctic fur seals was obtained from body reserves of the mothers while they are on land (Arnould and Boyd, 1995b). At least for this species, the longer the duration of the foraging trip, the greater the proportion of milk energy delivered to the pup is derived from body stores (Arnould and Boyd, 1995b). It remains to be tested whether this is true for all fur seals and sea lions. The most advantageous strategy for lactating females to maximise nutrient transfer to the pups, would be to produce and store energy rich milk while at sea and deposit excess nutrients as body lipids and protein to be converted it into milk while nursing on land (Arnould and Boyd, 1995b). Then on land, the rate of nutrient transfer to the pup is facilitated by maximizing milk production and the concentration of solids in the milk. It appears that otariids species making long foraging trips have maximized their energy transfer to the pup by secreting nutrient-rich milk while those making shorter trips produce a less concentrated milk (Figure 4).

In previous research papers interspecific comparisons between milk lipid concentration and duration of foraging trip have been made between data from 14 species (Georges *et al.*, 2001), and eight species (Trillmich and Lechner, 1986; Costa, 1991a) and seven species (Ochoa-Acuna *et al.*, 1999). In such comparisons care must be taken to account for factors such as

lactation stage, sampling bias and analytical methods that can obscure the interpretation of the data on milk composition (Oftedal, 1984b). Therefore, in interspecific comparison, criteria must be used to select the available data and minimize incorporation of bias into the comparison. In some studies there was a linear relationship between the mean duration of the period of absence and lipid concentration (Trillmich and Lechner, 1986; Costa, 1991a; Ochoa-Acuna *et al.*, 1999), while Georges *et al.* (2001) demonstrated an asymptotic relationship between the two parameters. It must be noted that the latter authors did not include data from subantarctic, Juan Fernandez and Guadalupe fur seals that are absent for very long intersuckling periods (Figure 4). The conclusions drawn from the analyses that have not included these species should be interpreted with caution.

Conclusion – Milk composition and attendance pattern

It is likely that there is a strong relationship between milk composition and attendance pattern in otariids for those otariids species making long foraging trip at sea; however, more work is needed in this area. The relationship between milk composition and attendance pattern in otariids species making short foraging trips is less clear. Latitude, distance to foraging ground and availability of food are factors that are likely to determine the attendance pattern of lactating females. The limited data and the lack of standardization of sampling make interspecific comparisons difficult. The fact that lactating otariids are absent for the longest inter-suckling period of any of the mammals, make this group of animal special for testing the central place foraging theory (Stephens and Krebs, 1986).

Milk composition and stage of lactation

The general effect of stage of lactation on milk composition seems to be consistent across species (Cook *et al.*, 1970; Oftedal, 1984b; El-Sayiad *et al.*, 1994; Jacobsen *et al.*, 2004; Tsiplakou *et al.*, 2006; West *et al.*, 2007);

however, there are differences between species in the degree of change in the milk composition as lactation progresses.

Phocids

The fat content of the milk of phocids increases as lactation progresses and pup growth rate reflects the extent of this increase (Kovacs and Lavigne, 1986). The general trend in milk composition variation in phocid species studied so far indicates that there is a low fat content in early lactation but in mid to late lactation the fat concentration is considerably higher (Table 3). As with the offspring of other mammalian species, phocids energy demands increase as lactation progresses (Kovacs and Lavigne, 1986) but it is difficult to envisage how the increase in demand could drive the increase in fat concentration of the milk. Moreover, in some phocids species, while the fat content of milk in early lactation is low that of protein is more constant throughout lactation (Table 3). The low concentration of protein in most phocid milk is associated with a relatively small rate of gain in the young's lean body mass. For example hooded seals and bearded seals have the lowest protein concentration of any mammalian milk (Bonner, 1984; Oftedal *et al.*, 1988) and in hooded seal pups this correlates with a low gain in lean body mass (Oftedal, 2000). The relationship between milk protein content and growth rate, and the protein demand of the offspring, need to be further investigated in comparative studies in pinnipeds (Oftedal, 1986).

A few phocid species have been shown to follow a trend in which the concentration of water decreases and fat increases as lactation progresses (Riedman and Ortiz, 1979; Lavigne *et al.*, 1982; Tedman and Green, 1987; Iverson *et al.*, 1993; Carlini *et al.*, 1994; Oftedal and Iverson, 1995; Oftedal *et al.*, 1996). Similarly, in harp seal milk the protein content remained constant and milk fat content increased throughout lactation (Lavigne *et al.*, 1982; Stewart *et al.*, 1983; Webb *et al.*, 1984). The relatively high concentration of water in early lactation is probably an adaptation to provide water for the pup, which is unable to obtain sufficient water from lipid catabolism since an adequate blubber layer has not yet been deposited. Therefore, milk provides

free water to the pup when it is needed most, consequently, the decline in water concentration in milk will coincide with the time the young is less dependent on free water (Lavigne *et al.*, 1982).

The large weaning mass seen in phocids is a combination of the transfer of very nutrient-rich milk and the rapid deposition of energy in the form of lipid in the blubber. The milk energy output rates are far greater in phocids than in terrestrial non-fasting mammals (Oftedal, 1984b). This is possible because the large maternal body mass of phocids enables them to store large quantities of fat, hence phocids can sustain the expensive energy cost of lactation by mobilization of stored fat (Bowen *et al.*, 1992). However, some phocids species such as harp seals, Weddell seals, bearded seals and harbour seals feed at some stage during the lactation period (Bowen *et al.*, 1992; Oftedal, 2000). Harbour seals are known to feed from mid lactation onwards (Boness *et al.*, 1994), most likely because energy reserves are depleted and hence they are unable to sustain lactation (Bowen *et al.*, 1992). It appears that maternal size in harbour seals constrains the proportion of body fat that can be stored (Boness *et al.*, 1994). Furthermore, lactating harbour seals depleted 33% of their body mass during the first 80% of the nursing period, and depleted their body reserves faster than other phocids (Bowen *et al.*, 1992). A limited amount of energy stored coupled with rapid energy depletion during lactation cannot be sustained without feeding (Bowen *et al.*, 2001). It has been suggested that it is likely that half of the phocid species may feed during lactation (Boness and Bowen, 1996); however, whether this occurs only in the smaller phocid species is still to be investigated.

Conclusion - Phocids, milk composition and stage of lactation

Phocid seals have developed for a lactation strategy in which a great amount of energy is transferred in a short time and deposited as body fat. In combination with this, the rapid growth rate of pups allows a rapid deposition of fat in the blubber that is essential for insulation and providing post-weaning

energy reserves. The needs of the neonate seem to parallel the milk composition and most phocids appear to follow the same trends, although more studies in milk composition and effect of lactation stage on composition should be undertaken. The hypothesis that phocids fast for the entire nursing period can no longer be sustained. The smallest phocids species cannot withstand the cost of lactation and maternal metabolism while fasting and must resume foraging as lactation progresses. Consequently, it has been suggested that several of the phocids might have adopted an "otariid-like foraging strategy", but it remains to be resolved exactly how many species belong to this group.

Otariids

Data on changes in milk composition throughout the whole lactation period for six out of 16 otariid species have been investigated, such as the South American fur seals (Ponce de Leon, 1984), California sea lions (Ofstedal *et al.*, 1987a), Antarctic fur seals (Arnould and Boyd, 1995a), Australian sea lions (Gales *et al.*, 1996), Australian fur seals (Arnould and Hindell, 1999); and subantarctic fur seals (Georges *et al.*, 2001). Less complete, but otherwise useful, data are available from the northern fur seals (Costa and Gentry, 1986) Galapagos fur seals and Galapagos sea lions (Trillmich and Lechner, 1986). The general trend in these species is that milk fat concentration increases progressively whereas protein content remains fairly constant throughout the lactation period.

Increase in foraging trip duration related to stage of lactation and/or change in food availability (Boyd *et al.*, 1991; Higgins and Gass, 1993; Lunn *et al.*, 1994) may be responsible for the changes in milk composition throughout lactation. It is likely that there is a joint effect of increased foraging trip duration, and stage of lactation or pup age on milk composition. In subantarctic fur seals, for example, stage of lactation explained most of the variation in milk composition (Georges *et al.*, 2001). Whereas in Antarctic fur seals and Australian sea lions, stage of lactation accounted for only a small proportion of the variation in milk composition (Kretzmann *et al.*, 1991;

Arnould and Boyd, 1995a). Kretzman *et al.* (1991) and Gales *et al.* (1996) found large variability in milk lipid concentration between and within individual Australian sea lions. However, they were unable to identify which factors contributed most to the variation in milk composition. In Antarctic fur seals days postpartum and maternal mass contributed to the variation in milk lipid and it was suggested that foraging trip duration also explained some of the variation (Arnould and Boyd, 1995a). These authors have recognized that extensive and systematic sampling is needed in order to describe milk composition in otariids and control for intraspecific variation in milk composition (Kretzmann *et al.*, 1991; Arnould and Boyd, 1995a).

The general trend in otariid milk composition during lactation is that milk lipid and gross energy increases during the first stages of lactation and reaches a maximum at mid lactation and then tends to decrease during the later stages of lactation (Table 4). During lactation water content in milk varies inversely with milk lipid concentration and milk protein concentration remains fairly constant throughout the lactation period (Georges *et al.*, 2001). Although not all otariid species follow these trends. Galapagos fur seals for example produce milk that decreases in fat concentration with pup age in early lactation (Trillmich and Lechner, 1986). Thus during the perinatal period they rely on their body reserves to produce milk with a high concentration of fat. By doing this they save body water and allow the neonate to build an insulation layer and an energy reserve in the form of fat (blubber) for the oncoming period of fasting. When the mother begins to forage her water balance improves and she can produce a more dilute milk that enhances the pup's ability to be active in the high temperatures of the Galapagos (Trillmich and Lechner, 1986). This study did not incorporate data for the whole lactation period therefore it remains to be seen if the trends described for milk composition throughout lactation in otariids are consistent with those for Galapagos fur seals.

Changes in protein concentration throughout lactation have been reported for a few species of otariids. For instance in Antarctic fur seals,

protein concentration decreased in one year but increased in the following two years (Arnould and Boyd, 1995a) and varied little in Australian fur seals (Arnould and Hindell, 1999). It is likely that increases in protein content in milk during lactation may benefit the pup by the transfer of essential nutrients to the pup, however, whether the composition of the protein changes has not been reported. Notwithstanding, it was reported in Galapagos fur seals that the proportions of whey and casein proteins changed throughout lactation (Trillmich, 1988). For instance, of the total protein in milk, whey proteins constituted 40% and casein 60% in early lactation and 25% and 75% in mid-lactation respectively. The implication of the changes in the proportion of whey and casein of the total protein in milk of Galapagos fur seals has not been investigated.

Increases in lipid and energy content of milk in early lactation have been associated with increases in the foraging trips and/or the stage of lactation in Antarctic fur seals, northern fur seals and Australian fur seals and sea lions (Costa and Gentry, 1986; Arnould and Boyd, 1995a; Gales *et al.*, 1996; Arnould and Hindell, 1999; Goldsworthy and Crowley, 1999). It was postulated that the main reason for the increase in milk lipid was associated with an increase in foraging trips but in some species foraging length was not correlated with milk composition, which indicates that other factors must be operating. Increased milk lipid during early lactation could be associated with the recovering of maternal body condition, which is significantly depleted following a long and demanding peri-natal period (Trillmich, 1986a; Georges and Guinet, 2000a). Body condition had a significant effect on milk lipid in subantarctic fur seals (Georges *et al.*, 2001) but it is not known whether this applies to other otariids species. The increasing demands of the growing pup may affect the maternal response and increase milk fat concentration (Georges *et al.*, 2001).

By the last month of the lactation period, milk fat tends to decrease as shown in Australian sea lions and Australian fur seals, and subantarctic fur seals (Gales *et al.*, 1996; Arnould and Hindell, 1999; Georges *et al.*, 2001). In subantarctic fur seals the data suggested that the relationship between milk

fat concentration and stage of lactation at the end of this period was best described by an asymptotic relationship i.e. decrease in lipid content. The lower milk fat at the end of lactation and the transfer of maternal body reserves to the pup at a lower rate at the end of lactation may be explained by the pup weaning process and/or with competitive demands on maternal resources for both lactation and gestation (Arnould and Hindell, 1999; Georges *et al.*, 2001). This hypothesis was advanced for subantarctic fur seals in which it was proposed that at the end of lactation they are able to direct their body reserves towards gestation (after a period of delayed implantation) rather than to milk production (Georges and Guinet, 2000b).

For the Antarctic fur seals, the rate of milk production decreased by the end of lactation (Arnould *et al.*, 1996a; Arnould, 1997), however, a concurrent decrease in milk lipid concentration has not been reported in late lactation (Arnould and Boyd, 1995a). This could be explained by the fact that there is not a concurrent active gestation (delayed implantation) during their relatively short four month lactation period (Boyd, 1991). Therefore it is likely that this species may have less of a limitation on resources allocated to milk production and in that way milk lipid concentration in late lactation is not affected (Arnould and Hindell, 1999). It remains to be tested in other otariids species whether the late lactation decrease in milk lipid concentration is caused by the weaning process or the demands of gestation. One way to investigate how maternal resources are partitioned between lactation and gestation is to measure the milk production of otariids with different gestation demands in later stages of lactation.

Conclusion – Otariids – milk composition and stage of lactation

Data on milk composition throughout lactation in otariids are needed for all species. As a consequence little is known about what changes may occur throughout this period or what factors may cause these variations. It has been postulated that demands of the growing pup, gestation, weaning process and changes in attendance patterns may be some of the more important

influences on milk composition but there is little empirical data to confirm this idea. Indeed, there are many inconsistencies between studies in terms of methodology and interpretation.

General Conclusion – Factors that influence milk composition in pinnipeds

It is likely that the extent to which temporal and maternal factors affect milk composition varies across otariid species. For instance, it is not clear if maternal age influences milk composition, however body condition has proven to be a predictor of milk composition in otariids. Another important factor that determines milk composition is the maternal attendance pattern and this factor seems to be less important in species with short foraging trips compared with species with long foraging trips. Stage of lactation in phocids and otariids influence milk composition greatly; however, both groups have shown different milk composition trends throughout the nursing period. There is a great degree of inconsistency between studies that have tested factors that affect milk composition, and this is probably due to the lack of data on milk composition throughout the whole lactation period in otariids. Therefore, when possible, sampling regimes should cover the whole lactation period, and if not conclusions should be made with caution.

FATTY ACIDS: A SOURCE OF INFORMATION

In ecology, information about diet is an important key to understand trophic interactions within an ecosystem such as predator-prey relationships, the structure of food webs and the foraging behaviour of individuals (Paine, 1980; Sih *et al.*, 1998). Determining the diet of free ranging marine mammals is a task that is usually unachievable. Direct observation of cetaceans and pinnipeds foraging behaviour at the surface and at depth is impossible and indirect methods must be applied. The latter methods are based on the recovery of hard prey remains that are resistant to digestion. The hard prey

remains are obtained from stomach lavage, faeces, stomach contents from stranded or bycatch animals, and regurgitations (Pierce and Boyle, 1991). These methods are subject to numerous biases and these methods have many limitations (Pierce and Boyle, 1991).

A technique that has shown to overcome many of these biases is the use of fatty acid (FA) profiles of body tissues to infer the predator's diet (Iverson, 1993; Budge *et al.*, 2006). The seasonal and inter-annual shift in diet has been demonstrated in lactating Antarctic fur seals using fatty acid signature analysis (FASA) (Iverson *et al.*, 1997a; Lea *et al.*, 2002; Staniland and Pond, 2005). The diets of Antarctic fur seals and of southern elephant seal were differentiated by determining their milk FA signatures and comparing these to potential prey species (Brown *et al.*, 1999). Milk from black bears was also used to detect shifts in diet through FASA (Iverson *et al.*, 2001b). Therefore the potential of FASA to study the foraging behaviour of predators is well documented. Recent advances in FASA have shown that it is possible to quantify the diet of marine predators such as pinnipeds through quantitative FASA or QFASA (Iverson *et al.*, 2004).

FAs are the building block of lipids and comprise the majority of lipids found in all organisms. There are about 14 FAs that are commonly found in any marine lipids but in total around 50 to 70 FAs have been identified and quantified (Ackman *et al.*, 1988; Ackman, 1989).

The peculiarity of marine lipids is that they contain high levels of long-chain polyunsaturated FAs (PUFAs) (Ackman, 1982; Ackman, 1989) in some cases they contain unusual or novel FAs. Predators, such as pinnipeds, are monogastric animals and therefore the dietary FAs consumed are deposited into the adipose tissue (blubber) and/or milk with little or no modification (Roy *et al.*, 2005; Budge *et al.*, 2006; Kloareg *et al.*, 2007). As a consequence body tissues of pinnipeds are high in PUFA and unusual FAs that only originated from the diet. Furthermore, these dietary FAs deposited in the predator tissues can be distinguished from those FAs that are biosynthesized by the

animal. Therefore the main advantages of FAs to infer diet are that it does not rely on the recovery of hard parts of prey and therefore prey without hard part are also identified. In addition, information about the recent or past diet consumed can be obtained depending on the samples analyzed, e.g. blubber, milk, blood.

This section of the review will be limited to discussion of the potential and limitations of FA techniques to identify diet in pinnipeds and the effect of diet on milk lipid composition in pinnipeds. For more details about FA techniques in general, Budge *et al.* (2006) have presented an excellent review about the study of the trophic ecology of marine ecosystem with particular emphasis on marine mammals and pinnipeds. The review includes details about the laboratory analysis and other important considerations.

The study of the foraging ecology and food webs can be approached in three ways. Firstly, the use of individual FAs as biomarkers or tracers such as an unusual FA found in a predator that can be trace to a single prey species. Secondly, determination of the FA composition of the prey in which an array of fatty acids is analysed to obtain a signature of the prey and then this is matched with the FA signature of the predator. The most recent approach is to use of signature FAs to quantitatively estimate the proportion of prey species in the diet of the predator by QFASA. Most FAs studies that infer diet in marine predators from body tissues have been qualitative, (Smith *et al.*, 1996; Iverson *et al.*, 1997a; Raclot *et al.*, 1998; Bradshaw *et al.*, 2003; Olsen and Grahl-Nilsen, 2003) by using FASA; however, more recently quantitative and semi quantitative analysis of the diet has been conducted by using QFASA (Cooper *et al.*, 2003; Iverson *et al.*, 2004; Cooper *et al.*, 2005).

FAs as biomarkers or tracer of diet

The presence of high concentrations of the long chain 20:1 and 22:1 in the oil of the North Atlantic fin whale (*Balaenoptera physalus*) separated this species geographically from those in the Antarctic (Ackman and Eaton, 1966).

An unusual methyl branched FA present in the blubber of sperm whale, *Physeter macrocephalus*, was also found in the lipids of ocean sunfish (Pascal and Ackman, 1975). The use of FAs as tracers is not exclusive to studies of food chains in marine mammals and has also been applied in other marine species.

The trophic relationship between seven species of shellfish and two species of squid were elucidated by the presence of an anomalous non-methylene interrupted FAs (NMIFAs) (Paradis and Ackman, 1977). The authors suggested that these unusual FAs could have greater potential to be used to study trophic relationships in marine food webs. The NMIFAs, which are synthesized by mollusc and benthic marine invertebrates (Paradis and Ackman, 1977), are also present in the lipid of their predators, bearded seals (*Erignathus barbatus*) and Pacific walruses. The NMIFAs were traced from bearded seals to adult polar bears indicating the diet preference of the polar bear (Thiemann *et al.*, 2005).

The unusual occurrence of elevated concentrations of FAs with odd chain lengths in adult smelt (*Osmerus mordax*) in winter was a result of consuming the amphipod, *Pontoporeia femorata* (Paradis and Ackman, 1976b; Paradis and Ackman, 1976a). Furthermore, it was found that these "odd chain FAs" varied with season and geographical locations of the smelt (Paradis and Ackman, 1976b) and in mullet (*Mugil cephalus*) (Deng *et al.*, 1976). Addison and Ackman (1970) suggested that the presence of odd chain FAs in these two species, indicated that they are feeding on the same prey, amphipod.

The presence of an unusual FA (octapentadecaenoic acid) found in marine dinoflagellates could be used as a tracer since it was present in various species of herbivorous copepods and carnivorous chaetognaths (Mayzaud *et al.*, 1976). For instance, some north Atlantic copepods synthesise a unique monounsaturated 22:n1n-11 and large concentrations of 20:1n-9 in fatty alcohols (wax esters) which when these FAs were traced to

the lipids of capelin, sand lance (*Ammodytes americanus*), Atlantic herring (*Clupea harangus*), and mackerel, (*Scomber scombius*) allowed the copepods to be identified as a food source for the fish (Pascal and Ackman, 1976; Ratnayake and Ackman, 1979; Ackman *et al.*, 1980).

A feeding experiment with Arctic copepods supported the idea of using FAs as tracers. The experiment consisted of feeding herbivorous copepods (*Calanus finmarchicus*, *C. hyperboreus* and *C. glacialis*) with diatoms (*Thalassiosira antarctica*) or a dinoflagellate (*Amphidinium carterea*) depending on the FAs composition of the copepods (Graeve *et al.*, 1994). The experiment demonstrated that a specific FA (16:1n-7), present in low levels in some copepods but high in the experimental diet, increased significantly in the copepods after being feed for 47 days (Graeve *et al.*, 1994). The predominant prey of ocean sunfish (*Mola mola*) was identified due to the presence of an unusual trans-6-hexadecenoic acid previously found in jellyfish (Hooper *et al.*, 1973). Interestingly this unusual FA tracer was originally found in the Atlantic leatherback turtle, *Dermochelys coriacea coriacea* (Ackman *et al.*, 1972), which also preys on jellyfish.

FASA: Fatty Acids Signature Analysis

FAs from prey that are >14 in carbon chain length are deposited in the predator tissue with little or no modification (Iverson, 1993). So instead of using one or a few unusual FAs from prey we can consider an array or signature FAs from the predator's tissue that should mirror, to some extent, the signature of the major items of prey in the predator's diet (Iverson, 1993). Furthermore, although both methods, FASA and FAs as tracers, can potentially identify various prey species, the advantage of using FASA is that the relative contribution of various preys to the overall diet can be assessed. Another important issue when using FAs as a tracer, and that has been overcome with FASA, is that if the prey does not contain an unusual FA that can be traced from the predator then information is potentially lost. An array of FAs, is potentially more useful because the FA signature can differ

significantly from prey to prey and the risk of losing prey information can be substantially minimized. FASA have been used to study the foraging ecology of pinnipeds and other marine species such as penguins, fish, cetaceans, black and polar bears (Iverson *et al.*, 1997a; Iverson *et al.*, 1997b; Kirsch *et al.*, 1998; Raclot *et al.*, 1998; Brown *et al.*, 1999; Kirsch *et al.*, 2000; Nozeres *et al.*, 2001; Bradshaw *et al.*, 2003; Grahl-Nielsen *et al.*, 2003; Iverson *et al.*, in press)

When using FASA there are certain aspects that should be taken into consideration, for instance, not all FAs are informative of diet, because there are certain FAs that are synthesized *de novo* by the predators. Iverson (1993) grouped FAs into three categories by whether they were *de novo* by the predator or could only originate from the diet ("indicators FAs"). These categories are according to Iverson (1993):

- I. FAs that are synthesized *de novo* by the predator and include short and medium FAs <C14 and also C16:0, C16:1, C18:0 and C18:1.
- II. FA that could be synthesized *de novo* but are most likely originated from the diet and includes FA C14:0, C20:1 and C22:1.
- III. FAs that could only originate from the diet including C18:2n-6, C20:1n-11, C20:5n-3, C22:1n-11, omega 3 and omega 6 FA, and many unusual FAs.

The FASA implies that a large number of FAs of the predator are used to compare or match to the signature of FAs of the prey. While simple in concept, Smith *et al.* (1997a) pointed out some potential problems for statistical analysis. By combining FASA and classification trees analysis, they introduced a new tool to study the foraging ecology of the predators. Classification trees analysis or Classification and Regression Tree (CART) has been used extensively in the analysis of FAs signature of pinnipeds (Iverson *et al.*, 1997a; Iverson *et al.*, 1997b; Brown *et al.*, 1999; Walton *et al.*, 2000; Lea *et al.*, 2002). However, the use of CART is controversial (Smith *et al.*, 1997b; Grahl-Nielsen, 1999; Smith *et al.*, 1999), and even further it has been argued that FA from predators cannot be used to infer diet (Grahl-

Nielsen and Mjaavatten, 1991; Grahl-Nielsen *et al.*, 2000; Grahl-Nielsen, 2001; Olsen and Grahl-Nielsen, 2003).

QFASA: Quantitative Fatty Acids Signature Analysis

FASA has been used to study trophic relationships, spatial and temporal differences in diet within and between species, in a qualitative manner. Whereas QFASA have been used by using the FAs signature of prey and predator, to estimate the predator's diet in a quantitative manner. QFASA was recently introduced by Iverson *et al.* (2004) and the method basically applies a statistical model that estimates the predator's diet by comparing the FAs signatures of potential prey with the FAs signature of the lipids of the predator. The model takes into consideration how the prey FAs are metabolized and deposited in the predator's tissue.

Iverson *et al.* (2004) pointed out that four fundamental requirements are needed in order to accurately estimate the predator's diet as follows:

- I. A quantitative statistical model and assessment of its performance that includes the potential for improvement.
- II. To understand and take into account the lipid metabolism and deposition in the predators tissues.
- III. Detailed information on the lipid content and FA composition of each potential prey, for this to be achieved adequate sampling and analysis is required and in addition to what extent to which prey species can be differentiated by their FAs signature should be determined.
- IV. Adequate sampling, storage and analysis of FA predator tissue to obtain accurate quantification of all FAs and precursors (for detailed information see Budge *et al.* (2006).

QFASA works in the following way, it applies *weighting factors* ("calibration coefficients" that take into account the way FAs are metabolized and deposited in the predator's tissue) to individual predator FAs. Thereafter the model calculates the average FA signature of each prey species and

takes the *weighted mixture* of the FAs signatures of prey, and then selects the weighting that minimizes the statistical distance between the prey species *weighted mixture* and the *weighted predator* FA signature. To estimate the proportional contribution of prey to the predator's diet, the average FA signature of each prey species are corrected to account for differences in fat content, and FA contribution between prey types. More specific details about the statistical model for QFASA, its applications and how it works can be found in Iverson *et al.* (2004), and Budge *et al.* (2006). QFASA has already been applied to estimate diet of predators including harbour and grey seals, Steller's sea lions, Hawaiian monk seal, mink and seabirds (Iverson, 1998; Cooper *et al.*, 2001; Iverson and Springer, 2002; Cooper, 2004; Iverson *et al.*, 2004; Beck *et al.*, 2005; Cooper *et al.*, 2005; Iverson *et al.*, 2005; Nordstrom *et al.*, 2005; Tollit *et al.*, 2006; Beck *et al.*, 2007). However, the general consensus is that more work remains to be done to improve its performance (Iverson *et al.*, 2004; Budge *et al.*, 2006). Despite the potential of QFASA, it has only been validated in grey seals (Iverson *et al.*, 2004; Cooper *et al.*, 2005), Hawaiian monk seals (Iverson, 1998; Iverson *et al.*, 2003; Iverson *et al.*, 2005) and Steller's sea lions (Tollit *et al.*, 2005) and thus validation of the method and determination of "calibration coefficients" in captive feeding experiments are needed for other pinnipeds.

The application of QFASA to quantitatively estimate predator's diet has received considerable criticism by Grahl-Nielsen *et al.* (2004) who claim that the creation of correction factors to account for the deposition of individual FAs into the predator's tissue is unrealistic. Grahl-Nielsen *et al.* (2004) suggested that since Iverson *et al.* (2004) found differences in the correction factors between grey seals and harp seal fed on the same diet that the correction factors vary according to the prey and predator. Consequently Grahl-Nielsen *et al.* (2004) reject the suggestion of Iverson *et al.* (2004) that estimates of calibration factors from the grey and harp seal feeding experiments could be used to estimate the diet of other predators. This issue is acknowledged by Iverson and colleagues, but they suggest that provided different predators have similar physiological and biochemical characteristics

then if they consume the same diet FA deposition and synthesis, while not identical, will be similar (Iverson *et al.*, 2004; Iverson *et al.*, 2005). Furthermore, they were confident that the calibration coefficients determined to account for the effect of the metabolism of one predator on FA deposition, could be used as a baseline for another predator. This, however, was not to be considered a definitive solution and further work would be needed to determine a set of calibration coefficient that is most suitable for given predators (Iverson *et al.*, 2004). Moreover, a feeding experiment with captive Steller's sea lions and Hawaiian monk seals has demonstrated that the calibration coefficients estimated for grey and harbour seals but could not be applied in Steller's sea lions or Hawaiian monk seal (Beck *et al.*, 2005; Iverson *et al.*, 2005; Tollit *et al.*, 2005; Tollit *et al.*, 2006).

There are also concerns about the possible effects of the prey's energy density, which varies in relation to its chemical composition and lipid content (Grahl-Nielsen *et al.*, 2004). The lipid content of prey can vary with age, reproductive status, and in a spatial and temporal scale. In addition, the digestive efficiency of pinnipeds changes according to the total lipid intake (Rosen and Trites, 2000; Trumble *et al.*, 2003). There are a series of factor associated with predator's age, activity level, food intake, body condition and health that may be relevant (Grahl-Nielsen *et al.*, 2004). In this regard, sex and seasonal and geographical differences in the diet of grey seals and Hawaiian monk seals have been found to have an effect (Bowen *et al.*, 2005; Iverson *et al.*, 2005). While the usefulness of QFASA has been questioned (Grahl-Nielsen *et al.*, 2004; Thiemann *et al.*, 2004), the overall consensus is that QFASA has the potential to be a useful method to quantify the composition of the diet of predators and that more work is needed (Grahl-Nielsen *et al.*, 2004; Iverson *et al.*, 2004; Cooper *et al.*, 2005; Nordstrom *et al.*, 2005; Tollit *et al.*, 2005; Tollit *et al.*, 2006).

Fatty acid analysis of predator tissues

Blood serum lipid fatty Acids

Blood serum is the medium for transporting lipids between the different organs and tissues. Long chain fatty acids, in the diet mainly in the form of triacylglycerol (TAG) (represent the common form of storage lipids and make up the majority of lipids found in adipose tissues and blubber), are digested, absorbed by the intestinal epithelial cells, re-esterified to form TAG, which are released into the lymphatic system with lipoproteins as chylomicrons. The lymphatic system empties the chylomicrons into the blood circulation which delivers the chylomicrons to all the tissues. Fatty acids carried by chylomicrons in the blood are then removed by tissues such as blubber and lactating mammary gland. However, not all FA are carried in the chylomicrons, short-medium chain FA <C14 are transported to the liver where they are oxidized (Nelson, 1992). The FA composition of TAG in chylomicrons reflects that of the diet (Ockner *et al.*, 1969; Harris *et al.*, 1988; Grundy and Denke, 1990; Gibney and Daly, 1994; Lambert *et al.*, 1996; Summers *et al.*, 2000). There are few data available on lipid digestion and metabolism in pinnipeds (Bailey *et al.*, 1981; Cooper *et al.*, 2003; Cooper *et al.*, 2005). The FA composition of the serum of Harp seal pups after weaning and during fasting reflected that of their own blubber (Bailey *et al.*, 1981). Further, Cooper *et al.* (2005) show that FA signatures of the chylomicrons reflected that of the diet. In both investigations, the authors acknowledged that not only diet or blubber contributed to the FA in blood lipid but other inputs from endogenous sources or loss via peroximal β -oxidation should be considered.

Because dietary long chain FA can be metabolised during absorption it is clear that this could the incorporation and metabolism of FA prior to deposition will affect the relationship between FA composition of the diet and adipose tissue and milk (Innis, 2004; Fleith and Clandinin, 2005). Recent work with grey seals has attempted to investigate the metabolism of FA prior to deposition (Cooper *et al.*, 2005). The main findings of this study were that

individual dietary FAs differed in their metabolism and rate of incorporation into the blood chylomicron and this may lead to differences in FA signature between the chylomicrons and the diet. These differences were minimal 3hrs post-feeding and this is important because by analysing the FA signatures in blood lipid the most recent meal of a seal can be determined. The potential use of QFASA with chylomicrons to infer diet was tested by Cooper *et al.* (2005) with juvenile non lactating grey seals and shown to be accurate when the differences in metabolism of different dietary FA were considered. What remains to be investigated is the relationship between dietary FAs, blood lipid, and milk FA in lactating pinnipeds and in particular otariids.

Milk fatty acids

Capital breeders (phocids), in contrast to otariids, fast during their lactation period and thus rely on the lipid storage (blubber) for the production of milk lipids. Therefore milk FAs are derived entirely from the mobilization of fat reserves (blubber), hence the milk FAs should mirror the FAs of the blubber (Iverson *et al.*, 1995). In contrast, milk collected at the arrival of an otariid mother to the colony should reflect at least in part the food consumed during the preceding feeding period (Brown *et al.*, 1999; Lea *et al.*, 2002). Whereas in the period of fasting most of the energy is derived from body reserves (blubber) and therefore milk and blood fatty acids should reflect the fatty acids of the blubber (Iverson, 1993; Iverson *et al.*, 1995).

There has been considerable research on lactating women to test theories about the effect of diet on the composition of milk FAs (Fleith and Clandinin, 2005). Mobilization and transfer of adipose tissue FA into the milk and endogenous synthesis of FAs that were incorporated into the milk have been demonstrated in lactating humans (Insull *et al.*, 1959; Finley *et al.*, 1985). The general consensus is that diet affects the FA profile of women's milk (Sanders and Reddy, 1992). Most studies have measured the changes in milk FAs on a long term basis (days) (Insull *et al.*, 1959; Mellies *et al.*, 1979; Harris *et al.*, 1984; Finley *et al.*, 1985; Silber *et al.*, 1988; van Beusekom *et al.*, 1990; Sanders and Reddy, 1992) while a few have study the changes within

hours (Hachey *et al.*, 1987; Francois *et al.*, 1998). Francois *et al.* (1998) demonstrated that specific fatty acid from the diet (vegetable oil and fish oil) given to lactating women appeared in the milk within 6 hours and remained significantly elevated for up to 72 hours. These findings support the idea that there is a rapid transfer of dietary fatty acids to women's milk. There are no comparable data for pinnipeds.

A long term feeding trial with two independent groups of captive lactating minks (*Mustela vison*) feed with different diets prior to and during the lactation period was conducted to determine the effect of dietary neutral lipids on the milk FA composition (Wamberg *et al.*, 1992). The milk of lactating mink was significantly different between the two dietary groups as shown by the different ratios of polyunsaturated and monosaturated fatty acids, whereas the changes over time in the classes of fatty acids was less obvious. Plasma samples collected during the feeding trial contained large amounts of long chain fatty acid that must have been derived from the dietary fat. These results confirmed that milk fatty acids were influenced by the dietary fatty acids. The sampling regime in this experiment meant it was not possible to estimate the time course for uptake of dietary FA into the milk; however, the large concentrations of long chain fatty acids in the plasma indicated that there was a rapid uptake from the intestine.

A recent investigation by Staniland and Pond (2004) has given some indication that the influence of dietary fatty acids on human and mink milk may also be true for otariids. The study consisted of a feeding trial in which a diet composed of krill and ice fish was fed to lactating Antarctic fur seals for 4 days and then the females was suckled for 3 days by the pup. Their experiment was intended to replicate an average foraging trip. Milk was collected every 12 hours during 3 days (after the 4 days of feeding) and the samples analysed for FAs. There were no significant differences in the milk FAs between collection times; however, there were significant differences between experimentally fed females (diet high in fish) and naturally feeding females (diet predominantly krill). These findings apparently conflict with the feeding experiment conducted

in lactating women. There are potential sources of variation that may have contributed to these results such as the sampling regime and feeding time was not adequate to detect a significant change in the milk fatty acids and that the difference in fatty acid signature between the fish (fed to the fur seal) and the krill were very similar. Detailed information on the turnover rates of dietary FAs within the mammary gland and the contribution of body tissues such as blubber are needed to interpret Staniland and Pond's (2004) results correctly. Ideally these questions should be addressed with feedings experiments with captive and free living animals.

Blubber fatty acids

Blubber as a fat/energy depot is important for two reasons. In the adult, since blubber FAs are derived from the diet and endogenous synthesis, they are expected to reflect at least in part dietary fatty acids. The second aspect is that the blubber of the pup is an energy reserve for the period post-weaning in phocid pups and during the fasting period between the foraging trips of the mothers of otariid pups. Since the only source of food for the pups is the milk then the FAs in their blubber should also reflect the FAs in the milk and any endogenous synthesis. Most researchers have focused their studies testing these theories on phocids while very little is known about otariids.

There is controversy about whether FA in blubber can be used to infer the diet of the adult or whether milk FA are deposited in the pup's blubber with little modification (Iverson *et al.*, 1995; Grahl-Nielsen *et al.*, 2000; Grahl-Nielsen, 2001). There are a number of factors that may affect the composition of the FAs of the milk such as the metabolism of dietary FAs within the mammary gland and blubber and endogenous synthesis of FAs in pinnipeds. However, it is well established that all monogastric mammals modified the pattern of FA in a way that is similar and predictable (Nelson and Ackman, 1988; Nelson, 1992), and thus are applicable to pinnipeds. However, it must be mentioned that the FA signature of the predator will be influence by the endogenous *de novo* synthesis of certain FAs while specific FAs may

deposited at different rates and digested specific FAs may be metabolized prior to deposition (Cooper *et al.*, 2003; Iverson *et al.*, 2004).

Hooded seal mothers lactate for 4 days and newborns are born with considerable amounts of adipose tissue in comparison with other mammals (Iverson *et al.*, 1995). The FAs composition of the adipose tissue of lactating hooded seal differs from that of the newborn and from the FA composition of her milk (Iverson *et al.*, 1995). At the termination of lactation in hooded seal the FA composition of the pup blubber was almost identical to that of its mothers' milk (Iverson *et al.*, 1995). Grahl-Nielsen and Mjaavatten (1991) undertook a feeding experiment with captive adult grey seals and harbour seals to compare the FA composition of the blubber with that of the diet. The FA composition of the diet was not reflected in the blubber and it was concluded that the FA composition of blubber of the seals could not be used to infer diet (Grahl-Nielsen and Mjaavatten, 1991). However, the seals were feed constantly and were not fasted to deplete the body reserves so that dietary FAs may have been catabolised and not deposited in the blubber (Iverson, (1993). The issue needs to be resolved with further experiments.

Similar and contrasting results were found in lactating grey seal leading to further confusion and controversy. Although, Grahl-Nielsen *et al.* (2000) found that the FA composition of the blubber of lactating grey seals differed from the milk FA, they did observe a systematic difference between the FA composition of the pups' blubber and that of the milk contrary to the results reported by Iverson *et al.* (1995) in hooded seals. Grahl-Nielsen *et al.* (2000) suggested that the differences in the results are due to a low number of mother-pup pairs and a simple statistical analysis in the investigation by Iverson *et al.* (1995). While Grahl-Nielsen *et al.* (1999) have challenged the application of of FA technique to study the diet of marine mammals, Staniland and Pond (2004) suggested that the amount of blubber collected is a source of bias in another study by Grahl-Nielsen *et al.* (2000).

Furthermore, a vertical stratification of FAs in the blubber of southern elephant seals such that the relative proportion of FAs in the inner layer and outer layer of the blubber differed with dietary FA found in higher proportions in the inner layer have been reported (Best *et al.*, 2003). Less obvious differences between the inner and outer layers have been reported in other species of phocids (Kakela *et al.*, 1993; Fredheim *et al.*, 1994; Kakela and Hyvarinen, 1996). Clearly there is differential mobilization and deposition of certain FAs into different layers of the blubber that may confound the interpretation of dietary composition from the analysis of a whole layer of blubber by obscuring a greater turnover of FAs in the inner layer. It was possible to describe the foraging ecology of southern elephant seals from an analysis of the blubber FAs (Bradshaw *et al.*, 2003). Further research is needed on the level and magnitude of stratification of FA in the blubber in addition to turnover rates of FA in the blubber of other species of phocids so that diet can be more accurately inferred from the blubber FA.

There has been considerable information about the anatomical distribution and structure of adipose tissue in phocids whereas little is known in otariids. Vertical stratification in phocids blubber is evident (Kakela *et al.*, 1993; Fredheim *et al.*, 1994; Kakela and Hyvarinen, 1996; Best *et al.*, 2003) and it has been suggested that the blubber is a continuous subcutaneous layer that covers the body core and that the mobilization of FA have been demonstrated to occur uniformly around the body (Nordy and Blix, 1985; Slip *et al.*, 1992; Beck and Smith, 1995).

There are data indicating significant vertical (depth) stratification and non-uniform distribution of blubber in two otariids species, Cape fur seals (Arnould *et al.*, 2005) and Steller's sea lions (Wilson *et al.*, 2005). There were differences in the proportion of monounsaturated, saturated and polyunsaturated FA between the inner and outer of the blubber of adult Cape fur seals females (Arnould *et al.*, 2005). Likewise, differences in the concentrations of many FAs were observed between blubber layers (Wilson *et al.*, 2005). Arnould *et al.* (2005) also reported regional differences in the proportions of FAs between the blubber from the neck, rump and mammary

gland area. The higher proportion of total monounsaturated and oleic acid (18:1n-9) in the blubber sampled from the mammary gland in comparison with blubber from the rump and neck suggested that FA are deposited and mobilized differently around the body in otariids. These findings indicate the need to determine the part of the body that is the most appropriate for tissue sampling in studies using FA profiles of blubber to infer dietary intake of FA (Arnould *et al.*, 2005; Wilson *et al.*, 2005).

General Conclusion – Fatty Acids: a source of information

The diet of predators can be traced from predator's tissues by using particular FAs that act as tracers or by using the whole FA profile. The later has shown to be a better method to infer diet. Most studies have estimated the diet of pinniped in a qualitative manner via FASA of blubber or milk. Recently a new method has shown that the diet can be determined in a quantitative manner by QFASA. For QFASA to be effective data such as the rate of the deposition and metabolism of FAs in the predator's tissue are required. Until the full potential of QFASA can be explored more work should focus on understanding the metabolism, deposition and turnover rates of FA that have received so little attention in published research.

GENERAL DISCUSSION

Three distinctive lactation strategies have been described in pinnipeds. The shortening of the lactation period by phocids has the advantage of reducing the time the pup is exposed to the hazards of the terrestrial environments. The daily energy output in the form of milk in phocids is greater than in otariids due to the limited time to transfer nutrients and energy to the pup. As a consequence phocids produce milk with very high concentrations of fat. The milk composition in phocids is also a direct response to reduce the effect of water stress in the fasting lactating female. Nevertheless, it seems that some phocids have adopted a strategy similar to that seen in otariids and feed during lactation probably due to the high cost per day during the short

lactation. An aquatic lactating strategy has been adopted by odobenids, in which the mother nurses the pup in the aquatic environment. By doing this maternal care is extended and increasing the chances of survival of the offspring. Otariids have extended their lactation period and the growth of the pup is gradual rather than accelerated as seen in phocids. The former produce a lower milk fat concentration and the daily energy output is less than in phocids. Milk fat concentration varies greatly among pinniped species and it is likely that such variation in milk fat concentration and the absence of lactose in otariid milk is a consequence of their lactation strategy and evolution.

The factors that affect milk composition throughout the lactation period in otariids have been identified. Factors such as stage of lactation, attendance pattern, and maternal body condition are predictors of milk composition in otariids, but because data throughout the nursing period are lacking in this group little can be said and conclusions about interspecies comparisons should be made with caution. Data on milk composition should be used from mid lactation, since milk secreted is at peak maximal production (Oftedal, 1984b). Most studies used milk fat concentration from early lactation. Studies on milk composition throughout the whole lactation period in otariids are scarce. Collecting milk samples from otariids generally occurs during the breeding period since animals are easy to access during this time. Furthermore, interspecific comparison must be restricted to species for which similar data are available (Gales *et al.*, 1996). Comparisons of the milk composition of pinnipeds are difficult due to lack of data and the poor quality of the data, which in most cases are taken from the early lactation phase, have a small sample size and are limited by the sampling timing. Until biases are minimized and sampling techniques are standardized, interspecies comparison should be interpreted cautiously. Additional data must be incorporated into the analysis for those species that have not yet been investigated. More attention should focus on the mechanisms by which otariids are able to change their rate of milk production and milk composition in relation to their maternal foraging strategy.

There is a great gap in the information regarding milk composition and the factors that determines its composition, especially in otariids. The collecting of such information will not come easily since most otariids are only accessible during the breeding season and in many cases they occur in remote places. Notwithstanding, great efforts have been made in the past to study the lactation of pinnipeds but a great deal remains to be elucidated. The composition of milk of NZSL has not been determined nor the factors that affect its composition. These and other aspect such as milk intake and milk energy output and lactation strategies carried out should be investigated in NZSLs. For instance, NZSLs live in an intermediate environment between high and low latitudes, travel long distance to their foraging ground (similar to subantarctic fur seals) but their foraging cycle duration is short (similar to Australian sea lions) and their lactation period is long relative to their latitude. For these reasons the NZSL is of special interest to test theories about the evolution of lactation strategies in pinniped.

How diet affects milk composition in NZSL has also not been tested. The effect can be twofold. Firstly it can affect the fat concentration in milk, and secondly the FA composition of the lipid. It has been hypothesized that changes in diet were responsible for the inter-annual variation in milk fat concentration in Antarctic fur seals (Lea *et al.*, 2002). This hypothesis has yet to be tested. Notwithstanding, it may be possible that changes in diet have an indirect effect on milk composition via changes in the nutritional conditions of the mother in years of poor food availability. A second effect is that dietary FA can change the lipid composition of milk and this has been demonstrated by using FASA. However, there are many unknown factors that contribute to the difference in the FA composition of the milk and that of the diet. For instance, only recently, an attempt to investigate the turnover rates of FA and deposition in the mammary gland of FA in pinnipeds has shown that the sources of FAs in milk and the factors affecting the overall composition are still not well understood (Staniland and Pond, 2004). Therefore the results obtained from FASA and QFASA to infer diet from predator's tissue such as milk should be interpreted with caution until some of these questions are answered.

LIFE HISTORY OF NEW ZEALAND SEA LION

The NZSL is considered one of the world's rarest pinniped and one of the least abundant of sea lions (Campbell *et al.*, 2006; Chilvers *et al.*, 2007). However, little is known about their biology, but in recent years great effort has been made to increase the knowledge of the biology of the species.

The NZSL is endemic to New Zealand and is classified as "Threatened" under the NZ Marine Mammals Protection Act 1978 and New Zealand threat classifications system (Hitchmough, 2002) due predominantly to its restricted number of breeding sites and distribution. In addition, it is classified as "Vulnerable" by the International Union for the Conservation of Nature (IUCN, 2004).

The main breeding sites are restricted to the subantarctic and include the Auckland Islands and Campbell Island (Wilson, 1979; Gales and Fletcher, 1999; McNally *et al.*, 2001; Childerhouse *et al.*, 2005). Almost 86% of the pups are born on Dundas Island, Enderby Island and Figure of Eight Island which are part of the Auckland Islands (Figure 5) (Chilvers *et al.*, 2007).

Their localized distribution and small population, around 10,000 to 13,000 individuals with pup production is in decline (Campbell *et al.*, 2006; Chilvers *et al.*, 2007), make this species vulnerable to the impact of commercial fisheries bycatch and disease (Woodley and Lavigne, 1993; Duignan, 1999; Manly *et al.*, 2002; Breen *et al.*, 2003; Duignan and Wilkinson, 2003; Wilkinson *et al.*, 2003; Duignan *et al.*, 2004; Roberston *et al.*, 2006; Wilkinson *et al.*, 2006; Castinel *et al.*, 2007a; Castinel *et al.*, 2007b; Castinel *et al.*, 2007c). Thus in the last decade three epidemics have caused not only severe mortality of pups but also adult mortality (Duignan, 1999; Wilkinson *et al.*, 2003). Furthermore, it has been demonstrated that the operations of the squid fisheries overlap with the feeding grounds of lactating NZSLs (Chilvers *et al.*, 2005, 2006b) and, therefore, there is great potential for competition for food sources (Meynier *et al.*, 2006b).

The diet of NZSL has been studied using traditional methods based on the identification of hard parts of prey recovered from regurgitates, faeces and stomach contents (Childerhouse *et al.*, 2001; Bando *et al.*, 2005; Meynier *et al.*, 2006a; Meynier *et al.*, 2006b). These studies indicate that teleost fishes are the predominant prey of NZSL, followed by the commercially important arrow squid. It is not known how much these conclusions are biased by the method of diet analysis, which underestimates the prey that lacks hard parts. However, it is possible to assess the relative importance of teleost fish and arrow squid (Bando *et al.*, 2005; Meynier *et al.*, 2006a; Meynier *et al.*, 2006b). Recently comparisons of the blubber fatty acid (FA) profiles of the predator and prey species have been investigated to study the diet of the NZSL and initial results have shown that NZSLs have a long term fish based diet (Meynier *et al.*, 2006a; Meynier *et al.*, 2006b).

In addition to bycatch of adult sea lions and the outbreak of disease, there are other factors that may have a negative effect on the growth of the population. A low and declining production of pups in the last 8 years add to the factors that are impeding the recovery of the population (Campbell *et al.*, 2006; Chilvers *et al.*, 2007). In order to support their pups, lactating females are maximizing their foraging effort by making amongst the deepest and longest dives of any species of sea lion (Gales and Mattlin, 1997; Chilvers *et al.*, 2006b). Studies on maternal foraging behaviour and energetics have demonstrated that lactating females are operating near their physiological limit (Gales and Mattlin, 1997; Costa *et al.*, 1998; Costa and Gales, 2000; Chilvers *et al.*, 2005, 2006b). It is not clear how this is affecting the reproductive success of the females.

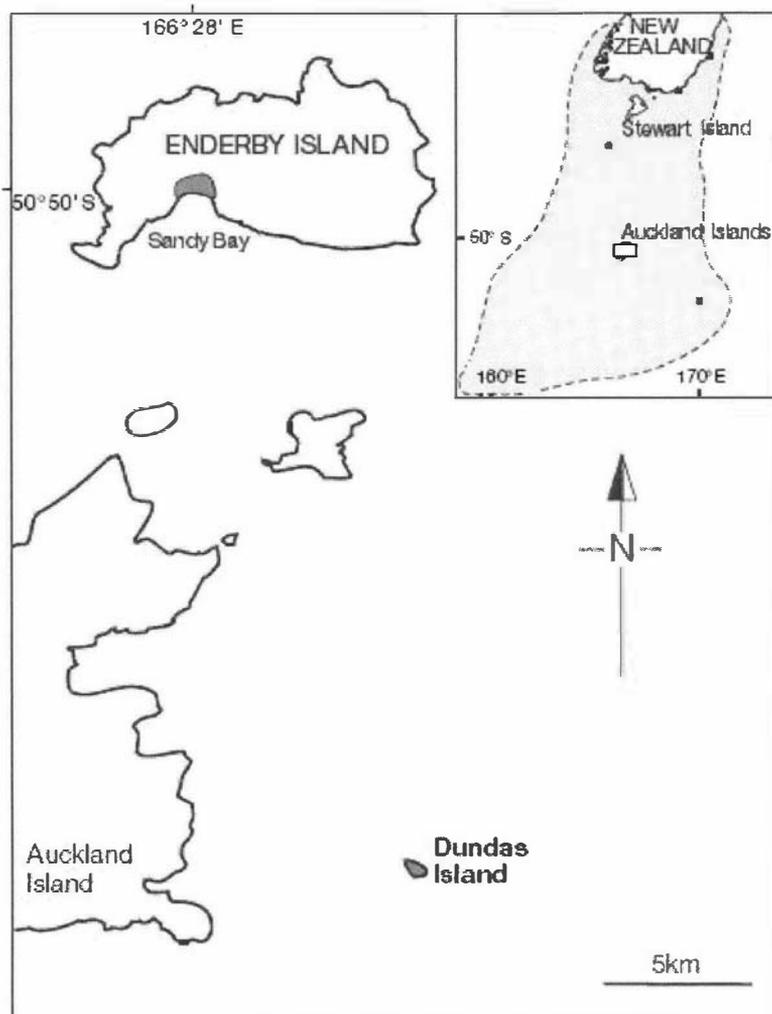


Figure 5. Northeast Auckland Islands showing the main breeding areas for New Zealand sea lion (NZSLs): Sandy Bay, Enderby Island (in grey, 50° 50' S, 166° 28' E) and Dundas Island, 8 km south (in grey). Inset: New Zealand's sub-Antarctic. Grey shaded area indicates NZSLs' current distribution. From Chilvers *et al.* (2006).

THESIS RATIONALE

Lactation is an important part of the biology of pinnipeds and is of particular interest because their lactation strategy is unique among mammals. In the studies of lactation it is crucial to adequately determine milk composition and quantity of milk secreted. The review of the literature presented in this chapter has shown that much of the data on the milk composition in pinnipeds is restricted or limited. Logistical constraints of working with these large wild animals such as pinnipeds and sometimes in remote places have meant that few milk samples have been collected. In

addition different analytical methods have been used and the effect of stage of lactation among other factors are often not considered or mentioned in the literature (Ofstedal *et al.*, 1987a; Schulz and Bowen, 2004). In lactation studies of pinnipeds the lack of extensive sampling has made inter-specific comparisons difficult. For otariid species, milk composition been analysed throughout the entire lactation period (in only three species) (Gales *et al.*, 1996; Arnould and Hindell, 1999; Georges *et al.*, 2001) and for inter-annual variation (Arnould and Boyd, 1995a; Lea *et al.*, 2002).

It is well established that lactation stage affects milk composition in pinnipeds (Ofstedal, 1984b); however, it is unclear what other factors affect it. A better understanding of lactation in pinnipeds and what factors affect the maternal reproductive success is an important part in the management of a species. Maternal reproductive success, which includes success in rearing her offspring, is directly associated with her lactational performance. The survival and well being of the offspring depends on the quality and quantity of milk secreted by the mother.

An aspect that has had considerable attention is how the diet affects the lipid composition of the milk in pinnipeds (Iverson, 1993). As otariid mothers alternate between nursing the pup ashore and foraging at sea, it is of special interest to understand the mechanism of how and at what rate dietary lipids are incorporated into the milk. Otariid mothers depend on local food sources during lactation and these food sources may be scarce due to natural factors such as severe oceanographic conditions (e.g. ENSO) or anthropogenic factors e.g. interactions with local fisheries. By understanding how dietary lipids influence the composition of milk lipid we can understand how local food resources affect maternal reproductive success.

THESIS OUTLINE

The aim of this investigation is to describe the milk composition of NZSLs at the Auckland Islands in early lactation and to identify factors affecting the composition and how they influence it. By undertaking this research a better understanding of the lactation strategy of NZSL will be gained and this information can be used for management of the species.

This thesis contains seven chapters divided in four sections;

- I. Introduction and Literature review (Chapter 1);
- II. Methods of milk analysis and milk composition (Chapter 2- 3);
- III. Factors that affect the milk composition (Chapter 4-6);
- IV. General Discussion and Conclusion (Chapter 7).

In section II, Chapter 2 validates the method of analysis used to analyze the gross chemical composition of the milk of the NZSLs. The validation is based on comparing the reference method of analysis against a method widely used in the dairy industry but new for milk of marine mammals. Chapter 3 describes the gross chemical composition of NZSL milk and describes the changes in milk composition that occurred during seven summer seasons.

In section III; Chapter 4 examines the maternal, offspring and temporal factors that affect the milk composition. Chapter 5 tests the theory of whether changes in the diet are reflected in the FAs of milk, blubber and serum.

Chapter 6 investigates the changes in the signature FA in milk in four years in relation to signature FAs of the diet from the most common prey consumed by NZSLs. The results of this chapter are discussed in relation to the changes in the signature FA of milk could be related to shift in local food resources between years. In addition, the differences in the signature FA of milk between two colonies, Enderby Island and Dundas Island, are investigated.

Finally, section IV (Chapter 7) discusses the finding of this thesis in a broader context and in relation to management plans and conservation priorities for NZSL population.

THESIS AIMS

The specific research objectives of this thesis are:

- I. To validate a new analytical method to determine the milk composition of NZSLs.
- II. To describe the gross chemical composition of NZSL milk from early lactation.
- III. To investigate the interannual differences over a period of seven years and the intra seasonal variation in milk composition in early lactation.
- IV. To identify the factor/s that influences the milk composition in NZSL.
- V. To test whether acute changes in diet are reflected in the FA composition of milk, serum and blubber.
- VI. To study the temporal and spatial difference in milk fatty acids signature by means of FASA. To relate the composition of milk FAs to the FA profiles of five potential prey species.

Table 1. Duration of lactation period in fur seals, sea lions and walruses.

| Species | Lactation period (months) |
|---|---------------------------|
| Northern FS <i>Callorhinus ursinus</i> ¹ | 3-4 |
| Antarctic FS <i>A. gazella</i> ² | 4 |
| South American SL <i>Otaria byronia</i> ³ | 5- 12 |
| California SL <i>Zalophus c. californianus</i> ⁴ | 6-12 |
| South American FS <i>A. australis</i> ⁵ | 6-24 |
| Juan Fernandez FS <i>A. philippii</i> ⁶ | 7-10 |
| Subantarctic FS <i>A. tropicalis</i> ⁷ | 10 |
| Australian FS <i>A. pusillus doriferus</i> ⁸ | 11 |
| Guadalupe FS <i>Arctocephalus townsendii</i> ⁹ | 9-11 |
| Steller's SL <i>Eumetopias jubatus</i> ¹⁰ | 11-12 |
| New Zealand FS <i>A. forsteri</i> ¹¹ | 11-12 |
| New Zealand SL <i>Phocarctos hookeri</i> ¹² | ~12 |
| Galapagos SL <i>Zalophus c. wollebaeki</i> ¹³ | ~12 |
| South African FS <i>A. pusillus pusillus</i> ¹⁴ | ~12 |
| Walrus <i>Odobenus rosmarus</i> ¹⁵ | 12-36 |
| Galapagos FS <i>A. galapagoensis</i> ¹⁶ | 12-36 |
| Australian SL <i>Neophoca cinerea</i> ¹⁷ | 15-18 |

SL= sea lion, FS = fur seal. Sources: ¹(Doidge *et al.*, 1986; Wickens and York, 1997; Donohue *et al.*, 2002); ²(Lunn *et al.*, 1993; Wickens and York, 1997; Goldsworthy, 1999); ³(Bonner, 1984; Oftedal *et al.*, 1987a); ⁴(Stewart and Yochem, 1984; Oftedal *et al.*, 1987b; Melin *et al.*, 2000); ⁵(Trillmich and Majluf, 1981; Bonner, 1984; Trillmich *et al.*, 1986b); ⁶(Francis *et al.*, 1998); ⁷(Georges *et al.*, 1999; Goldsworthy and Crowley, 1999); ⁸(Arnould and Hindell, 1999); ⁹(Wickens and York, 1997); ¹⁰(Pitcher and Calkins, 1981; Higgins *et al.*, 1988; Pitcher *et al.*, 2001); ¹¹(Bonner, 1984; Goldsworthy and Shaughnessy, 1994; Mattlin, 1998); ¹²(Cawthorn, 1990); ¹³(Trillmich and Lechner, 1986; Oftedal *et al.*, 1987a); ¹⁴(Bonner, 1984; David and Rand, 1986); ¹⁵(Bonner, 1984; Oftedal *et al.*, 1987a; Kovacs and Lavigne, 1992); ¹⁶(Bonner, 1984; Trillmich and Lechner, 1986; Wickens and York, 1997); ¹⁷(Higgins and Gass, 1993; Gales *et al.*, 1996).

Table 2. Duration of lactation in phocids.

| Species | Lactation period (days) |
|--|-------------------------|
| Pack ice whelping seals | |
| Hooded seal <i>Cystophora cristata</i> ¹ | 4 |
| Harp seal <i>Phoca groenlandica</i> ² | 12-13 |
| Crabeater seal <i>Lobodon carcinophagus</i> ³ | 14-21 |
| Bearded seal <i>Erignathus barbatus</i> ⁴ | 12-24 |
| Grey seal <i>Halichoerus grypus</i> ⁵ | 16 |
| Caspian seal <i>Phoca caspica</i> ⁶ | 21 |
| Ribbon seal <i>Phoca fasciata</i> ⁷ | 21-28 |
| Spotted seal <i>Phoca largha</i> ⁸ | 28 |
| Leopard seal <i>Hydrurga leptonyx</i> ⁹ | ~30 |
| Fast ice whelping seals | |
| Weddell seal <i>Leptonychotes weddelli</i> ¹⁰ | 35-42 |
| Ringed seal <i>Phoca hispida</i> ¹¹ | 36-41 |
| Baikal seal <i>Phoca sibirica</i> ¹² | 60-75 |
| Land whelping seals | |
| Harbor seal <i>P. v. richardsi</i> ⁶ | 21-35 |
| Southern elephant seal <i>Mirounga leonine</i> ¹³ | 23 |
| Northern elephant seal <i>M. angustirostris</i> ¹⁴ | 28 |
| Harbor seal <i>Phoca vitulina vitulina</i> ¹⁵ | 28-42 |
| Harbor seal <i>P. v. concolor</i> ⁶ | 33 |
| Hawaiian monk seal <i>Monachus schauinslandi</i> ¹⁶ | ~ 42 |
| Mediterranean monk seal <i>M. monachus</i> ¹⁷ | 42-49 |
| Harbor seal <i>P. v. stejnegeri</i> ⁶ | 90 |

Sources: ¹(Oftedal et al., 1993); ²(Kovacs and Lavigne, 1985); ³(Shaughnessy and Kerry, 1989); ⁴(Iverson et al., 1993); ⁵(Oftedal et al., 1987a); ⁶(Burns, 1981); ⁷(Bonner, 1979b); ⁸(Oftedal et al., 1987a; Gjertz et al., 2000); ⁹(Hofman, 1979); ¹⁰(De Master, 1979); ¹¹(Hammill et al., 1991); ¹²(Popov, 1979); ¹³(Laws, 1979; Hindell et al., 1994); ¹⁴(Riedman and Ortiz, 1979; Puppione et al., 1996); ¹⁵(Bonner, 1979a); ¹⁶(Brenton, 1979a; Kenyon, 1981); ¹⁷(Boulva, 1979; Kenyon, 1981)

Table 3. Milk composition in phocids.

| Species | Milk composition | | | | |
|---|-------------------------|-----------------------|-------------------------|------------------------|-------------------|
| | Fat (%) | Water (%) | Protein (%) | Sugar (%) | Ash (%) |
| Harp seal ^{1,2,3,4} | 35.8±1.8 ^a | 51.4±1.8 ^a | 10.4±0.5 ^a | 0.69-0.79 ^a | 0.61 |
| | 35.4 ^b | 32.4±0.4 ^c | 7.7±0.2 ^c | 0.65 ^c | -- |
| | 57.1±0.5 ^c | -- | -- | -- | -- |
| Hooded seal ^{5,6} | 56.3 ^a | 49.8 | 6.2 ^a | 0.86 ^a | 0.86 |
| | 61.0 ^b | -- | 4.7 ^b | 1.05 ^b | -- |
| | 61.1 ^c | -- | 5.1 ^c | 0.99 ^c | -- |
| Grey seal ^{7,8} | 39.8±2.8 ^a | 45.0±2.1 ^a | 11.2±0.8 ^a | 0.7 ^a | 0.69 |
| | 55.6±1.6 ^b | 33.0±1.4 ^b | 9.4±0.14 ^{b,c} | 0.8 ^{b,c} | -- |
| | 60.0±1.86 ^c | 28.6±1.3 ^c | -- | -- | -- |
| Bearded seal ⁵ | 49.5 | 46.4 | 6.8 | 0.05 | 0.6 |
| Weddell seal ^{9,10} | 53.6 ^a | 43.6 | 14.1 | 0.02 | -- |
| Harbour seal ¹¹ | 50 | -- | -- | -- | -- |
| Southern elephant seal ^{12,13} | 16.1 ± 7.0 ^a | 70 ^a | 12.6 ±2.3 ^a | 0.28 ±0.10 | -- |
| | 39.5 ±15.2 ^b | 33 ^c | 10.7 ±2.8 ^c | - | -- |
| Northern elephant seal ¹⁴ | 24 ^a | 75 ^a | 5-12 | <0.25 | -- |
| | 47 ^b | 35 ^c | -- | -- | -- |
| | 54 ^c | -- | -- | -- | -- |
| Crabeater seal ^{15,16} | 35 ^a | -- | 10 ^a | 1.1 – 1.9 | 1.04 ^a |
| | 50 ^b | -- | 10.8 | -- | 0.93 ^b |

^a early lactation; ^b mid lactation; ^c late lactation.

Sources: ¹(Cook and Baker, 1969); ²(Lavigne *et al.*, 1982); ³(Webb *et al.*, 1984); ⁴(Ofstedal, 1996 #121); ⁵(Bonner, 1984); ⁶(Ofstedal *et al.*, 1988); ⁷(Baker, 1990); ⁸(Iverson *et al.*, 1993); ⁹(Ofstedal and Iverson, 1995); ¹⁰(Tedman and Green, 1987); ¹¹(Boness and Bowen, 1996); ¹²(Carlini *et al.*, 1994); ¹³(Hindell *et al.*, 1994); ¹⁴(Riedman and Ortiz, 1979); ¹⁵(Messer *et al.*, 1988); ¹⁶(Green *et al.*, 1993).

Table 4. Milk composition of otariids and walrus.

| Species | Milk composition | | | | |
|---------------------------------|------------------------|------------------------|------------------------|-----------|----------|
| | Fat (%) | Water (%) | Protein (%) | Sugar (%) | Ash (%) |
| Australian SL ^{1*} | 28.35 ^a | 56.9 ±9.9 | 9.9 ±2.5 | — | 0.9 ±0.3 |
| | 47.15 ^b | — | — | — | — |
| | 55.4 ^c | — | — | — | — |
| Steller SL ² | 24 | — | — | — | — |
| South American SL ³ | 38.6 ±3.1 ^a | 48.9 ±3.1 | 11.1 ±1.2 | --- | 0.8 ±0.1 |
| California SL ⁴ | 31.7 ^a | 59.0 | 8.5 | 0.3 | — |
| | 43.7 ^b | — | — | — | — |
| Galapagos SL ⁵ | 32.4 ^a | — | 9 | — | — |
| | 25.1 ^b | — | — | — | — |
| Galapagos FS ⁶ | 29.4 ±5.9 ^a | — | 9.9 ±1.4 ^a | 0.1 | 0.9 ±0.1 |
| | — | — | 14.0 ±0.9 ^b | — | — |
| Guadalupe FS ⁷ | ~41 | — | — | — | — |
| Juan Fernandez FS ⁸ | 41.4 ±5.8 | — | 11.9 ±2.0 | 1.2 ±0.4 | 0.7 ±0.1 |
| Subantarctic FS ⁹ | 45.0 ±3.7 ^a | 40.7 ±4.5 ^a | 13.4 ±1.4 ^a | — | — |
| | 51.9 ±4.9 ^b | 33.3 ±4.0 ^b | 11.6 ±1.3 ^b | — | — |
| | 52.3 ±6.0 ^c | 33.3 ±4.9 ^c | 11.5 ±1.2 ^c | — | — |
| South American FS ¹⁰ | 36.5 ±4.2 | — | 9.1 ±0.8 | — | — |
| Australian FS ^{11*} | 32.7 ^a | 54.6 ^a | 9.9 ^a | — | 0.7 ±0.1 |
| | 47.7 ^b | 39.1 ^b | 11.0 ^b | — | — |
| | 47.9 ^c | 44.3 ^c | 12.3 ^c | — | — |
| Cape FS ¹² | 23.2 ^a ±8.2 | 58.1 ±6.8 | 10.8 ±1.2 | — | 2.0 ±0.6 |
| Northern FS ¹³ | 45.6 | 36.4 | 12.4 | 0.1 | 0.6 |
| Antarctic FS ¹⁴ | 39.8 ±6.7 | 41.3 ±9.3 | 18.1 ±5.8 | — | 0.7 ±0.1 |
| Walrus ¹⁵ | 24.1 | 59.9 | 7.8 | — | 0.59 |

^a early lactation; ^b mid lactation; ^c late lactation. SL= sea lion, FS = fur seal. * values estimated from regression equations see Arnould and Hindell (1999); † values were averaged.

Sources: ¹ (Kretzmann *et al.*, 1991; Gales *et al.*, 1996); ² (Higgins *et al.*, 1988); ³ (Werner *et al.*, 1996); ⁴ (Oftedal *et al.*, 1987b); ⁵ (Trillmich and Lechner, 1986); ⁶ (Trillmich and Lechner, 1986; Trillmich, 1988); ⁷ (Georges *et al.*, 2001); ⁸ (Ochoa-Acuna *et al.*, 1999); ⁹ (Georges *et al.*, 2001); ¹⁰ (Peaker and Goode, 1978); ¹¹ (Arnould and Hindell, 1999); ¹² (Gamel *et al.*, 2005); ¹³ (Dosako *et al.*, 1983; Bonner, 1984); ¹⁴ (Arnould and Boyd, 1995a; Goldsworthy and Crowley, 1999); ¹⁵ (Fay, 1982).

Table 5. Mineral constituents of milk of different species with emphasis on that of marine mammals.

| Species | Minerals in milk (mg/kg) | | | | | | | | | | | | | |
|---|--------------------------|------|------|------|-----|-----|-----|------|-----|-----|-----|-----|-----|-----|
| | Ca | P | Na | K | Mg | Fe | Zn | Cl | Al | Cu | Ba | Cr | Mn | Cd |
| Harp seal <i>Phoca groenlandica</i> ¹ | 950 | 708 | 699 | 456 | 104 | 53 | 3.6 | | 3.6 | 1.8 | 1.8 | 0.6 | 0.6 | 0.1 |
| Giant panda | 1.3 | | 0.8 | 1.7 | 0.2 | | | | | | | | | |
| <i>Ailuropoda melanoleuca</i> ² | | | | | | | | | | | | | | |
| Southern elephant seal <i>Mirounga leonine</i> ³ | | | 990 | 1360 | | | | | | | | | | |
| Northern elephant seal <i>M. angustirostris</i> ⁴ | | | 770 | 720 | | | | | | | | | | |
| Northern FS <i>Callorhinus ursinus</i> ⁶ | 567 | 1193 | 521 | 838 | 141 | 3.5 | 9.0 | 1191 | | 3.7 | | | | |
| Juan Fernandez FS <i>A. philippii</i> ⁵ | 731 | 872 | | | | | | | | | | | | |
| Galapagos FS <i>A. galapagoensis</i> ⁷ | 630 | | | 1670 | | | | | | | | | | |
| California SL <i>Zalophus californianus</i> ⁸ | 885 | 1003 | 1060 | 2030 | | | | | | | | | | |
| Southern SL <i>Otaria flavescens</i> | | | | | | | | | | | | | | |
| Polar bear ⁹ | 290 | 230 | | | | | | | | | | | | |
| Black bear ⁹ | 410 | 280 | | | | | | | | | | | | |
| Sea otter <i>Enhydra lutris</i> ¹⁰ | 1060 | 1250 | | | | | | | | | | | | |

Table 5. (Continued).

| Species | Minerals in milk (mg/kg) | | | | | | | | | | | | | |
|--|--------------------------|------|-----|------|-----|----|----|------|----|----|----|----|----|----|
| | Ca | P | Na | K | Mg | Fe | Zn | Cl | Al | Cu | Ba | Cr | Mn | Cd |
| Spinner porpoises ¹¹ | | 1070 | | | | | | 455 | | | | | | |
| Spotted porpoises ¹¹ | | 1250 | | | | | | 468 | | | | | | |
| Blue whale <i>Balaenoptera musculus</i> ¹² | 310 | 210 | 80 | 130 | 20 | | | 110 | | | | | | |
| Pygmy sperm whale <i>Kogia breviceps</i> ¹³ | 1500 | 1700 | | | | | | | | | | | | |
| Weddell seal ⁸ | | | 530 | 570 | | | | | | | | | | |
| Donkey <i>Equus asinus</i> ¹⁴ | 1200 | 700 | | | | | | | | | | | | |
| Horse <i>E. caballus</i> ¹⁴ | 800 | 500 | | | | | | | | | | | | |
| Common zebra <i>E. burchelli</i> ¹⁴ | 800 | 500 | | | | | | | | | | | | |
| Cow ¹⁵ | 1250 | 960 | 580 | 1380 | 120 | | | 1030 | | | | | | |
| Human ¹⁵ | 330 | 150 | 150 | 550 | 40 | | | 430 | | | | | | |

¹(Webb *et al.*, 1984); ²(Nakamura *et al.*, 2003); ³(Peaker and Goode, 1978); ⁴(Riedman and Ortiz, 1979); ⁵(Ochoa-Acuna *et al.*, 1998); ⁶(Dosako *et al.*, 1983); ⁷(Trillmich and Lechner, 1986); ⁸(Ofstedal *et al.*, 1987a); ⁹(Jenness *et al.*, 1972); ¹⁰(Jenness *et al.*, 1981); ¹¹(Pilson and Waller, 1970); ¹²(Gregory *et al.*, 1955); ¹³(Jenness and Odell, 1978); ¹⁴(Ofstedal, 1988); ¹⁵(Jenness, 1974).

Table 6. Temporal parameters of attendance pattern in otariids.

| Species | Time to 1 st | | | Time absent (%) |
|------------------------------------|-------------------------|--------------------|----------------------|-----------------|
| | Departure (days) | Time Absent (days) | Time Presence (days) | |
| Australian FS ¹ | --- | 5.0 | -- | -- |
| South American FS ² | --- | 4.6 | 1.3 | 78 |
| Guadalupe FS ³ | --- | 11.5 | 5.0 | 70 |
| Subantarctic FS ⁴ | --- | 15.9 ±4.6 | 3.8 ±1.1 | 81 |
| South African FS ⁵ | 4.3 ±3.3 | 3.0 ±2.5 | 2.4 ±1.4 | 56 |
| California SL ⁶ | 5-8 | 4.3 ±0.5 | 1.4 ±0.1 | 75 |
| Steller SL ⁷ | 5.8 ±0.6 | 1.5 ±0.1 | 0.86 ±0.05 | 64 |
| Galapagos SL ² | 6.8 ±2.1 | 0.5 | 0.6 | 47 |
| Antarctic FS ⁸ | 6.9 | 4.2 ±0.8 | 1.8 ±0.5 | 67 |
| Galapagos FS ² | 7.4 ±1.2 | 1.3 | 1.0 | 57 |
| Northern FS ⁹ | 7.4 | 5.9 | 2.2 | 73 |
| New Zealand SL ^{10*} | 8.6 ±0.2 | 1.7-2.7 | 1.2 | 57-69 |
| New Zealand FS ¹¹ | 9.7 | 4.2 | 1.8 | 70 |
| Australian seal lion ¹² | 9.8 ±1.8 | 2.0 ±0.5 | 1.4 ±0.3 | 59 |
| Juan Fernandez FS ¹³ | 11.3 ±3.4 | 12.3 | 5.3 | 70 |

* attendance pattern were recorder at early lactation. SL= sea lion, FS = fur seal

¹(Arnould and Hindell, 1999); ²(Trillmich, 1986b); ³(Figueroa-Carranza, 1994); ⁴(Georges and Guinet, 2000b); ⁵(David and Rand, 1986); ⁶(Antonelis *et al.*, 1990; Melin *et al.*, 2000); ⁷(Higgins *et al.*, 1988; Hood and Ono, 1997); ⁸(Doidge *et al.*, 1986; Costa *et al.*, 1989; Arnould and Boyd, 1995b); ⁹(Gentry and Holt, 1986); ¹⁰(Gales and Mattlin, 1997; Chilvers *et al.*, 2005, 2006); ¹¹(Goldsworthy and Shaughnessy, 1994; Mattlin, 1998); ¹²(Walker and Ling, 1981; Kretzmann *et al.*, 1991; Higgins and Gass, 1993); ¹³(Francis *et al.*, 1998).

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CHAPTER 2

DETERMINING THE GROSS COMPOSITION OF THE MILK OF
NEW ZEALAND SEA LIONS
USING FOURIER TRANSFORM INFRARED SPECTROMETRY

ABSTRACT

The milk composition of the New Zealand sea lion, *Phocarctos hookeri*, was determined with Roesse-Gottlieb, Kjeldahl, total solids gravimetric standard methods and with a Milkoscan (FT 120) and with an Accelerated Solvent Extractor (ASE) for fat, protein and total solids concentration. Agreement between analytical methods for fat, protein and total solids was assessed using different measures of statistical fitness such as; Pearson correlation coefficient (r), coefficient of determination (R^2), concordance correlation coefficient, relative prediction error and intraclass correlation coefficient. The repeatability and reliability of the methods used to determine the concentration of fat in New Zealand sea lion milk were excellent. There was a significant correlation between the results of the Milkoscan (FT 120) and the standard methods Roesse-Gottlieb for milk fat determination ($r = 0.86$, $P < 0.05$) and gravimetric ($r = 0.88$, $P < 0.05$) for total solids determination; whereas the correlation between Milkoscan (FT 120) and the Kjeldahl standard method for milk protein determination was not significant ($r = 0.79$, $P > 0.05$). Precision of the methods, level of agreement between standard methods and test methods and accuracy ranged from excellent to acceptable. This study demonstrated that the results obtained from the analysis of milk from New Zealand sea lion obtained with an appropriately calibrated Milkoscan (FT 120) are comparable with those obtained with standard methods for fat, protein and total solids. The Milkoscan (FT 120) is fast and cost-effective and is widely used in dairy laboratories around the world which should make them readily accessible to ecologists/biologists.

INTRODUCTION

When studying lactation strategies in mammals it is crucial to have data on the composition of the milk as well as the quantity consumed to be able to determine the nutrient intake of the young (Ofstedal, 1981; Ofstedal and Iverson, 1995). This can be problematical for non-domestic animals in which sampling bias, low number of milk samples and analytical and methodological difficulties may potentially obscure the results. While analytical methods are well established for the analysis of cows' milk, their suitability for the analysis of milk from non-domestic species must be verified (Ofstedal and Iverson, 1995).

The standard methods for estimating the concentration of milk fat for pinnipeds include the Roesse-Gottlieb (R-G) method (International Dairy Federation, 1987a, 1996), the Dole and Meinertz procedure (Dole and Meinertz, 1960) and a stoichiometric (elemental CHN analysis) method (Gnaiger and Bitterlich, 1984). The Kjeldahl method is widely accepted as the standard method for protein determination (International Dairy Federation, 2001). Although automated versions of the R-G (Lee *et al.*, 1989; Matheson and Otten, 1999) and Kjeldahl methods are available, these methods can be tedious and time consuming, especially, if large numbers of samples need to be analyzed.

The R-G method has been the most commonly used method to determine the fat concentration in pinniped milk and also the milk of cetaceans and sirenians (Lauer and Baker, 1969; Pilson and Waller, 1970; Jenness and Odell, 1978; Pervaiz and Brew, 1986). Lipid is extracted with a solvent such as ethanol and diethyl ether after the fat globules have been disrupted with ammonium hydroxide (International Dairy Federation, 1987a, 1996). However, if significant hydrolysis of the triacylglycerols occurs in the milk due to inappropriate storage or if the milk was obtained from the stomach contents of pups then the total fat concentration may be underestimated (Ofstedal and Iverson, 1995). Alternative methods to the R-G method use chloroform and methanol to extract lipids (Folch *et al.*, 1957; Bligh and Dyer,

1959) but it has been argued that these methods are unsuitable to accurately determine the milk fat concentration of pinnipeds (Ofstedal and Iverson, 1995).

Since the late 1940's the dairy industry has developed automated infrared instruments, primarily for quantitative analysis of multicomponent systems such as milk (Conn, 1960; Gunzler and Gremlich, 2002). Quantitative infrared instruments for the determination of fat, protein, and lactose in milk are based on the absorption of infrared energy at specific wavelengths (Goulden, 1964). Quantitative analysis was improved remarkably with the development of Fourier transform infrared (FT-IR) spectroscopy in the 1960s (Griffiths and Haseth, 1986). Later with the incorporation of powerful data handling systems, FT-IR spectroscopy has become the dominant method used to determine milk composition in the dairy industry (Gunzler and Gremlich, 2002). By comparison, with conventional infrared spectroscopy, FT-IR is more efficient in light collection and the time to process data is more rapid (Martel and Paquin, 1990).

FT-IR spectroscopy has been evaluated extensively for the analysis of cows' milk in comparison with traditional analytical methods (Ng-Kwai-Hang *et al.*, 1988; Luinge *et al.*, 1993), and compared to other automated instruments, (Van de Voort *et al.*, 1992; Lefier *et al.*, 1996) and found to be robust and accurate. However, despite the advantages of FT-IR spectroscopy instruments, there are some drawbacks associated with establishing and maintaining their accurate calibration (Biggs, 1978; Van de Voort *et al.*, 1992; Remillard *et al.*, 1993).

The pressurized or accelerated solvent extraction (ASE) is another method that has been shown to be reliable for the determination of fat concentration in bovine milk (Richardson, 2001). It has been applied to various milk products, and fulfils the dairy industry requirements for a rapid and cost-effective method (Richardson, 2001). Conventional solvents are used and in contrast to other fat extraction methods, it operates at elevated temperatures and pressures, which permits the extraction of fat at a much faster rate. Analogous to the R-G method, ASE is fully automated and can be used for a variety of milk products (Richardson, 2001). However there are no

reports on the application of this method to determine milk fat concentration in pinniped milk.

In this chapter analytical procedures that are widely used in the dairy industry were applied to milk samples from New Zealand seal lions (NZSL) and the results compared with those obtained using AOAC (Association of Official Analytical Chemists) standard methods such as R-G method, Kjeldahl method and gravimetric method for totals solids.

The objectives of this study were:

- a. to compare the FT-IR spectroscopy method by using a Milkoscan (FT 120) against the R-G, Kjeldahl and gravimetric standard methods for milk fat, protein and total solids determination, respectively;
- b. to assess the repeatability of the methods for milk fat determination;
- c. to assess the repeatability and reliability of the Milkoscan (FT 120) to determine the fat, protein and total solids concentrations in the milk of the New Zealand sea lion (NZSL), *Phocarctos hookeri*.

MATERIALS AND METHODS

Study site and Animals

Milk samples were collected from NZSL as part of a study conducted at Sandy Bay, Enderby Island, the Auckland Islands (50° 30'S, 166° 47'E), during early lactation (January and February). Milk samples for the analyses described in this paper were collected in the 2002/3 and 2004/5 austral summer seasons. Adult NZSL females known to have a pup were selected for collection of milk samples from a pool of branded/tagged individuals of known age.

Animals were only captured after they had been ashore for at least three hours to minimize risk of regurgitation while under general anaesthesia (see Chilvers *et al.* 2005 for handling details). Sea lion females were captured

using a specially designed hoop-net (Furhman Diversified, TX, USA) with a multi-layered head end to impede vision and a hole at the apex of the net to allow for the sea lion's nose to protrude. Animals were physically restrained by two handlers once the mouth and the nose were safely in position inside the hole at the apex. Thereafter, the animals were anaesthetized with isoflurane/oxygen delivered by a face mask from a portable field anesthetic gas (Isoflurane) vaporizer (Acoma MK III, Japan) (Gales and Mattlin, 1998). Anaesthetized animals were transferred to a restraining board and then measured with a tape to the nearest centimeter and weighed to the nearest 0.5 kg. Oxytocin (0.5 - 2.0 IU/kg) was injected intramuscularly into the gluteal region and milk samples (5-30ml) were collected from one or more teats (into a 50 ml sterile container) either by manual expression or using a vacuum pump, constructed from the barrel of a 60 ml plastic syringe. The milk samples stood for a few hours at ambient temperature (5 – 10 °C) to let any solids (sand) to settle to the bottom, and were subsampled into 2 ml cryovials. The milk was completely recovered and only the sediment consisting mainly of sand was left behind. The samples were stored at -196 °C in liquid nitrogen. On return from the field site, the milk samples were stored at -80 °C until immediately before analysis, when they were stored overnight in a refrigerator at 4 °C. Samples were then thawed at 32°C in a water bath for 15 minutes and gently mixed thoroughly by inverting 2 ml cryovials by hand to ensure homogeneity.

Calibration of Milkoscan (FT 120)

The Milkoscan (Milkoscan FT 120, Foss A/S, Hillerod, Denmark) was calibrated for fat, protein and total solids in the milk of NZSLs with 10 milk samples from 5 individual from each year 2003 and 2005 early lactation in the. Four to five subsamples (2 ml cryovials) from each 10 individual were pooled together to produce 10 bulk samples (8-10ml) for the calibration procedure. Each bulk sample contained milk from one individual female. For each milk bulk sample, fat, protein, non protein nitrogen (NPN) and total solids concentration were determined with reference methods: for fat, the Roes-

Gottlieb method (International Dairy Federation, 1987a, 1996); for protein, the Kjeldahl method (International Dairy Federation, 2001); for NPN, a semi-micro Kjeldahl method (Rowland, 1938) and the total combustion method (AOAC, 1995) and a gravimetric method for total solids (McDowell, 1972; Boon, 1979; International Dairy Federation, 1987b). Before the calibration of the Milkoscan, the same 10 milk samples were run in the Milkoscan (FT 120) (replication by samples two and sequence was three successively per sample) for fat, protein and total solids.

The data from the 10 milk samples on milk fat, protein and total solids concentrations obtained from the reference methods were entered into the PC (connected to the Milkoscan, FT 120). The data from the Milkoscan (FT 120) were used to develop partial least-squares regression equations that were the basis for the calibration of the Milkoscan (FT 120) for the major components of milk (fat, protein and total solids). A coefficient of determination $R^2 = 0.94$ was the minimum criterion for calibration of the parameters.

The ASE method

Milk fat was extracted in an Accelerated Solvent Extractor ASE-200, Dionex Corporation, Sunnyvale, CA, with 11 ml stainless steel extraction cells and 40 ml glass receiving vials, using a mixture of solvents in the ratio of 3:2:1 petroleum-ether/acetone/isopropanol, as described by Richardson (2001) for liquid milk (cream). The five operator-adjustable instrumental parameters of the ASE-200 were adjusted to the settings for cream due to the high fat content of NZSL milk. The operational parameters were set as follows: 1) extraction temperature at 120 °C; 2) extraction pressure at 1500 psi; 3) static extraction time 2 min; 4) number of cycles 3 and; 5) flush volume at 100%. Samples of NZSL milk (0.5-1.0 g) were pipetted into an extraction cell containing 1.0-1.2 g of Hydromatrix on a tared balance (Richardson, 2001). The total amount of solvent recovered after the extraction was ~ 16 ml and the solvents were dried under a constant nitrogen flow in a heat block at 65 °C to 110 °C (for details see Richardson, 2001).

Validation of Milkoscan (FT 120) and ASE

A total of 10 milk samples (each 8 to 10ml) and not including those used for calibration were used for the validation of the Milkoscan (FT 120) and ASE method. Milk fat, protein and total solids concentrations were determined for these samples with the reference methods: for fat, the R-G method (International Dairy Federation, 1987a, 1996); for protein, the Kjeldahl method (International Dairy Federation, 2001); and for total solids, the Total Solids Gravimetric method (McDowell, 1972; Boon, 1979; International Dairy Federation, 1987b). All milk samples analyzed for fat concentration by the R-G method were run in duplicate and a standard (Pam's whole milk powder 26.56% fat concentration) was also analyzed to test for error in the procedures. The same 10 milk samples were then analyzed with the Milkoscan (FT 120) for fat, protein and total solids and by the ASE method for fat concentration. To determine the reliability and repeatability of the Milkoscan (FT 120) and the ASE, the results from 10 milk samples from NZSLs, were compared with those obtained by the reference methods. In addition, to determine the reliability and repeatability of the Milkoscan and the ASE, 10 and 11 milk samples not including those for calibration and validation were run in duplicate with the Milkoscan (FT 120) and with the ASE method, respectively.

Variance between aliquots

Analysis of variance was used to test the reliability of the procedure for subsampling the milk in the field, three aliquots from each of 15 different females were analyzed with the Milkoscan (FT 120) for fat, protein and total solids concentration.

Data analysis

Scatter plots were produced by plotting the results obtained from the ASE and Milkoscan (FT 120) against those from the standard methods respectively for fat, protein and total solids. Duplicated results for fat, protein and total solids

were plotted against each other. Estimates of the regression lines were obtained using the REG procedure of SAS (Statistical Analysis System, version 9.1; SAS Institute Inc., Cary, NC, USA). The intercept of the linear model was tested for significance at 0.05. Agreement between analytical methods for fat, protein and total solids was assessed using different measures of statistical fitness. For this purpose, it was assumed that y_i was the assessment of concentration of fat, protein or milk solids in the i^{th} sample using a new method; e.g., Milkoscan (FT 120), and x_i was the assessment of the same milk component in the same i^{th} sample using the standard method (e. g., R-G). The measures of statistical fitness are described below.

a) Coefficient of determination, R^2 , is a measure of model precision and was calculated as:

$$R^2 = \frac{\left[N \sum_{i=1}^N x_i y_i - \left(\sum_{i=1}^N x_i \right) \left(\sum_{i=1}^N y_i \right) \right]^2}{\left[N \sum_{i=1}^N x_i^2 - \left(\sum_{i=1}^N x_i \right)^2 \right] \left[N \sum_{i=1}^N y_i^2 - \left(\sum_{i=1}^N y_i \right)^2 \right]}$$

where N is the total number of paired observations (Shieh, 2001). A value of $R^2 = 1$ indicates 100% precision between the testing and the standard method.

b) The concordance correlation coefficient (CCC) was calculated to determine the overall level of agreement between methods of measuring fat, protein, and total solids.

The CCC is calculated as:

$$\text{CCC} = \text{BCF} \times r$$

where BCF is the bias correction factor from the regression line and r is the Pearson correlation coefficient (Lin, 1989, 1992, 1997; Steichen and Cox, 1998). The Pearson correlation is the squared root of R^2 as calculated above. The r value measures the degree of variation around the $y=x$ line and BCF (Bias Correction Factor) measures the deviation from the $y=x$ line.

BCF was calculated as:

$$BCF = \frac{2}{v + \frac{1}{v} + \mu^2}$$

where

$$\mu^2 = \frac{(\bar{x} - \bar{y})^2}{\sqrt{\sigma_x^2 \sigma_y^2}},$$

$$v = \frac{\sigma_x^2}{\sigma_y^2},$$

\bar{x} , σ_x^2 , \bar{y} and σ_y^2 are mean and variances of assessments obtained using analytic method x or y, respectively. Values of CCC and their significance are shown in Table 7.

c) Relative prediction error (RPE) which was calculated according to Rook *et al.* (1990) as:

$$RPE = \frac{\sqrt{\frac{1}{N} \sum_{i=1}^N (x_i - y_i)^2}}{\bar{x}}$$

Fuentes-Pila *et al.* (1996) suggested that a RPE lower than 10% is an indication of satisfactory prediction, whereas a RPE between 10% and 20% indicates a relatively acceptable prediction, and a RPE greater than 20% indicates poor prediction.

d) The intraclass correlation coefficient (ICC) was used to assess the level of accuracy of the ASE and Milkoscan (FT 120). The ICC was obtained using the MIXED procedure of SAS (Statistical Analysis System, version 9.1; SAS Institute Inc., Cary, NC, USA) fitting a linear model with concentration of a milk component as the dependent variable and method as class effect.

$$ICC = \frac{\sigma_B^2}{\sigma_B^2 + \sigma_W^2}$$

where σ_B^2 is the variance between methods and σ_W^2 is the variance within a method. A value for ICC equal to 1 indicates that two different analytic methods will estimate the same concentration of a milk component when measured in the same sample (Zar, 1999). Therefore the closer the values to

1 the smaller the difference between pairs of x_i and y_i values. ICC indicates the relative part of the between method error in the whole error.

e) Bland and Altman (1995) test. The mean of the paired measurements (x-axis) were plotted against their difference (y-axis). The 95% limits of agreement were calculated as the mean difference plus or minus 1.96 times the standard deviation of the differences (Bland and Altman, 1995; Steichen and Cox, 1998).

RESULTS

Ten additional NZSL milk samples were analyzed by the Milkoscan (FT 120) previously calibrated with NZSL milk and standard methods for gross chemical composition (Table 8). Means for milk fat, protein and total solids, standard deviation, minimum and maximum values are given in Table 8 for each analytical test. Agreements between the test method and the standard method were assessed (Table 9).

The variance between aliquots for fat, protein and total solids concentration was 4% (ICC=0.96), 6% (ICC=0.84) and 4% (ICC=0.96) respectively.

Table 7. Values corresponding to the concordance correlation coefficient (CCC) and the description and significance.

| CCC | Description |
|-----------|----------------|
| 0.21-0.40 | Fair |
| 0.41-0.60 | Moderate |
| 0.61-0.80 | Substantial |
| 0.81-1.00 | Almost perfect |

Milk fat concentration

The mean milk fat concentrations obtained with Milkocan, R-G and the ASE methods are shown in Table 8.

There was a significant correlation ($r = 0.86$, $P < 0.05$) between the results of the Milkoscan (FT 120) and the standard method R-G for milk fat

determination (Figure 6a). The agreement between these two methods according to Lin's CCC was 0.85 (95% confidence interval = 0.75, 0.73) indicating an almost perfect agreement (Table 7); and BCF of 0.99 indicated that the line of best fit was very close to equal to the perfect agreement line, whereas the R^2 was 0.74 (Table 9 and Figure 6a). The mean milk fat difference between the two tests according to Bland and Altman test was 0.30% (SD 4.17) and 95% (limits of agreement) of the pairs of results differed by less than ± 2.98 % (Table 9). A RPE of 20.99% indicated poor predictability of the Milkoscan (FT 120) and an ICC of 0.71 indicated an acceptable accuracy (Table 9).

Table 8. Descriptive statistics of New Zealand sea lion, *Phocarctos hookeri*, milk composition determined with Roese Gottlieb, Kjeldahl, gravimetric standard methods and the Milkoscan and ASE test methods.

| Method | Mean (%) | SD | Minimum (%) | Maximum (%) |
|-------------------------------|----------|-------------|-------------|-------------|
| Milk Fat (n=10) | | | | |
| Milkoscan (FT 120) * | 22.17 | 8.67 | 13.08 | 39.18 |
| Roese Gottlieb ¹ * | 22.47 | 7.62 | 13.80 | 36.70 |
| ASE ² | 19.13 | 8.95 | 8.39 | 35.93 |
| Milk Protein (n=9) | | | | |
| Milkoscan (FT 120) * | 9.47 | 1.57 | 7.21 | 11.92 |
| Kjeldahl ¹ | 10.02 | 1.99 | 7.34 | 13.02 |
| Milk total solids (n=10) | | | | |
| Milkoscan (FT 120) * | 31.45 | 9.26 | 18.97 | 47.96 |
| Gravimetric ¹ | 32.95 | 7.38 | 24.81 | 45.40 |

¹ Standard methods of analysis for milk components; ² Accelerated solvent extraction (ASE) method for fat determination; * Analysis in duplicate.

Similar agreement between the results obtained by the methods ASE and standard R-G for fat determination was found. A strong and significant correlation ($r = 0.99$, $P < 0.05$) was found between the results of the two methods (Figure 6b). The agreement (CCC = 0.86) and fit to the perfect line of agreement (BCF = 0.87) between ASE and R-G results were acceptable while precision was excellent ($R^2 = 0.99$) (Table 9 and Figure 6b). The mean difference in milk fat concentration between ASE method and the R-G method was 4.11% (SD 3.37) with a 95% limit of agreement of ± 2.41 (Table 9). A

satisfactory prediction was shown by a RPE of 4.84%, which is lower than 10% and an acceptable accuracy (ICC=0.71) (Table 9).

There was strong agreement (CCC= 0.99, Table 3) between duplicates in both the R-G ($r= 0.99$, Table 9) and ASE methods ($r= 0.99$, Table 9). Both methods had excellent precision ($R^2=0.99$) and acceptable accuracy as indicated by a RPE below 10% (RPE=7.86%, R-G; RPE= 3.55%, ASE, Table 9). Both methods (BCF=1.0) had the line of best fit close to the perfect agreement line and excellent accuracy as indicated by the ICC of 0.99 (Table 9). The mean difference between the replicates for ASE was 0.33% (SD 0.56) and for R-G was 0.20% (SD 0.72) of the mean with 95% limits of agreement of ± 0.40 and ± 0.51 , respectively (Table 9). Overall the performance of the ASE was similar to that of the R-G method for the determination of the fat concentration in NZSL milk.

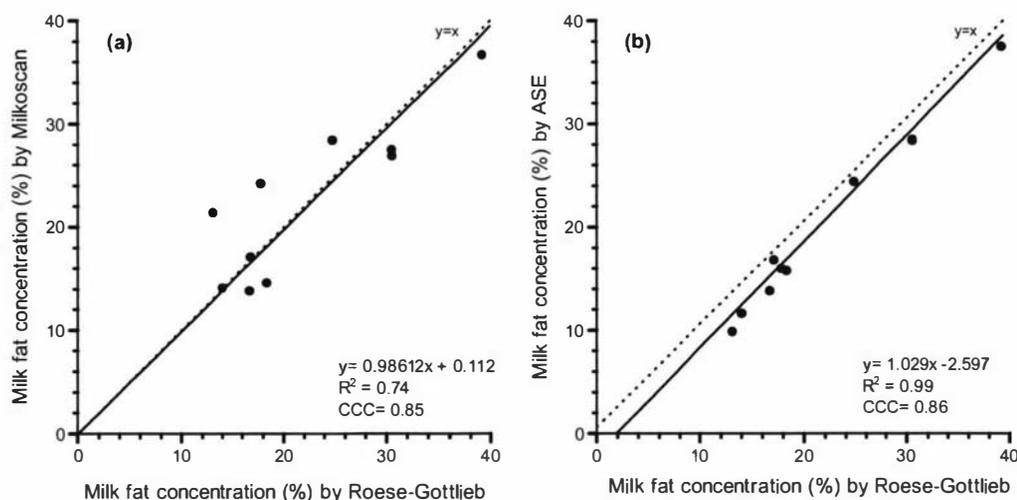


Figure 6. New Zealand sea lion, *Phocarctos hookeri*, milk fat concentration, (a) determined with the Roese-Gottlieb method versus the Milkoscan (FT 120) method, (b) determined with the Roese-Gottlieb method versus the Accelerated solvent extraction method, with the regression equation and line of best fit (solid line) and line of equality $x=y$ (broken lines). CCC =Concordance Correlation Coefficient.

The results from the replicated analysis of the fat concentration in NZSL milk with the Milkoscan (FT 120) were highly repeatable as indicated by $R^2= 0.99$ for CCC, R, BCF (Table 9, Figure 8a). The Milkoscan (FT 120) had satisfactory prediction with a value RPE (5.43%) below 10%, but a low accuracy as shown by an ICC of 0.30 (Table 9).

The Bland and Altman method of assessing agreement indicated that the mean difference between the milk fat concentrations of replicated samples analyzed with the Milkoscan (FT 120) was 0.24% (SD=1.07) while the limit of agreement shown that 95% of the pairs differed by less than $\pm 0.77\%$. As shown in Figure 9a, 60% of the pairs of replicates fell within the calculated limits of agreement. The highest difference between pairs was 1.93% (Figure 9a). Similarly, replicated milk samples analyzed with R-G for milk fat concentration had a mean difference of 0.20% (SD 0.72) with a 95% confidence interval of $\pm 0.51\%$ (Table 9).

Milk protein

The mean milk protein concentrations obtained with the Milkoscan (FT 120) and the Kjeldahl standard method are shown in Table 8. As shown by Figure 7a, there was an acceptable significant correlation ($r = 0.79$) between the results of the Kjeldahl and Milkoscan (FT 120) methods for milk protein determination, and there was low agreement between the two methods (CCC=0.72). The line of best fit was very close to the perfect agreement line (BCF=0.91), whereas precision ($R^2 = 0.61$) was classified as substantial (Table 9). The estimates for prediction (RPE=10.99%) and accuracy (ICC=0.74) were within the predetermined limits of acceptability (Table 9).

The mean difference in protein concentration between the two methods was 0.55% (SD 3.35) with 95% confidence limits of agreement of $\pm 2.40\%$ (Table 9).

There was a strong and significant correlation between replicated samples for protein concentration ($r=0.96$) as shown in Figure 8b. The replicated samples were in very good agreement with CCC = 0.97 (Table 9). The results for replicated samples of protein determined with Milkoscan (FT 120) had a satisfactory prediction as shown by a value of 5.92% for RPE (Table 9) and a value of 0.98 for r and a good precision ($R^2 = 0.96$) (Table 9). The fit to the perfect line of agreement was almost perfect (BCF=0.99, Table 9). The accuracy of the Milkoscan (FT 120) to determine milk protein concentration

was very good according to the ICC (0.97) (Table 9). The Bland and Altman method of assessing agreement indicated that the mean difference between the milk protein concentration replicated samples was 0.19% (SD 0.52) while the 95 % limit of agreement shown that 95% of the pairs differed ± 0.37 %. Fifty percent of the pairs fell within the 95% limits of agreements (Figure 9b).

The mean NPN concentration in the 10 milk samples was 0.02% \pm SD 0.02 while the total nitrogen concentration was 1.32% \pm SD 0.26. To test whether there was a significant improvement in the regression equation or agreement between the methods the data for protein was corrected for NPN. No significant improvement was observed. Thus the data remained uncorrected for NPN and the Milkoscan (FT 120) was not calibrated for NPN.

Milk total solids

The mean concentration of total milk solids obtained with the Milkoscan (FT 120) and the total solids reference method are shown in Table 9. The prediction and accuracy were acceptable (RPE=14.25, ICC=0.74), respectively (Table 9).

The correlation between the results from the Milkoscan (FT 120) and the total solids reference method was relatively strong and significant ($r= 0.88$, Figure 7b). There was an almost perfect agreement between the methods as indicated by a CCC of 0.85 (Table 7 and 9). Precision was acceptable ($R^2=0.78$) while there was a considerable fit to the perfect line of agreement (BCF=0.95, Table 9, Figure 7b). The mean difference between the standard test and the new test was 1.50% (SD 4.19) while 95% of the pairs differed by approximately ± 2.73 % or less (Table 9).

Replicated values of total solids determined with Milkoscan (FT 120) had an excellent agreement (CCC = 0.99) and a significant and strong correlation ($r=0.99$, Figure 8c). The precision and the fit to the perfect line of agreement were excellent ($R^2 = 0.99$, BCF = 0.99, respectively; Figure 8c and Table 9). The prediction of the Milkoscan (FT 120) method was satisfactory when determining the total milk solids (RPE = 2.70%, Table 9). The ICC accounted

for 0.80 (Table 9) showing very good accuracy for the method. Bland and Altman method of agreement indicated that the mean difference between replicate samples of total solids accounted for 0.01% (SD 0.80) with a 95% limit of agreement $\pm 0.57\%$ (Table 9, Figure 9c). Figure 9c shows that only 40% of the pairs fell within the limits of agreement.

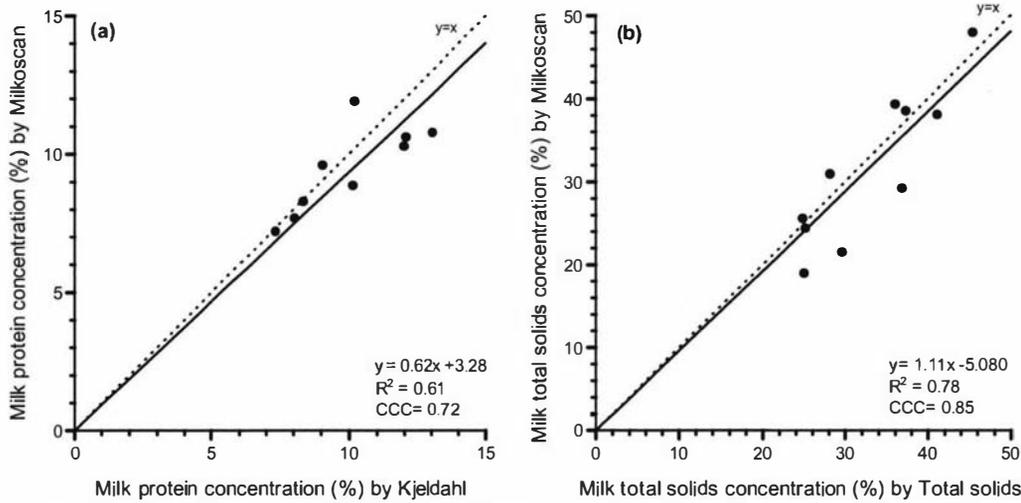


Figure 7. The concentration in New Zealand sea lion, *Phocarctos hookeri*, milk of (a) protein (%), and (b) total milk solids (%), determined by the Milkoscan (FT 120) (y axis) versus by the standard methods, Kjeldahl for protein and a gravimetric method for total milk solids (x axis), with the regression equation and line of best fit (solid line) and line of equality (broken lines). CC=Concordance Correlation Coefficient.

Table 9. Measures of statistical fitness to assess agreement between Roese Gottlieb, Kjeldahl and Total solids gravimetric standard methods and alternative Milkoscan (FT 120) and Accelerated Solvent Extraction (ASE) test methods to determine the composition of New Zealand sea lion's, *Phocarctos hookeri*, milk fat, protein and total solids.

| | Measures of statistical fitness | | | | | | | |
|---|---------------------------------|-------------|-------------|-------------------------------|-----------------|-------------|-------------|------------|
| | R^2 | Concordance | Pearson | Bias | Mean | Relative | Intraclass | y= |
| | | Correlation | correlation | Correction | Difference | Prediction | Correlation | |
| | Coefficient | Coefficient | Factor | ($\pm 95\%CI$) ⁶ | Error (%) | Coefficient | | |
| Milk fat method | | | | | | | | |
| Milkoscan (FT 120) vs. Roese-Gottlieb | 0.74 | 0.85 | 0.86 | 0.99 | 0.30 \pm 2.72 | 20.99 | 0.71 | 0.98x +0.1 |
| ASE vs. Roese-Gottlieb | 0.99 | 0.86 | 0.99 | 0.87 | 4.11 \pm 2.20 | 4.84 | 0.71 | 1.03x -2.6 |
| ASE vs. ASE ² | 0.99 | 0.99 | 0.99 | 1.0 | 0.33 \pm 0.35 | 7.86 | 0.78 | 1.07x -5.9 |
| Roese-Gottlieb vs. Roese-Gottlieb | 0.99 | 0.99 | 0.99 | 1.0 | 0.20 \pm 0.49 | 3.55 | 1.00 | 0.98x +0.7 |
| Milkoscan (FT 120) vs. Milkoscan (FT 120) | 0.99 | 0.99 | 0.99 | 0.99 | 0.24 \pm 0.70 | 5.43 | 0.30 | 0.98x +0.3 |
| Milk protein method | | | | | | | | |
| Milkoscan (FT 120) vs. Kjeldahl | 0.61 | 0.72 | 0.79 | 0.92 | 0.55 \pm 2.19 | 10.99 | 0.74 | 0.62x +3.3 |
| Milkoscan (FT 120) vs. Milkoscan (FT 120) | 0.96 | 0.97 | 0.98 | 0.99 | 0.19 \pm 0.34 | 5.92 | 0.97 | 1.09x +1.0 |
| Milk total solids method | | | | | | | | |
| Milkoscan (FT 120) vs. Total solids | 0.78 | 0.85 | 0.88 | 0.95 | 1.50 \pm 2.73 | 14.25 | 0.74 | 1.11x -5.1 |
| Milkoscan (FT 120) vs. Milkoscan (FT 120) | 0.99 | 0.99 | 0.99 | 0.99 | 0.01 \pm 0.52 | 2.70 | 0.80 | 1.02x -0.6 |

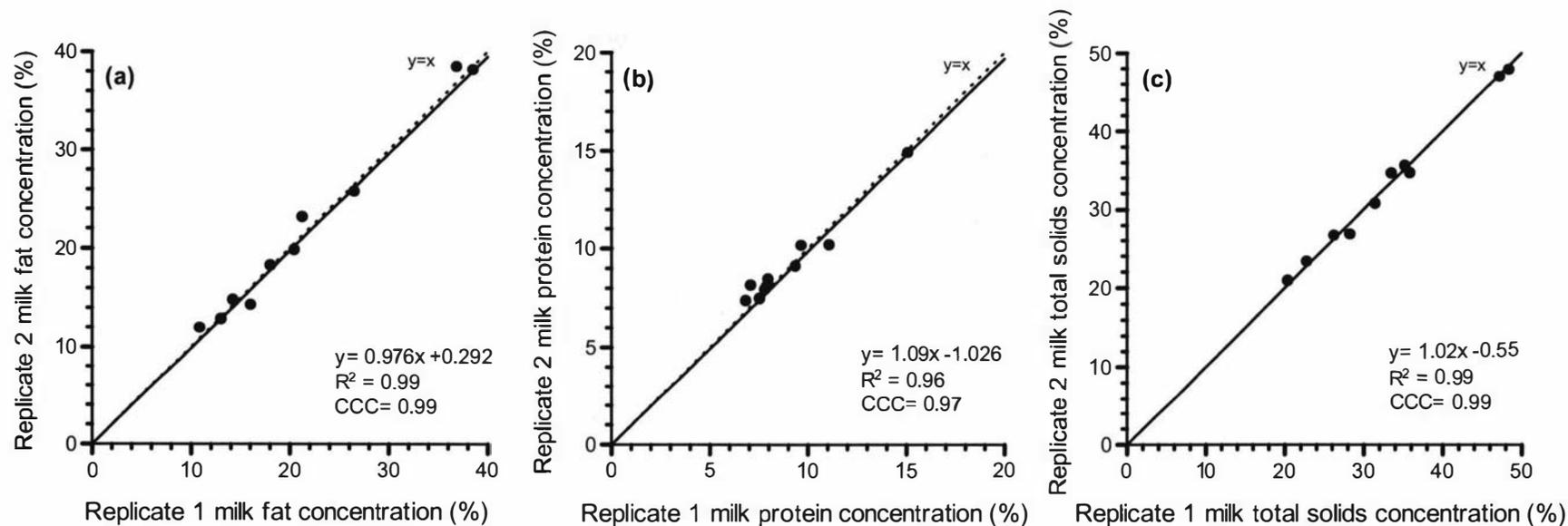


Figure 8. Results of replicated analyses of New Zealand sea lion, *Phocartos hookeri*, milk for (a) fat concentration (%), (b) protein concentration (%), (c) total solids concentration (%), determined with the Milkoscan (FT 120), with the regression equation and line of best fit (solid line) and with the line of equality (broken lines). CCC=Concordance Correlation Coefficient.

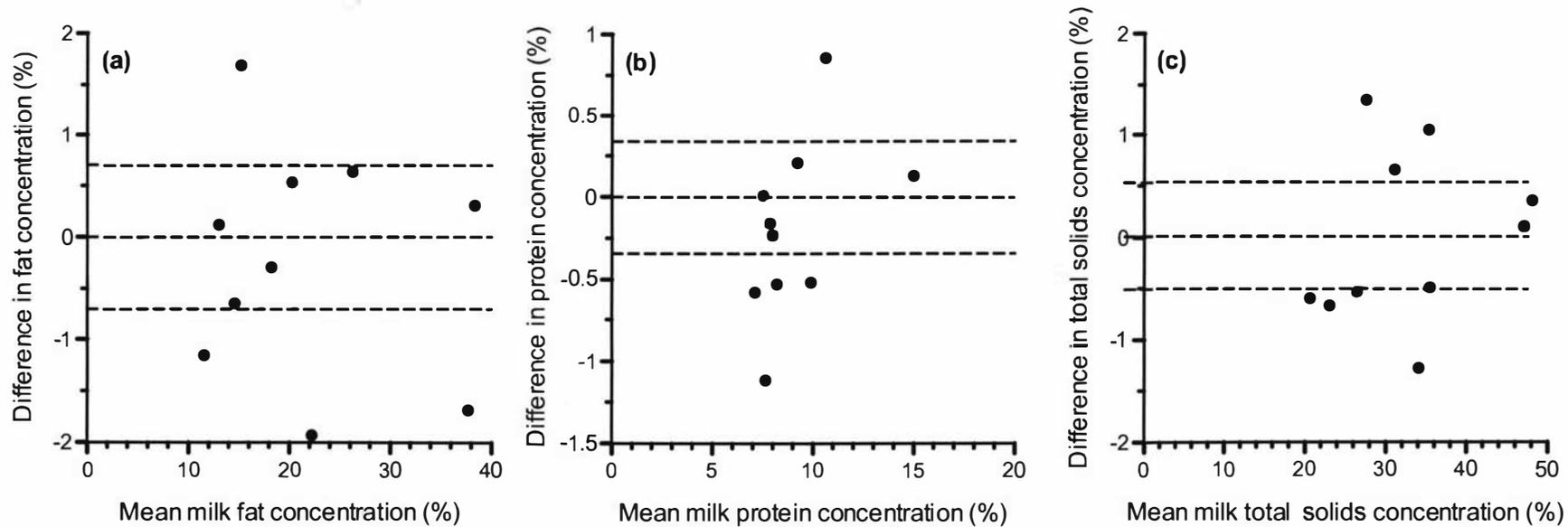


Figure 9. Difference between the results of replicated analyses of New Zealand sea lion, *Phocarctos hookeri*, milk for (a) fat concentration (%) against mean milk fat concentration (%), (b) protein concentration (%) against mean milk protein concentration (%), (c) total solids concentration (%) against mean milk total solids concentration; determined with Milkoscan (FT 120) method, with the 95% limits of agreement (broken lines).

DISCUSSION

The repeatability, reliability, accuracy and precision of the Milkoscan (FT 120) were excellent when determining the concentration of fat, protein and total solids in the milk of the NZSL. Moreover, the performance of the Milkoscan (FT 120) was satisfactory when compared with the standard methods for determining the concentration of fat, protein, and total solids in NZSL milk. However, there were differences between the reference methods and the Milkoscan (FT 120). Such differences could be a result of various effects such as individual, stage of lactation, year of sampling; sample temperature, homogenization and storage, calibration and instrumental, etc, that may have contributed to the overall source variation. Therefore their importance and relevance will be discussed.

Milk fat determination

The repeatability and reliability of all three methods used to determine the concentration of fat in NZSL milk in this study were excellent. Furthermore the comparison between the results from the standard R-G method, and those from the Milkoscan (FT 120) indicated that the Milkoscan (FT 120) is a satisfactory alternative to the R-G method. The mean difference of 0.30% between the milk fat concentrations estimated by the two methods was minimal (Table 9). The replication of repeated analyses was excellent for both methods and although the mean difference between pairs of results from the Milkoscan (FT 120) was higher, this was considered to be non-significant. Notwithstanding, the main difference between the R-G method and the Milkoscan (FT 120) method was shown by a RPE of 20.99% that suggested a poor prediction by the Milkoscan (FT 120) and an acceptable ICC and R^2 . It could be suggested that the prediction by the Milkoscan (FT 120) may be improved by increasing the number of samples that are used for calibrating the instrument or there could be inbuilt instrumental factor that may not be related to having representative samples or a sampling error.

In the analysis of cows' milk, fatty acid composition and the size and the variation in the size of fat globules were the main factors that contributed to the departure of the results between the reference and infrared technique (Sjaunja, 1984a). The homogenization of the milk samples could affect the repeatability of the method. The milk of NZSLs has a wide range of fat content and since the efficiency of homogenization is negatively related to fat content and size of fat globules (Mulder and Walstra, 1974) it may be more difficult to homogenize the milk of NZSLs. Furthermore, failure to homogenize the milk sample will result in variation of the size of the homogenized fat globules and may produce more light scattering (Sjaunja, 1984c) since light scattering increases with the particle size/wavelength ratio (Goulden, 1964). This in turn may have contributed to the discrepancy between the R-G and Milkoscan (FT 120) results and the poor prediction of the latter method. Notwithstanding, NZSL milk has a high content of fat compared to cows' milk and in some of the samples some fat tended to rise rapidly to the surface. Thus great effort and care was taken to thoroughly mix the milk samples by warming and careful manipulation. That this was successful is indicated by the intraclass correlation of 0.96 for the measurement of fat concentration on duplicate samples. Furthermore, there was good agreement between the ASE and R-G methods for determination of fat concentration (Table 9 and Figure 6b) suggesting that sub-sampling was not a problem and that the cause of the discrepancy in the results between the Milkoscan (FT 120) and R-G must lie elsewhere.

Deterioration of milk samples with the release of free fatty acids (lipolysis) due to poor preservation of samples can cause variation between methods in the analysis of cow's milk (Ng-Kwai-Hang *et al.*, 1988). Lipolysis of NZSL milk samples was very unlikely since milk samples were frozen and kept in liquid nitrogen in the field and during transportation to the university and stored at minus 80 °C at the university. The analyses of the bulked samples by the reference method and the alternative method were analysed within 48 hours during which time the samples were kept under refrigeration. If there was any deterioration it could be associated with the freezing of the samples and length of time that they were stored. Storage of frozen milk

samples for long periods of time can cause chemical and physical deterioration, in particular of fat emulsions. When milk samples freeze expanding ice crystals cause pressure that is exerted on the fat globules (Keeney and Kroger, 1974), which eventually results in the destruction of fat globules. This is most obvious in milk with a high concentration of fat such as cream (Sjaunja, 1984b). Such damage might cause fat droplets to coalesce and rise rapidly to the surface causing difficulty in obtaining a representative sub-sample but as discussed above the results from the ASE and R-G indicate sub-samples were representative and repeatable.

The stage of lactation can affect fat determination by the Milkoscan (FT 120) through changes in fatty acid composition (Sjaunja, 1984a). The milk samples for calibration and validation in this study were collected in the same year, from the same pool of females and at the same stage of lactation (early). Therefore it is anticipated that any effect of stage of lactation or season on milk fat composition was minimized and is not responsible for the discrepancies between the results for R-G and the Milkoscan (FT 120). Nevertheless the fatty acid composition was not measured in these samples and future studies should explore the effect of variation in fatty acid composition of NZSL milk on the analysis of fat concentration with the Milkoscan (FT 120).

The performance of the ASE was similar to that for the R-G method. The mean difference between the pairs of results from the ASE method was slightly higher than for R-G method, indicating a higher variability and suggesting that ASE was less efficient at lipid extraction than the R-G method. Nevertheless the results indicate that the ASE is a satisfactory alternative method for determining the fat concentration in pinniped milk. In comparison with the standard R-G method the ASE method has a more rapid extraction time, however, the recent development of an automated version of the R-G method has been described, which increases the speed of analysis significantly (Lee *et al.*, 1989; Matheson and Otten, 1999). The automated R-G can analyze a total of 48 samples in 10 h (Matheson and Otten, 1999) whereas in the present study only 40 samples were analyzed in 10 h with the

ASE. A significant advantage of the ASE method is the reduction of solvent use, and hence cost. In addition, the ASE method does not use hazardous solvents such as chloroform and diethyl ether. Thus while the R-G method will remain the preferred method for calibrating the Milkoscan (FT 120), the ASE method may be used for measuring the fat concentration in milk samples when a Milkoscan (FT 120) is not available or cannot be adequately calibrated.

Milk protein determination

Milk proteins are chemically and physically more stable than milk fat (Kinsella, 1984; Rousseau, 2000); however, the results from the Milkoscan (FT 120) for protein concentration were not identical to those from the Kjeldahl method but still acceptable. The Milkoscan (FT 120) tended to underestimate the protein concentration (Figure 7a) and this could be a product of a systematic change in milk protein quality. For example, proteolysis has been reported to underestimate protein content when using infrared techniques such as the Milkoscan (FT 120) (Sjaunja *et al.*, 1984). Calibration and validation samples were treated in the same manner and stored at the same temperatures, thus any difference in the quality of protein between samples should have been minimal.

The Kjeldahl method determines the protein concentration of milk indirectly by measuring the total nitrogen content including the NPN, therefore variation in the NPN content can affect the estimate of the protein concentration. The Milkoscan (FT 120) was not calibrated for NPN, because the NPN concentration was less than 1.5% of the total nitrogen content of the milk and any variation in the content of NPN would have a negligible affect on the estimation of protein concentration by the Milkoscan (FT 120) for milk protein. The reason for the discrepancies between the Kjeldahl method and the Milkoscan (FT 120) in the estimation of protein concentration is not apparent.

Nevertheless the Milkoscan (FT 120) was very robust for measuring the milk protein concentration and differences between replicates were minimal. We were unable to replicate samples with the Kjeldahl method due to lack of

milk volume and therefore could not assess if this standard method performed equally to the Milkoscan (FT 120). While the Kjeldahl method is still the reference method for calibration, the Milkoscan (FT 120) is a satisfactory alternative for convenient and accurate analysis of the protein concentration in NZSL milk.

Milk total solids determination

The mean difference between results from the Milkoscan (FT 120) and the standard method for the estimation of the concentration of total solids was substantially higher than those observed for the concentrations of fat and protein. However, a Relative Prediction Error (RPE) indicated an acceptable prediction and precision.

The discrepancy between the methods could be associated with systematic errors. The reference method for total solids determination involves heating a sample to dryness and then weighing it and it performs well with both skim milk and whole milk of cows (McDowell, 1972; Boon, 1979). However, it has been suggested that accelerated heating could cause the formation of an impermeable skin on the sample as it dries that may impede complete drying and hence bias in the results. While this has not been identified as a problem with samples of cows' milk (McDowell, 1972; Boon, 1979) and was not observed to happen in with NZSL milk during the present work¹, it cannot explain some of the discrepancy between the results of NZSL milk samples for the Milkoscan (FT 120) and the standard method.

Replicated analyses for total solids demonstrated that the Milkoscan (FT 120) provided accurate and replicable results. Owing to limitations in availability of milk samples the analysis of total solids with the reference method was not replicated, so data on the performance of the standard method for total solids with NZSL milk are not available.

Advantages of the Milkoscan (FT 120)

The Milkoscan (FT 120) has the ability to rapidly analyze milk samples from non domestic animals, such as marine mammals (60 samples/h)¹ with only 1 g of milk required. The Milkoscan (FT 120) does not require the post-sample weighing needed by both the R-G and total solids method, and it provides simultaneous results for fat, protein and total solids, rather than having to process three samples to obtain these data. Milkoscan (FT 120) can be calibrated for NPN and for lactose in the case of milk with lactose content such as reported for some cetacean species (Pilson and Waller, 1970). Although an expensive piece of equipment, the cost of analysis is minimal since no chemical solvents are required for analysis. The Milkoscan (FT 120) is widely used in dairy laboratories around the world which should make them readily accessible to ecologists/biologists that are studying the milk composition of wild animals.

Despite the above mentioned advantages of the automated apparatus, such as that of Milkoscan (FT 120), there are inbuilt instrumental factors that contribute to discrepancies between the reference methods and Milkoscan (FT 120) that have been reported in analysis of cows' milk (Sjaunja *et al.*, 1984). A limitation of the Milkoscan (FT 120) for wild animal studies is that it requires calibration with milk from the species under investigation for which at least 10 milk samples are needed with sufficient (approx. 10 ml) milk to run the standard methods for fat, protein and total solids. Given the difficulties associated with the capture and handling of wild animals under field conditions it may not always be possible to collect adequate milk samples to run such analyses. Nonetheless, for the present thesis (see Chapter 3), more than 300 milk samples¹ were available and the Milkoscan (FT 120) was the method of choice in terms of cost and speed of analyses. As shown by the results the variances created by the methods were not great and the results were within acceptable limits, despite the poor prediction between the

¹ Unpublished data provided by Federico G. Riet Sapriza, IVABS, Massey University, Private Bag 11 222, Palmerston North, New Zealand.

Milkoscan and the R-G method for milk fat determination (Table 9). There is a need to investigate the factors that affects the analysis of milk from wild animals such as that of the NZSL. This study has confirmed that the Milkoscan (FT 120) can be used to analyze the milk of the NZSL and could be used to determine the milk composition of other pinniped species.

Conclusion

The data in this study demonstrate that results from the analysis of milk from NZSL obtained with an appropriately calibrated Milkoscan (FT 120) are comparable with those obtained with standard methods for fat, protein and total solids. This suggest that the Milkoscan (FT 120) could determine the milk composition of other marine mammals including cetaceans, sirenians, phocids or polar bears whose milk contains high concentrations of fat and proteins. The main advantages of the Milkoscan (FT 120) are that a small amount of milk (1 gram of milk) is needed which is suitable for the study of lactation in non domestic animals; however, a minimum of 10 samples are needed for calibration. Furthermore, the Milkoscan (FT 120) is neither time consuming nor uses hazardous chemical substances. Furthermore, cost of analysis is minimal and Milkoscan (FT 120) equipment is very common in dairy laboratories making this technology generally available for ecologists and biologists that are interested with the milk composition of non domestic animals such as marine mammals.

Data also presented in this work indicate that results from the ASE method for milk fat determination are comparable with those using the standard R-G method. In addition, in comparison with the R-G method, ASE extracts fat more rapidly and uses smaller quantities of solvents that are also less hazardous. Thus, it too is a suitable alternative method for use with pinniped milk.

Acknowledgments

The research undertaken in this chapter was supervised by Pádraig J. Duignan (IVABS, MASSEY UNIVERSITY), Nicolas Lopez-Villalobos (IVABS, MASSEY UNIVERSITY), Louise B. Chilvers (Marine Conservation Unit, Department of Conservation), Ian S. Wilkinson (Department of Environment and Climate Change, Australia), Duncan D. S. Mackenzie (IFNHH, MASSEY UNIVERSITY) and Alastair MacGibbon (Fonterra Research Centre). I would like to thank the Department of Conservation (DoC) Southland, and also A. Castinel, W. Hockley and all DoC staff for their assistance in the field. We are grateful to E. Conaghan, R. Richardson, Y. van der Does (Fonterra Research Centre), and to W. Desai and F. Jackson (Institute of Food Nutrition and Human Health, Massey University, Palmerston North) for their invaluable help and their technical support with laboratory analysis.

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CHAPTER 3

GROSS COMPOSITION AND INTER-ANNUAL VARIATION OF THE MILK OF NEW ZEALAND SEA LIONS

ABSTRACT

The gross milk composition and inter-annual variation in lipid and protein concentration was investigated in New Zealand sea lions (*Phocarctos hookeri*) breeding on Enderby Island, Auckland Islands. A total of 384 early lactation milk samples were collected from 288 lactating sea lions over seven austral summer breeding seasons (1997, 1999-2003, 2005). The lactating females' ages ranged from 3 to 23 years. Early lactation is identified as being from approximately four weeks to eight weeks post partum. The mean (\pm SD) of milk components were lipid (19.9 \pm 8.24%), protein (9.4 \pm 2.43%), water (69.1 \pm 9.04%), and ash (0.5 \pm 0.06%). Concentrations of specific minerals such as Ca, K, Mg, Na, and P were determined. Based on the concentration of lipid and protein in the milk, New Zealand sea lions produce the least energy-dense milk (9.8 \pm 3.20 kJ/g) at early lactation than any otariid reported in the literature. There was a significant effect of year of sampling on the concentration of lipid in milk but not on the concentration of protein. Milk fat concentration in the year 2001 was significantly lower than in other years, whereas 2005 was significantly higher. The yearly variation in milk fat concentration could be associated with annual variation in food availability around the Auckland Islands, shift in the energy content of the diet and likely changes in maternal body condition.

INTRODUCTION

The lactation period is the most energetically demanding stage in a mammal's life cycle, and pinnipeds are no exception (Higgins and Gass, 1993; Gales *et al.*, 1996). Pinnipeds, true seals (Family Phocidae) and eared seals (Family Otariidae), secrete milk with a higher fat concentration than that of any terrestrial mammal (Ofstedal, 1988) and other marine mammals including dolphins (Pilson and Waller, 1970; Pervaiz and Brew, 1986) and whales (Gregory *et al.*, 1955). Otariids produce lipid-rich energy-dense milk to enable their pups to deposit blubber as an insulating thermoregulatory layer and as a depot of reserves to sustain them while the mothers are foraging at sea (Bonner, 1984; Trillmich and Lechner, 1986; Arnould and Boyd, 1995a; Werner *et al.*, 1996; Goldsworthy, 1999; Georges *et al.*, 2001). Unlike phocids, that generally fast for the duration of a short lactation period during which milk is produced from stored fat reserves, otariids lactate for a much longer period during which they alternate periods ashore feeding their pup with periods at sea foraging. These two strategies have been referred to as capital (phocid) and income (otariid) breeders (Boyd, 2000).

In otariids, the duration of lactation is highly variable between species (4 months to 3 years) with equally variable milk fat concentration. Marked interspecific variation in attendance patterns between otariid species also accounts for some of the variation in milk fat concentration (Arnould and Boyd, 1995b; Ochoa-Acuna *et al.*, 1999). Antarctic fur seal (*Arctocephalus gazella*) (Arnould and Boyd, 1995b) and northern fur seals (*Callorhinus ursinus*) (Costa and Gentry, 1986) represent high latitude species that have short lactation, long foraging trips, and produce milk with high fat concentration for an otariid (Trillmich and Lechner, 1986). Gentry *et al.* (1986) proposed a latitudinal decline in temporal patterning of energy transfer from mother to pup. The short lactation in high latitude species is associated with the high seasonal productivity of the environment; whereas temperate latitude species such as the Australian sea lion (*Neophoca cinerea*) live in an environment where food is scarce and patchy and are, therefore, obliged to extend their lactation

period (15 to 18 months) (Higgins and Gass, 1993; Gales *et al.*, 1996). The lactation strategy adopted by Australian sea lions results in a long pup dependency during which females make short foraging trips, and produce milk low in fat (Kretzmann *et al.*, 1991), and therefore, daily rate of energy transfer to the pup is low. The New Zealand sea lion (NZSL), *Phocarctos hookeri*, inhabits slightly higher latitudes than the Australian sea lions and, therefore, it is likely that their lactation strategy is intermediate between lower-latitude and higher-latitude otariid species. Taken into account these, we would expect that NZSL would secrete high energy rich milk. Therefore the objectives of this study were (i) to determine the gross chemical composition of NZSL milk and (ii) to investigate the variation in milk composition in early lactation over seven breeding seasons.

MATERIALS AND METHODS

Study Site and animals

The study was conducted at Sandy Bay, Enderby Island, the Auckland Islands (50° 30'S, 166° 47'E), on NZSL during early lactation (January and February) over seven summer seasons, 1997, 1999 to 2003, and 2005. Lactating females in 1997 were captured as part of another project (for details see Costa and Gales, 2000). In 1999, 2000, and 2001 adult females were selected from those on the beach observed to be suckling a pup, whereas in 2002, 2003 and 2005 adult females known to have a pup were selected from a pool of branded/tagged individuals of known age. Animals were only captured after they had been ashore for at least three hours to minimise risk of regurgitation while under general anaesthesia. Captures were made using a specially designed hoop-net (Furhman Diversified, TX, USA) with a multi-layered head end to impede vision and a hole at the apex of the net to allow for the sea lion's nose to protrude. Animals were physically restrained by two handlers once the mouth and the nose were safely in position inside the hole at the apex. Thereafter, the animals were anaesthetized with

isoflurane/oxygen delivered by a face mask from a field portable anaesthetic gas (Isoflurane) vaporizer (Acoma MK III, Japan) (Gales and Mattlin, 1998). Anaesthetised animals were transferred to a restraining board and then measured with a tape to the nearest centimetre and weighed to the nearest 0.5 kg. Oxytocin (0.5 - 2.0 IU/kg) was injected intramuscularly into the gluteal region and milk samples (5-30ml) were collected from one or more teats either by manual expression or using a vacuum pump constructed from the barrel of a 60ml plastic syringe, into a 50ml container. The small amount of milk collected from females did not potentially have an affect on pup. This was based on the fact that e.g. California sea lion (*Zalophus californianus*) pup mean daily milk intake ranged from 609-723 g (Ofstedal *et al.*, 1987b); Antarctic fur seal's pup consumed more than 3000 gram of milk per suckling bout (1-to 2 days feeding bouts) (Arnould *et al.*, 1996) and Northern fur seal's pup consumed 3400ml to 6780 ml per suckling bout (Donohue *et al.*, 2002). Milk samples stood for a few hours at ambient temperature (5 – 10 °C) to let the solids (sand) settle to the bottom, and were subsampled into 2 ml cryovials. The milk was completely recovered and only the sediment, consisting mainly of sand, was left behind. The samples were stored at -196°C in liquid nitrogen. On return from the field site, the milk samples were stored at -80°C. Prior to analysis, samples were stored overnight in a refrigerator at 4°C and then warmed to 32°C in a water bath for 15 minutes. To ensure homogeneity, the milk samples were gently and thoroughly mixed by inverting the cryovials by hand.

Analytical Method

The milk fat, protein and total solids were determined by Fourier transform infrared spectroscopy with a Milkoscan FT 120 (Foss electric A/S, Hillerod, Denmark). The Milkoscan FT 120 was calibrated and validated for sea lion milk (see Chapter 2) with reference methods, for fat with the Roese-Gottlieb standard method (IDF, 1987, 1996), for protein with the Kjeldahl method (New Zealand Dairy Board, 2001) and totals solids by the standard method (McDowell, 1972; Boon, 1979). The data obtained from the reference method

and from the Milkoscan were used to develop a partial least-squares regression and were the basis for the calibration model for the major components of milk (fat, protein and total solids). A coefficient of determination R^2 ranging from 0.94-0.97 indicated an acceptable calibration for the parameters.

In this study, protein content in skim milk was estimated for two reasons. Firstly, because the concentration of protein in skim milk is a better indicator of its concentration in the secretion released by the secretory vesicles; and secondly, because the concentration of protein in the whole milk is inversely related to the concentration of fat, which is affected not only by the amount of fat secreted but also by whether foremilk or hind milk is sampled (Mephram, 1977).

Gross energy content (kJ/g) of the milk was calculated by multiplying the derived chemical composition by standard values for the energy density of lipid (38.12kJ/g) and protein (23.64kJ/g) (Perrin, 1958). Milk was analysed for minerals (Ca, K, Mg, Na, and P) by elemental analysis using inductively coupled plasma optical emission spectrometry (AOAC, 2000). Milk samples ($n= 25$), five for each year from 1999 to 2003 and from females ranging in age from 4 to 16 year old, were selected to obtain a representative sample to cover the age range for mineral analyses.

Data analysis

All statistical analyses were performed using SAS (Statistical Analysis System, version 9.1; SAS Institute Inc., Cary, NC, USA). The concentration of individual components in milk were analysed using the MIXED procedure with a linear model that included the fixed effects of year and the random effect of animal. Correlation coefficients (r) between variables were estimated using the CORR procedure. Correlations were considered to be significant if $P<0.05$ when testing the null hypothesis $r=0$.

RESULTS

A total of 384 milk samples were collected from 288 sea lions aged between 3 and 23 years (Table 10), the age for most of the females captured in 1997 was 6 years old. The females were aged from sections taken from the first post canine tooth (Childerhouse *et al.*, 2004). Fifty females were sampled twice and in the latter four years, the first samples were collected mid January and the second in mid-February. Females in 1997 were captured in January and February.

The mean values for the gross chemical composition of milk in early lactation over seven seasons are summarized in Table 11. The sum of all the components measured in the milk (fat + protein, + minerals+ the estimated water content) accounted for a mean of $99.24 \pm 3.37\%$ (mean \pm SD; $n=381$) of the milk mass. This indicates that the milk contained only a small amount of carbohydrate. The milk samples in this study were not analysed for lactose or other carbohydrates. There was more than a 7-fold range in the values for fat content and a 6-fold range for protein content (Table 11). On a fat-free basis (skim milk), the range of protein concentrations was approximately 10 fold (Table 12). The molar concentrations of the ions are presented in Table 12, and the mean ratio of $K^+ : Na^+$ was 0.89.

There was a strong negative correlation between the water content and lipid concentration ($r = -0.90$, $P < 0.01$, see Figure 10a) whereas the correlation between water content and protein concentration was not significant ($r = -0.59$, $P > 0.01$, see Figure 10b). The correlation between water content and milk energy content was negative and significant ($r = -0.93$, $P < 0.01$, see Figure 10c).

Year had a significant effect on the gross energy content of NZSL milk (ANOVA, $P < 0.001$) (Figure 11a). Year 2005 had significantly higher milk energy content than other years (ANOVA, $P < 0.05$), in contrast 2001 had the lowest energy content and was significantly different from other years

(ANOVA, $P < 0.05$). Consequently, the energy content in 1997, 1999, 2002 and 2003 were not significantly different from each other (ANOVA, $P < 0.05$). The energy content in 1997 was significantly higher than in 2000 (ANOVA, $P < 0.05$).

Table 10. Number of lactating New Zealand sea lions, *Phocarctos hookeri*, sampled either once or twice in early lactation and collected over seven summer seasons at Sandy Bay, Enderby Island, Auckland Islands and the number of milk samples analysed.

| Year | Females sampled frequency | | Samples analysed |
|--------|---------------------------|-------|------------------|
| | Once | Twice | Number |
| 1997 | 27 | 7 | 41 |
| 1999 | 56 | - | 56 |
| 2000 | 85 | - | 85 |
| 2001 | 81 | 3 | 87 |
| 2002 | 4 | 18 | 40 |
| 2003 | 18 | 13 | 44 |
| 2005 | 13 | 9 | 31 |
| Totals | 284 | 50 | 384 |

The average concentrations of lipid in whole milk and protein in the skim milk of the sea lions varied with year (Figure 11b). The concentration of lipid in the milk collected in 2001 was significantly lower than all other years (ANOVA, $P < 0.05$). The concentration of lipids in milk samples for the year 2000 was significantly lower than that from 2005 (ANOVA, $P < 0.05$) but not significantly different from that of other years except for 2001 (ANOVA, $P > 0.05$). The concentrations of lipids in milk collected in 1997, 1999, 2000, 2002 and 2003 were not significantly different from each other (ANOVA, $P < 0.05$).

There was an effect of year of sampling on the concentration of protein in skim milk (ANOVA, $P < 0.001$) (Figure 11c). The protein concentration in skim milk in 1997 was significantly higher than in 2000, 2001, 2002 and 2003 (ANOVA, $P < 0.05$) but not significantly different from 1999 and 2005 (ANOVA, $P > 0.05$). The lowest concentration of protein in skim milk was observed in the season of 2002 followed by 2003; however, the difference was not significant

(ANOVA, $P > 0.05$) (Figure 11c). In three consecutive years 2001, 2002 and 2003 the concentration of protein in skim milk was significantly lower than the concentration in 2005 (ANOVA, $P < 0.05$).

Table 11. Mean gross chemical composition of whole milk of New Zealand sea lions, *Phocarctos hookeri*, collected in early lactation over seven seasons at the breeding site of Sandy Bay, Enderby Island, Auckland Islands.

| | n | Mean | Standard deviation | Range |
|---------------------|-----|--------|--------------------|---------------|
| Lipid (g/kg) | 382 | 19.9 | 8.24 | 6.03 – 44.81 |
| Protein (g/kg) | 381 | 9.4 | 2.43 | 3.24 – 20.83 |
| Water (g/kg) | 384 | 69.1 | 9.04 | 37.61 – 88.42 |
| Total Solids (g/kg) | 384 | 30.8 | 9.12 | 11.58 – 82.39 |
| Gross Energy (kJ/g) | 381 | 9.8 | 3.20 | 4.83 - 22.22 |
| Ash (k/kg) | 25 | 0.5 | 0.06 | 0.39 - 0.59 |
| Calcium (mg/kg) | 25 | 764.1 | 161.66 | 520 - 1090 |
| Potassium (mg/kg) | 25 | 1530.4 | 395.16 | 875 - 2430 |
| Magnesium (mg/kg) | 25 | 132.3 | 29.77 | 75.9 - 156 |
| Sodium (mg/kg) | 25 | 1015.1 | 206.55 | 668 - 1430 |
| Phosphorus (mg/kg) | 25 | 1326.5 | 223.86 | 962 - 1760 |

Table 12. Concentrations of protein and major cations in skim milk of New Zealand sea lions, *Phocarctos hookeri*, in the early lactation period collected over seven seasons at Sandy Bay, Enderby Island, Auckland Islands.

| | n | Mean | Standard deviation | Range |
|-----------------------------|-----|------|--------------------|-------------|
| Protein in skim milk (g/kg) | 381 | 11.9 | 3.73 | 3.63-36.06 |
| Calcium (mM) | 25 | 22.9 | 6.12 | 14.55-35.55 |
| Potassium (mM) | 25 | 46.2 | 10.91 | 24.66-70.50 |
| Magnesium (mM) | 25 | 6.7 | 1.83 | 3.59-10.0 |
| Sodium (mM) | 25 | 51.7 | 8.77 | 31.98-83.37 |

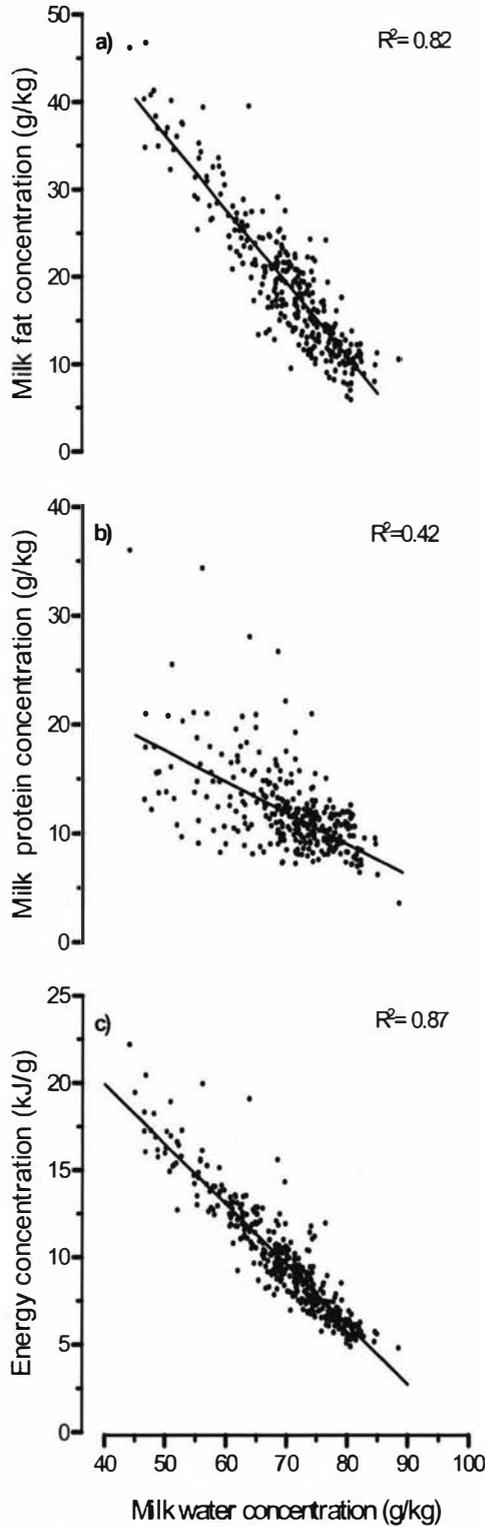


Figure 10. The correlation between a) lipid (g/kg), b) protein in skim milk (g/kg) and c) gross energy (kJ/g) and the water content (%) of milk from New Zealand sea lions, *Phocarcos hookeri*, in early lactation over 7 years (1997-2005) at Sandy Bay, Enderby Island, Auckland Islands.

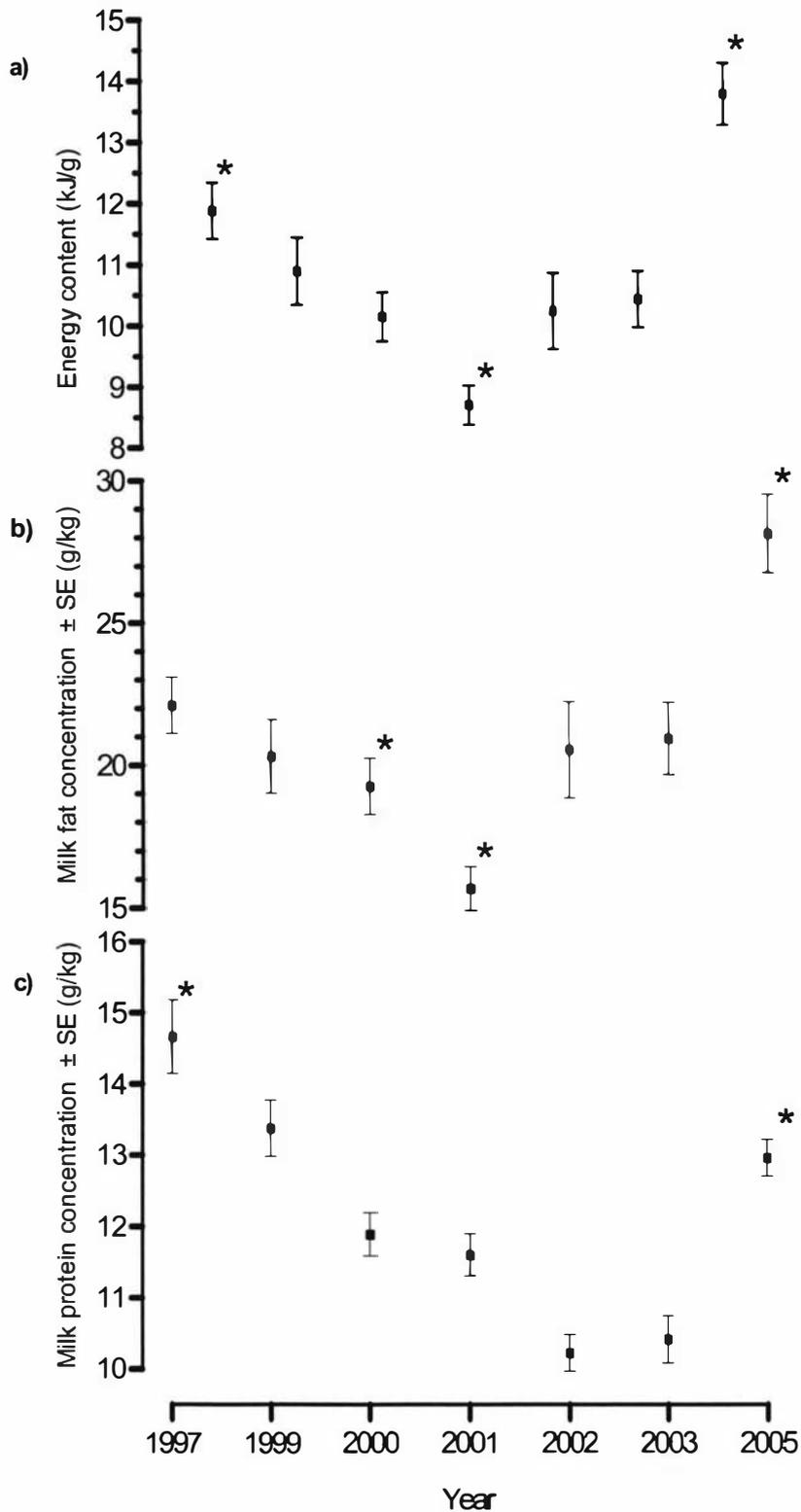


Figure 11. The mean concentrations (\pm SE) for a) gross energy (triangle) content (kJ/g), b) lipid (square) in the whole milk and c) protein (circle) in the skim milk of milk of New Zealand sea lions, *Phocartos hookeri*, in early lactation over seven summers from 1997, 1999 to 2003, and 2005 at Sandy Bay, Enderby Island, Auckland Islands.

DISCUSSION

This is the first investigation of milk composition in the NZSL based on a large number of free living animals sampled over a seven year period. The most significant finding is that the mean milk lipid concentration and mean energy content in early lactation is the lowest reported for any otariid while protein levels are comparable to other species (Figure 12). NZSL, Galapagos sea lions (Gentry *et al.*, 1986; Trillmich and Lechner, 1986) and Australian sea lion (Kretzmann *et al.*, 1991) are consistent with the theory that species that make short foraging trips and have relatively long lactation periods produce milk with a low concentration of fat. Both lactating NZSL and the Steller's sea lion (*Eumetopias jubatus*) (Steller's sea lions) make short foraging trips (36 hrs) and produce a milk with a low content of fat, and Steller's sea lions deliver small amount of milk energy per visit (Higgins *et al.*, 1988). By contrast, lactating northern fur seals (northern fur seals) make foraging trips of 7 days and forage at a distance of 100 km offshore (similar distance travelled by NZSL) and deliver a larger amount of milk energy (Gentry and Holt, 1986). However, while making shorter trips to sea may be energetically economical for NZSL it is not for northern fur seals, as the larger size of the sea lion allows them to travel at higher speed than fur seals (Gentry *et al.*, 1986) and thus reduce the ratio of travel cost to energy gained in the foraging area (Trillmich, 1986b). It is possible that NZSL have adopted an intermediate lactation strategy between polar and tropical species as in temperate fur seals species. Gentry *et al.* (1986) suggested that temperate fur seals are closer to "tropical" species in their lactation strategy; it is unclear if NZSL are consistent with this theory.

The fact that, NZSL lactating females perform deep dives, near their physiological limits (Costa and Gales, 2000; Chilvers *et al.*, 2006), in an environment where there is great potential for competition for food resources with fisheries suggests that NZSL may be living in a marginal habitat (Chilvers *et al.*, 2006), and this may explain their tendency to produce low-energy milk. However, this may not be the only explanation for the low fat milk of NZSL;

therefore, other factors such as stage of lactation, maternal body condition, age, attendance patten that may influence the milk composition should be considered.

Carbohydrates and minerals

The milk samples were not analysed specifically for carbohydrates because they are generally a minor component of otariid milk (Dosako *et al.*, 1983; Arnould and Boyd, 1995a; Urashima *et al.*, 2001). Previous studies have shown that they may be contribute to 0.70% of the total mass of the milk unaccounted for by the sum of water, lipid, protein and ash content. Because lactose and other carbohydrates are in low concentration in pinniped's milk (Peaker, 1977; Dosako *et al.*, 1983; Urashima *et al.*, 2001) it is thought other solutes must be present to maintain osmotic equilibrium across the epithelium. Therefore, it is likely that, in comparison with terrestrial mammals, ions such as K^+ , Na^+ and Cl^- make a greater contribution to the osmotic pressure of the milk (Peaker, 1977). Our results are consistent with this hypothesis in that the concentrations of K^+ (1530 mg/kg) and Na^+ (1015 mg/kg) are high and similar to those reported in other pinniped species: California sea lions (*Zalophus californianus*), northern fur seals (Dosako *et al.*, 1983), Galapagos fur seals (*Arctocephalus galapagoensis*) (Trillmich and Lechner, 1986), northern elephant seal (*Mirounga angustirostris*) (Boeuf and Ortiz, 1977; Riedman and Ortiz, 1979), harp seal (*Phoca groenlandica*) (Webb *et al.*, 1984). The mean molar concentrations of K^+ and Na^+ , in the skim milk were 46 and 52 mM, respectively, which, together with associated anions, would account for approximately 200mOsm of osmotically active solutes in the milk. While this is a substantial proportion of that expected, there would also be some contribution from the Ca^{++} and Mg^{++} that are not bound to proteins. It appears there is still a shortfall from the expected osmolality of approximately 280 – 300 mOsm, but solutes that contribute to the balance of the osmotic pressure remain unidentified.

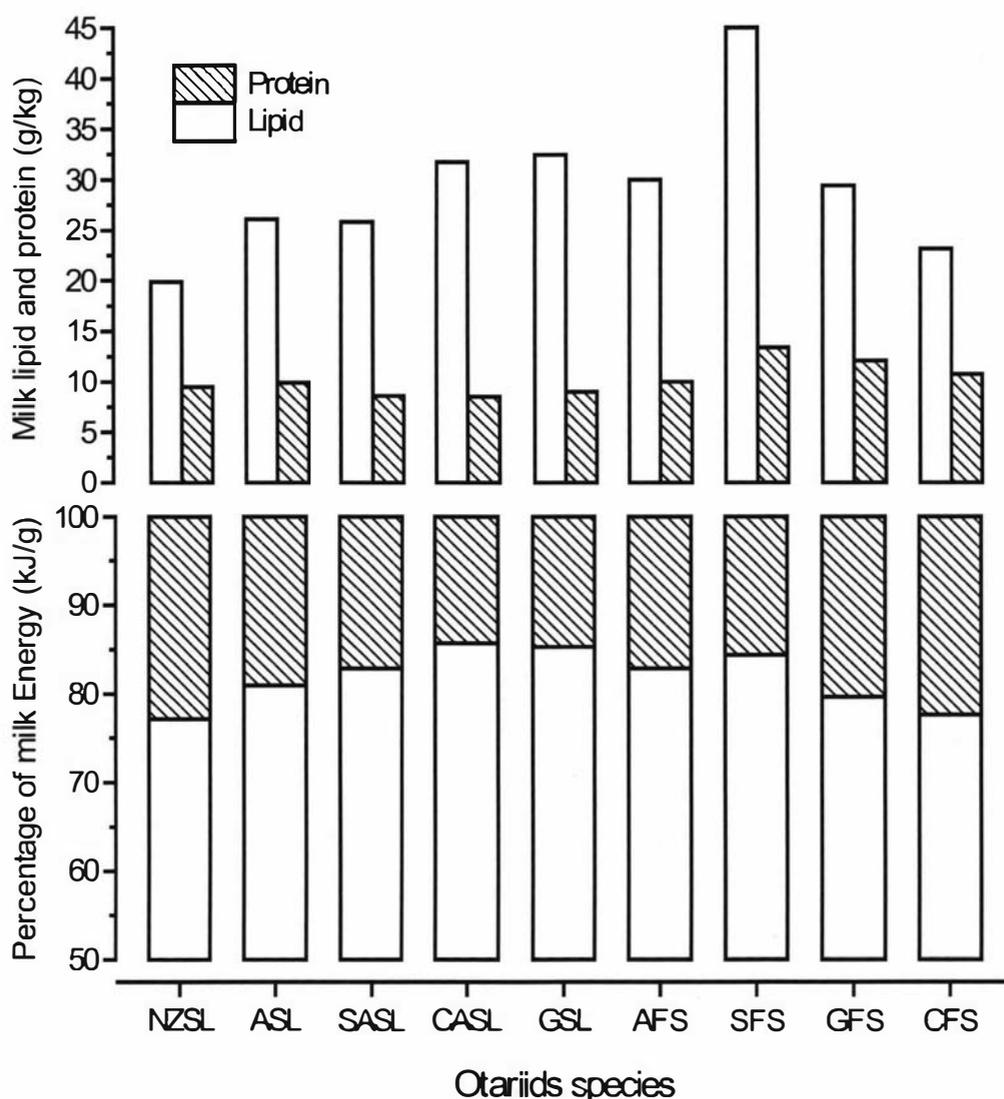


Figure 12. Milk lipid and protein concentrations (g/kg) and relative contribution of lipid and protein to total milk energy (kJ/g) in some otariids (sea lions and fur seals) species. The energy content of the milk was calculated from standard caloric values of lipid (38.12 kJ/g) and protein (23.64 kJ/g) (Perrin, 1958). Lipid and protein values for each species were obtained from the literature as follow: NZSL, New Zealand sea lions, *Phocarctos hookeri* (this study); ASL, Australian sea lions, *Neophoca cinerea* (Gales *et al.*, 1996); SASL, South American sea lions, *Otaria byronia* (Werner *et al.*, 1996); CASL, California sea lions, *Zalophus californianus* (Oftedal *et al.*, 1987a); GSL, Galapagos sea lions, *Zalophus californianus wollebaeki* (Trillmich and Lechner, 1986); AFS, Australian fur seals, *Arctocephalus pusillus doriferus* (Arnould and Hindell, 1999); SFS, Subantarctic fur seals, *A. tropicalis* (Georges *et al.*, 2001); GFS, Galapagos fur seals, *A. galapagoensis* (Trillmich and Lechner, 1986); CFS, Cape fur seals, *A. pussillus pussillus* (Gamel *et al.*, 2005).

In many terrestrial species, milk contains a molar ratio of K^+ to Na^+ of approximately 3:1, which is similar to that of intracellular fluids (Peaker, 1977). A lower ratio suggests that there is substantial leakage across the glandular epithelium through paracellular pathways (Peaker, 1977, 1978). The K^+ : Na^+

ratio in the present study was 0.89:1 which is similar to that for California sea lion and northern fur seal (Dosako *et al.*, 1983). The coefficient of variation for sodium concentration was 17%, which is lower than that for potassium (23%), calcium (27%) and magnesium (27%) indicating that the variance of sodium concentration was not exceptional. It is considered unlikely that the concentration of sodium is elevated by contamination with exogenous salt (from sand) during collection of the milk sample. Care was taken to prevent this from occurring and the sample sizes were generally large, in excess of 15 ml therefore any contamination with exogenous salt, if any, would have been diluted and minimised by large amount of milk collected.

Milk sampling bias

A number of factors associated with the collection of milk samples from animals, including pinnipeds, captured in the wild can potentially lead to biases in the results. The fat concentration of the milk is of particular concern in that it is well established for terrestrial mammals that the milk first released ('fore milk') from the mammary gland has a lower fat concentration than the milk released last (Oftedal, 1984). However from the few data available it is not clear if this applies for pinnipeds (Oftedal, 1984; Oftedal *et al.*, 1987a). The sampling regime of this study was consistent between years and with the methods used on other pinniped species, therefore, comparisons are considered valid.

To avoid the problems associated with variation in the amount of milk removed, it has been suggested that milk collection should be conducted at a standardised time after last milk removal by the pup, and the amount collected should be the same as in normal suckling conditions (Oftedal, 1984). In this study, female sea lions were allowed three hours onshore before capture to minimise the risk of regurgitation during anaesthesia. We are confident that the interval between the arrival of the female ashore and the milk collection time allowed for the pup to suckle the fore milk. As a result, our milk samples

were unlikely to be from the fore milk and did represent the true milk composition of NZSL.

Inter-annual variation in milk composition

There has been only three previous report on the inter-annual variation in milk composition in otariids, which was for Antarctic fur seals and Cape fur seals (Arnould and Boyd, 1995a; Lea *et al.*, 2002; Gamel *et al.*, 2005). Lea *et al.* (2002) associated the inter-annual variability in milk lipid concentration to diet whilst Gamel *et al.* (2005) did not report any significant changes in milk lipid concentration. Arnould and Boyd (1996a) reported that the highest mean concentration of fat and energy content in milk of Antarctic fur seals over the three year period corresponded to the year of low food availability and was associated with significantly longer maternal foraging trips in that year (Lunn *et al.*, 1993). In the poor food year, daily milk energy consumption and milk production was lowest and the high lipid concentration in milk did not compensate for longer absence of the mothers (Arnould *et al.*, 1996; Arnould, 1997). There were consequences associated with low food availability such maternal weight and body condition, reduced pup growth and weaning mass, and higher pup mortality due to starvation (Lunn and Boyd, 1993; Lunn *et al.*, 1993; Arnould *et al.*, 1996; Arnould, 1997).

It is likely that concentration of lipids in NZSL milk will be affected by the stage of lactation and by the length of foraging trips as in other otariid species. An increase in milk lipid concentration during lactation has been reported in the California sea lions (Oftedal *et al.*, 1987a); Antarctic fur seals (Arnould and Boyd, 1995a); South American fur seals, (*Arctocephalus australis*) (Ponce de Leon, 1984); Australian fur seals (Arnould and Hindell, 1999); and Australian sea lion (Gales *et al.*, 1996). The increase in milk lipid concentration in these species was ascribed to the increased time spent in foraging at sea with the progression of lactation and the need to conserve body water when on an extended trip (Boyd *et al.*, 1991; Higgins and Gass, 1993). Data on the effect of stage of lactation on the length and frequency of foraging trips in relation to

milk composition in the NZSLs have been tested to see if they conform to this general pattern (see Chapter 4).

There was a considerable annual variation in milk fat concentration but not in protein concentration and this may suggest a qualitative or quantitative variation in prey availability (Figures 11b and 11c). In 2002 and 2003 milk fat concentration was not different from that of 1997, 1999 and 2000, but the milk protein concentration was at its lowest for the 7 years (Figures 11b and 11c). The low concentration of protein in the milk suggests that females were malnourished during lactation while the increase in fat concentration is consistent with them still being able to draw on body lipid reserves. A fall in protein concentration while maintaining fat concentration in the milk is indicative of serious underfeeding in dairy cows that are in good or moderate body condition early in lactation (Grainger *et al.*, 1982; Thomson *et al.*, 1997). Therefore, if lactating NZSLs followed the same pattern observed in dairy cows, limited food resources during early lactation may explain the low protein concentration. An important point that should be considered is that in 2002 and 2003 the energy content of milk did not differ from 1997, 1999 and 2000 which indicated that females compensated for the lower energy provided by the lower protein content in the milk was by secreting milk with higher fat content. Thus it appears that during periods of food scarcity, female sea lions will compensate for reduced intake by drawing on body resources to maintain milk quality. At lower latitudes the opposite appears to occur in the “tropical” (not a truly tropical species) Galapagos fur seal that lives in an unpredictable (due to frequent El Niño events) but highly productive upwelling environment. In response to the constant threat of starvation, lactating Galapagos fur seals appear to limit energy expenditure to increase their own survival (Trillmich and Kooyman, 2001). Furthermore it has been predicted that temperate and “tropical” fur seals secrete low milk fat and the onshore energy cost of milk production is lower than for sub polar fur seal species such as northern fur seals (Gentry *et al.*, 1986). It could be predicted that this is true for sea lions and that lactating NZSL devoted small amounts of energy for the production of milk.

In 2001, lipid and energy content in milk was the lowest of any year and protein concentration in skim milk was not significantly different from that in 2002 and 2003 when protein concentration was also at the lowest yearly averages recorded. Furthermore these concentrations of protein were significantly lower than those in 1997 and 2005 (Figure 11c). In dairy cows the relationship between body condition, feed intake and milk composition has been extensively studied; low fat and protein concentrations are indicative of an animal in low body condition that is currently underfed (Grainger *et al.*, 1982; Thomson *et al.*, 1997). In addition to data on milk composition, information on pup growth and body condition of the females could be used as a proxy for food availability around the Auckland Islands and sub Antarctic latitudes during the summer of 2001.

In concordance with the poor quality of milk in 2001, pup growth rates were lower than in the subsequent two summers (2002 and 2003) and were below the average for growth rates for females and males pups (Chilvers *et al.*, 2007). The most significant cause of offspring mortality is trauma while starvation is the second most significant cause of mortality in pups (17.0%) in non-epidemic years and in 2001 malnutrition accounted for 25% of pup deaths which was the greatest in the four subsequent seasons but lower than 2000 (38%) (Castinel, 2007). During a strong El Niño event (1989) lactating South American fur seals spent longer at sea searching for food and less time nursing their pup ashore. High pup mortality during the first 3 weeks (42%) of life, and poor body condition of the pup was an indication that the short time spent ashore nursing their pup coupled with possible lower milk quality and production did not compensate for the longer absence at sea (Trillmich, 1986a). Starvation and low growth in NZSL pups supports the hypothesis that, there was no compensation for the low milk fat, because it is very likely that daily milk energy consumption and milk production were not maximised and lactating NZSL did not extend their stay on land nursing their pup. These hypotheses could be tested by investigating the attendance patterns, daily milk production and consumption in NZSL.

The growth rates of NZSL's pup were studied in the first 3 months after birth and during the summer season of 2001, 2002 and 2003 (Chilvers *et al.*, 2007). In contrast to 2001, the average pup growth rates in 2002 were the highest followed by 2003 (Chilvers *et al.*, 2007). Despite the observation that protein concentration was the lowest in 2002, the average pup growth rates in 2002 were the highest followed by 2003 and 2001 (Chilvers *et al.*, 2007). We hypothesized that pup growth rates were better in 2002 but because of the low milk protein concentration, pups were still underfed. Mothers were also underfed during early lactation as suggested by the low protein concentration compared to 1997 and 2005; however they had sufficient body reserves (i.e., better body condition) to be able to draw on lipids to produce high milk fat. A possible scenario is that milk yield was depressed and that pup growth rates may be sub maximal. Decrease in the milk yield or milk output but not a significant change in the milk composition has been observed in California sea lions during a period of low food availability (El Niño event in 1983-84) (Iverson *et al.*, 1991). The milk yields in NZSL in relation to local food sources wait to be determined.

Milk energy content was significantly higher in both 2002 and 2003 than 2001 and may explain the higher pup growth rates. Interestingly, 2002 had a significantly lower birth rate and high pup mortality compared to previous seasons (Wilkinson *et al.*, 2006). The main causes of high mortality in 2002 were, in order of magnitude, bacterial infection, trauma and starvation (12.5%) (Castinel, 2007). Therefore it appears that underfeeding was not a major problem for pups in 2002, compared to 2001 and 2003, as demonstrated by their growth rates. The growth rates in 1997 and 2005 (years of highest energy content) were unknown and therefore could not be compared with 2002. Starvation in 2005 caused 16% of the pup mortality the highest in seven years (Castinel, 2007) this may indicated a decrease in milk yield in that year. Offspring mortality rate (31.1%) in 2002 was the highest reported in seven years (Castinel, 2007) and thus females that lost their pup or did not reproduce in 2002, should have been in better body condition to nurse their pup in 2003, as they were not burdened with the energetic costs of lactation in 2002. This argument may not apply to the situation in 2002, mortality rates in

2001 were within the acceptable (10.9%) therefore this may not be responsible for the mother being in better body condition as it was suggested in data on maternal body condition is available to test this predictions.

The 2002 and 2003 season had similar milk energy content; however, growth rates were significantly higher in 2002 indicating that the costs of lactation and gestation had consequences for the performance of lactating females in 2003. This explanation is contradictory because 2002 had high early pup mortality thus females who lost their pup should have been in better condition the following season. A viable explanation for the lower growth rates of pups in 2003 may be that females were unable to deliver the quantity of milk to meet the demands of the pup in that year and/or that gestation may have a high cost for females. The cost of lactation and the influence of NZSL maternal body condition in milk composition and production may answer some of these questions.

In 2001 the low milk fat concentration (Figure 12) and low pup growth rates (Castinel, 2007) indicated that females pupped in poor body condition and were unable to draw on body reserves to produce energy rich milk and normal milk yield. Although total pup mortality rate was considered normal in 2001 (8.7%), starvation claimed 25% of the pup mortality, one of the highest level recorded (Castinel, 2007), may support the idea of underfeeding of pup and/or lower milk yield.

In 1997 and 1999 milk fat and protein concentration were above average. We have no data on growth rates of 1997 and 1999 and mortality rates of 1997. The lowest mortality rates (6.4%) in seven years for the 1999 season in addition to the high fat and protein concentration in milk support the idea that mothers were in good body condition and were well fed during early lactation. This was further supported by the fact that pup production and pup mortality (60%) were high in 1998 (Wilkinson, 2003) and females that lost their pups would be expected to be in good body condition in the next season (1999). Thus, it is suggested that the females were well fed during gestation and lactation and did not draw extensively on body reserves in early lactation.

The last year of this study, 2005 could be considered as a normal year and the milk composition demonstrates that NZSL are able to produce high fat milk and within the range reported for other species of otariid in early lactation (Figure 12). In that year pup production was low and pup mortality was considered within the normal range while starvation was responsible for 16% of the total deaths (Castinel, 2007). It is unknown whether the highest fat concentration and energy content in 2005 milk was a consequence an increase in foraging trip duration in relation to local food shortage as reported for Antarctic fur seals (Lunn *et al.*, 1993; Arnould and Boyd, 1995a). It could be suggested that that availability of food increased in 2005 and this idea could be supported with data in fish catch around the Auckland Islands.

There is also evidence to indicate that NZSL live in a marginal habitat where they compete for food with the squid fishery, and there are inter-annual fluctuations in food availability. If this is indeed the case, then reproductive success in this species may be compromised by the annual variation in the quantity and quality of milk provided to pups. Management recovery plans for the NZSL population should consider the potential for, and implications of resource competition, and further research should examine the lactation strategy of NZSL.

Conclusion

The data in the present study indicates that milk composition of NZSLs is generally similar to the milk of other otariid species; however, the milk fat and hence the energy content of milk is the lowest reported in this group of pinnipeds. There is strong evidence that milk composition of NZSLs is affected by environmental factors associated with year effect and likely other unidentified factors. The differences in milk composition coupled with data on pup mortality and growth across years over the period from 1997 to 2005 provide strong evidence to support the concept that milk yield was severely compromised in some years.

Acknowledgments

The research undertaken in this chapter was supervised by Pádraig J. Duignan (IVABS, MASSEY UNIVERSITY), Nicolas Lopez-Villalobos (IVABS, MASSEY UNIVERSITY), Louise B. Chilvers (Marine Conservation Unit, Department of Conservation), Ian S. Wilkinson (Department of Environment and Climate Change, Australia), Duncan D. S. Mackenzie (IFNHH, MASSEY UNIVERSITY) and Alastair MacGibbon (Fonterra Research Centre). The research undertaken in this chapter was carried out in collaboration with Dan Costa (Department of Ecology and Evolutionary Biology, University of California, USA) and Nick Gales (Australian Antarctic Division, Australia) who provided the milk samples and data from females and pup in 1997. I would like to thank A. Castinel and all DoC staff who assisted in the field. We are grateful to E. Conaghan, R. Richardson, D. Kreegher and Y. van der Does (Fonterra Research Centre) for their invaluable help and their technical support with laboratory analysis at Fonterra Research Centre, Palmerston North, New Zealand.

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CHAPTER 4

RELATIONSHIP BETWEEN THE GROSS MILK COMPOSITION AND MATERNAL CHARACTERISTICS IN NEW ZEALAND SEA LIONS

ABSTRACT

The relationship between maternal characteristics (age, body mass, length and body condition index), offspring characteristics (pup age and sex), maternal attendance (time at sea and ashore) and milk composition were investigated in New Zealand sea lions, *Phocarctos hookeri*, at the subantarctic Auckland Islands. The intra annual and the monthly variation in maternal characteristics were also investigated. Records were collected during 7 years including 1997, 1999-2003 and 2005. A maternal body condition index (BCI) was calculated as the difference between the actual value of body weight and the predicted value from the linear regression of body mass (kg) on body length (cm). The maternal attendance pattern was measured using radio telemetry (VHF transmitter). The means (\pm SD) of maternal age, BCI, body weight and length for seven years in early lactation were 10.7 ± 4.0 years old ($n=397$), -0.001 ± 10.1 kg/cm ($n=403$), 112.0 ± 15.30 kg ($n=406$) and 177.6 ± 7.3 cm ($n=403$), respectively. Milk lipids were found to be correlated with protein concentration in skim milk ($r=0.46$), milk total solids concentration ($r=0.89$) and milk energy content ($r=0.98$). BCI was significantly correlated with milk lipid concentration ($r=0.19$, $P<0.01$). There was a significant effect of month on concentration of lipids in milk, BCI, and maternal weight. The sex of the pup had a significant ($P<0.05$) effect on maternal body condition and to some extent in milk composition. There was a significant ($P<0.05$) variation in maternal body condition, body weight and body length. Older mothers and those in good body condition produced milk of higher lipid content suggesting that individual experience in rearing a pup, contributed to enhanced milk quality. Mothers nursing male pups had lower maternal body condition and produced milk with lower concentration of lipids suggesting that mothers were poorly prepared to invest more in a male pup than in a female pup. Maternal body condition varied between years suggesting that local food resources or other sources influenced the reproductive success of NZSL.

INTRODUCTION

Lactation studies of pinnipeds (seals, sea lions and fur seals, and walrus) have been based on measuring the gross chemical composition of milk during lactation and relating changes to the patterns of maternal foraging and variations in body condition (Oftedal, 1984). However the relationships between milk composition and the effect of various factors such as dietary constraints, physiological adaptations and offspring size are also likely to be important and are not well understood.

The milk composition of most species of otariids has been reported, however several authors (Oftedal and Iverson, 1995; Schulz and Bowen, 2004) reviewing the data have concluded that further data are required. Changes in milk composition throughout lactation (Arnould and Boyd, 1995a) and in relation to the maternal attendance pattern (Arnould and Boyd, 1995b) have been reported, but the influence of other factors such as maternal body condition, maternal age and offspring age and sex, which may potentially influence the composition of milk (Landete-Castillejos *et al.*, 2003; Landete-Castillejos *et al.*, 2004), have not been investigated in detail in otariids.

Maternal attendance patterns describe how lactating females partition their time between feeding at sea and nursing their pup on shore. Few studies have investigated the changes in milk composition in relation to attendance pattern in otariids (Costa and Gentry, 1986; Trillmich and Lechner, 1986; Costa, 1991; Arnould and Boyd, 1995b) or in terrestrial mammals (Kunz *et al.*, 1995; Tilden and Oftedal, 1997). Maternal attendance patterns reflect maternal investment in the young and they may provide an indirect measure of the foraging conditions during lactation (Melin *et al.*, 2000). For example, in years of low food supply lactating females of Antarctic fur seals (*Arctocephalus gazella*) increased their foraging duration (Lunn *et al.*, 1994) although survival, growth rate and weaning mass of the pups were still low (McCafferty *et al.*, 1998). The concentration of lipid in the milk was influenced by the duration of foraging trips in the Antarctic fur seals (Arnould and Boyd,

1995b) and it has been proposed that this is an adaptation to maximize the quantity of energy which is eventually delivered by the female to the pup (Trillmich and Lechner, 1986).

New Zealand sea lions (NZSLs) lactate for about 9 to 12 months while making short foraging trips to sea (on average 2.9 days at sea in summer) (Cawthorn, 1990; Gales and Mattlin, 1997; Chilvers *et al.*, 2006). Early in lactation they produce low energy milk compared with that of other otariids species at the same stage (see Chapter 4). Because of the latitude of their distribution and their “intermediate” maternal strategy (intermediate between polar and tropical lactation strategies), it is considered that NZSL are closer to temperate-zone otariid species such as Australian sea lions (*Neophoca cinerea*) subantarctic fur seals, New Zealand fur seals (*Arctocephalus forsteri*) than to high latitude species. In recent years considerable advances have been made in the knowledge of pup growth rates (Chilvers *et al.*, 2007), maternal attendance patterns (Chilvers *et al.*, 2005), maternal diving-foraging patterns (Chilvers *et al.*, 2005, 2006), milk composition (see Chapter 4) which have assisted in the description of the maternal strategies of NZSL. Nevertheless, the factors affecting milk composition are still poorly understood. Therefore the objectives of this chapter were to investigate a) the relationship between milk components, maternal characteristics (age, body mass, length and body condition) and offspring characteristics (age and sex); and b) the temporal changes in milk composition and maternal characteristics in early lactation (January and February) in relation to the duration of the maternal foraging trips preceding milk sampling and the duration of the maternal nursing period ashore.

MATERIALS AND METHODS

Study site and Animals

The study was conducted at Sandy Bay, Enderby Island, the Auckland Islands (50° 30'S, 166° 47'E), on NZSL during early lactation (January and February) over seven summer seasons, 1997, 1999 to 2003, and 2005. Lactating females in 1997 were captured as part of an earlier foraging project (for details see Costa and Gales, (2000). In 1999, 2000, and 2001 adult females were selected from those on the beach observed to be suckling a pup, whereas in 2002, 2003 and 2005 adult females known to have a pup were selected from a pool of branded/tagged individuals of known age. See Table 13 for total number of lactating females studied in this chapter.

See Chapter 2, MATERIALS AND METHODS section, for details about capture of sea lions, collection, handling and storage of milk samples in the field prior to analysis.

Analytical method

Analysis of milk was conducted as per Chapter 2, MATERIALS AND METHODS section.

Maternal age classes

Adult females (Table 13) were aged from sections taken from the first post canine tooth (Childerhouse *et al.*, 2004). Females were grouped in two age classes according to their growth rates. By age 10, the annual increase in length was less than 1%, although their weight continued to increase, and it was assumed that they had reached maturity (Childershouse unpublished data). Therefore lactating females aged from 3 to 10 years were grouped together (young mothers) and the second group consisted of all older animals aged from 11 to 26 years (older females).

Offspring sex and age

The age and sex of the pup was determined for four hundred and one females captured in 1997, 1999, 2000, 2001, 2002, 2003 and 2005 (Table 13). The Sandy Bay colony was monitored and surveyed daily during the pupping period and therefore birth dates of pups from branded and tagged females were recorded. The age in days of the pup was calculated from the birth date to the date of capture of the females. As part of another study on pup growth and survival, pups were sexed (Chilvers *et al.*, 2006). For a number of females for which the age of the pup was known, the relationship between milk composition and the age of her pup at the time of milk sampling was investigated.

Maternal body condition index

The Body Condition Index (BCI) is a simple method which has been used as a relative indicator of the nutritional condition and of the amount of energy reserves in pinnipeds (Costa *et al.*, 1989; Arnould, 1995). BCI provides information about potential survival, reproductive success and “health” of a population of animals (Kirkpatrick, 1980). BCI can be used to monitor changes in pinnipeds body condition during reproduction, growth, moult and in relation to environmental changes (Costa *et al.*, 1989; Boyd and Duck, 1991; Boyd *et al.*, 1993; Oftedal *et al.*, 1993). In subantarctic fur seals (*Arctocephalus tropicalis*) lactating females in good body condition had better maternal performance than females in poor body condition (Georges and Guinet, 2000a; Georges and Guinet, 2000b).

Body condition of lactating females was quantified with a body condition index (BCI). The BCI for each animal was calculated as following Guinet *et al.* (1998):

$$\text{BCI} = y_a - y_p$$

where y_a is the actual measure of body weight and y_p is the predicted body weight using the following regression equation:

$$y_p = 1.5875134 \times (\text{body length}) - 169.9046$$

This regression equation was derived using the data collected in this study (Figure 13). The allometric relationship between body weight and body was best described with a power function (Childerhouse, unpublished data) for all ages, including juvenile animals. In this study, which included females only between 3 and 26 years of age, a linear regression gave a better fit than a power function whereas in (Childerhouse, unpublished data) a greater range of ages was used.

Attendance pattern: time at sea and time ashore

The relationship between milk composition and maternal attendance behaviour (time ashore and duration of preceding foraging trip) was investigated in early lactation for a number of females for which a) the time of haul-out to when milk was sampled (time ashore) was known and b) the duration of the preceding foraging trip (time at sea) was known. The maternal attendance behaviour at Sandy Bay was measured using radio-telemetry. Sixty-five of females captured in 2001, 2002, 2003 and 2005 were fitted with VHF transmitters (70mm x 30 mm x 15 mm, Sirtrack, Havelock North, New Zealand) (Table 13). To locate the sea lion by VHF transmitters Sandy Bay was scanned a) manually with a receiver three times (9 am, 12 pm and 6 pm) each day; b) and with an automatic scanning receiver and data logger (Model R2000, Advanced Telemetry Systems Inc., Isanti, MN, USA). Arrival and departure time of sea lion females were compared and complemented with the data from manual and automated methods.

Data analysis

All statistical analyses were performed using SAS (Statistical Analysis System, version 9.1; SAS Institute Inc., Cary, NC, USA). The variables BCI, body length, body weight, and concentration of milk components were analysed using the MIXED procedure with a linear model that included the

fixed effect of maternal age, year of sampling, month of sampling and sex of offspring and the random effect of animal. Some relevant interactions between the main fixed effects were included in the model and retained when they were significant.

Multivariate analysis

Multivariate analysis of variance among BCI, body weight, milk fat concentration, protein concentration in skim and whole milk, total solids and energy content, pup age, time ashore and time at sea was run with the GLM procedure using a linear model that considered the fixed effect of year and maternal age and the random effect of animal. A Bonferroni correction for multiple tests was used (Rice, 1989; Narum, 2006).

Regression analysis

Some specific regression analyses were performed to establish the effect of offspring variables on milk composition using the GLM procedure.

The linear model included the milk components as dependent variables and pup age, time ashore and time at sea as independent variables with the fixed effect of year.

RESULTS

Descriptive statistics

The number of females and offspring that were included in this study are presented in Table 13. Descriptive statistics of the variables considered are presented in Table 14 and Table 23 in Appendix. Morphometric data of lactation NZSLs are shown in Table 15.

Table 13. Number of lactating New Zealand sea lions, *Phocarctos hookeri*, in early lactation at Enderby Island, Auckland islands from 1997, 1999 to 2003 and 2005 for which age was known, VHF fitted, body condition index (BCI kg/cm) calculated, length and weight recorded and pups sexed and their age was recorded in each of the seven years of the study

| Year | Number of females | | | | | Number of pups | |
|-------|-------------------|----------|-------------|-------------|-------------|----------------|------------|
| | Age (Years) | With VHF | BCI (kg/cm) | Length (cm) | Weight (Kg) | Sexed (M/F) | Age (days) |
| 1997 | 5 | 24 | 32 | 33 | 34 | 15/18 | - |
| 1999 | 70 | - | 64 | 64 | 64 | 49/22 | - |
| 2000 | 90 | - | 72 | 72 | 72 | 45/43 | - |
| 2001 | 97 | - | 101 | 101 | 101 | 42/40 | - |
| 2002 | 13 | 12 | 40 | 40 | 41 | 17/22 | 27 |
| 2003 | 24 | 21 | 61 | 61 | 61 | 27/27 | 55 |
| 2005 | 20 | 8 | 29 | 35 | 35 | 30/4 | 34 |
| Total | 319 | 65 | 399 | 406 | 408 | 225/176 | 116 |

Table 14. Descriptive statistics for maternal characteristics, pup age, attendance pattern, and milk composition of New Zealand sea lions, *Phocarctos hookeri*, in early lactation at Enderby Island, Auckland islands from 1997, 1999 to 2003 and 2005. Means values are expressed with standard deviation, sample size and with maximum and minimum values.

| Traits | n | Mean | SD | Minimum | Maximum |
|---------------------------------|-----|--------|-------|---------|---------|
| Maternal characteristics | | | | | |
| Body condition index (kg/cm) | 403 | -0.001 | 10.1 | -25.46 | 38.78 |
| Weight (kg) | 406 | 112.0 | 15.3 | 76.5 | 162.2 |
| Length (cm) | 403 | 177.6 | 7.3 | 157.0 | 197.0 |
| Age (years) | 397 | 10.7 | 4.0 | 3 | 26 |
| Pup age (days) | 116 | 33.0 | 14.43 | 5 | 64 |
| Time on land (hrs) | 64 | 12.0 | 11.7 | 0 | 56.64 |
| Time at sea (hrs) | 41 | 41.7 | 24.7 | 6.48 | 96.0 |
| Milk composition* | | | | | |
| Lipids (g/kg) | 382 | 13.95 | 8.24 | 6.03 | 44.81 |
| Protein Whole milk (g/kg) | 381 | 9.44 | 2.43 | 3.24 | 20.83 |
| Protein Skim milk (g/kg) | 381 | 11.96 | 3.73 | 3.63 | 36.06 |
| Total Solids (g/kg) | 384 | 30.82 | 9.12 | 11.58 | 82.39 |
| Water (g/kg) | 384 | 69.17 | 9.04 | 37.61 | 88.42 |
| Energy (kJ/g) | 381 | 9.81 | 3.20 | 4.83 | 22.22 |

*Data on milk composition was obtained from Chapter 3.

Table 15. Morphometric data of lactating female New Zealand sea lions, *Phocarctos hookeri*, in early lactation at Enderby Island, Auckland islands from 1997, 1999 to 2003 and 2005. Body length (cm) was adjusted for age, random effects of animal, month and year whereas age (years) was adjusted for random effects of animal, month and year. Mean values are expressed with standard errors and with sample sizes (n).

| Year | Length (cm) | n | Age (years) | n |
|------|-------------|----|-------------|-----|
| 1997 | 179.8 ±3.5 | 5 | 5.4 ±1.9 | 6 |
| 1999 | 177.2 ±1.1 | 62 | 9.5 ±0.5 | 70 |
| 2000 | 177.8 ±0.9 | 70 | 10.6 ±0.4 | 92 |
| 2001 | 176.2 ±0.8 | 98 | 11.4 ±0.4 | 102 |
| 2002 | 178.1 ±1.2 | 31 | 11.1 ±0.5 | 33 |
| 2003 | 178.3 ±0.9 | 61 | 12.1 ±0.4 | 62 |
| 2005 | 180.2 ±1.9 | 30 | 13.5 ±0.5 | 32 |

Milk composition

The correlation between milk components; maternal factors, pup age, attendance and milk components are presented in Table 16.

Monthly variation in maternal characteristics

The mean maternal weights in January and February respectively were significantly different ($P < 0.05$) (Table 17). The lactating females had significantly ($P < 0.05$) better body condition in February than in January (Table 17). Milk secreted in February had significantly higher lipid content and energy content than in January (Table 17). The milk protein concentration and skim milk protein concentration did not differ significantly ($P < 0.05$) between January and February (Table 17).

Females were grouped based on whether they lost weight or gained weight during early lactation (between January and February). For females that lost body weight, the average weight lost accounted for 4.26 ± 0.88 kg and for females that gained body weight the average weight gained was 5.09 ± 0.54 kg. And this difference was significant ($P < 0.001$).

NZSL females that reared a female pup between January and February and lost weight, lost 3.50 ± 1.40 kg ($n=6$) and those that gained weight, gained,

4.37 ±0.99kg (n=12). Whereas females that reared male pups between, January and February, lost 5.03 ±1.21kg (n=8) and those females that gained weight, gained 5.48 ±0.73kg (n=22) body weight.

Maternal Age

Younger females (3-10 years old) were significantly ($P<0.05$) lighter shorter and were in poorer body condition than older females (11-26 year old) (Table 17).

Older females produced milk with slightly higher lipid content and energy content than younger mothers but the differences were very small and were not significantly different ($P>0.05$) (Table 17). The milk protein and milk skim protein produced by younger mothers and older mothers did not differ significantly ($P>0.05$) (Table 17).

Offspring age and sex

Offspring age

A multivariate analysis of the variance indicated that the relationship between the age of the pup (days) and milk lipid concentration ($r=0.30$, $P=0.004$) energy content ($r=0.30$, $P=0.005$) total solids ($r=0.28$, $P=0.008$) was significant, whereas there was no significant relationship with protein concentration in skim milk ($r=0.07$, $P=0.51$) or in whole milk ($r=-0.07$, $P=0.51$) (Table 16). The general linear model indicated that there was a positive relationship between lipid concentration (Lipid g/kg=23.47x + 0.166, $R^2=0.265$, $n=89$, $P<0.05$), protein concentration in whole milk (protein in whole milk g/kg=9.51x -0.01, $R^2=0.11$, $n=89$, $P<0.05$), energy content (energy content kJ/g=11.19x +0.06, $R^2=0.30$, $n=89$, $P<0.05$) and total solids (total solids=32.62x + 0.16, $R^2=0.17$, $n=89$, $P<0.05$) and pup age (days), while protein concentration in skim milk (protein in skim milk g/kg=12.69x +0.01, $R^2=0.27$, $n=89$, $P<0.05$) declined with pup age (days).

Offspring sex

Lactating females that reared a female pup were heavier but shorter than females that nursed a male pup; however, these differences in body weight and length were not significant ($P>0.05$) (Table 17). The lactating females that nursed a female pup between January and February gained on average more body weight (0.43 ± 0.85 kg, $n=18$, $p>0.05$) and had significantly better body condition ($P<0.05$) (Table 17) than females (weight gained: 0.22 ± 0.70 kg, $n=30$) that nursed a male pup.

Lactating females that nursed a male pup produced milk with slightly lower lipid and energy content than females that nursed a female pup but these differences were not significant ($P>0.05$) (Table 17). Lactating females that nursed a female pup produced milk with significantly higher concentrations of protein in whole milk ($P<0.05$) and skim protein ($P<0.05$) than females that nursed male pups (Table 17).

Annual variation in maternal characteristics

Maternal Body Weight

There were significant inter-annual differences in maternal body weight (ANOVA $F_{6,98}=3.81$, $P<0.05$). In 1999, 2000, 2003 and 2005 females were significantly heavier than in 2001 ($P<0.05$) but the mothers were not significantly heavier in 2002 ($P>0.05$) than in 2001 (Figure 14a).

Maternal Body Condition

There were significant inter-annual differences in maternal body condition (ANOVA $F_{6,109}=2.23$, $P<0.05$). The maternal body condition was significantly higher in 1999 than all other years ($P<0.05$) (Figure 14b).

Attendance pattern: time at sea and time ashore

There was a weak and non significant relationship between milk composition and the time females spent ashore (lipid (g/kg)= $32.026x - 0.155$, $n=48$, $P=0.47$, $R^2=0.076$; total solids g/kg= $42.64x - 0.20$, $n=49$, $P=0.54$, $R^2=0.047$; energy kJ/g= $14.46x - 0.059$, $n=48$, $P=0.37$, $R^2=0.092$). There was a weak but significant relationship between time ashore and protein concentration in whole milk ($y=9.54x - 0.003$, $n=48$, $P=0.0008$, $R^2=0.25$) and protein concentration in skim milk ($y=13.98x - 0.025$, $n=48$, $P=0.01$, $R^2=0.25$). Protein concentration in skim milk ($y=13.81x - 0.0079$, $n=31$, $P<0.05$, $R^2=0.38$), total solids concentration ($y=42.53x - 0.11$, $n=31$, $P=0.42$, $R^2=0.097$) and energy content ($y=14.65x - 0.04$, $n=31$, $P=0.17$, $R^2=0.097$) were not significantly related to preceding foraging trip (time at sea) whereas protein concentration in whole milk had a significant relationship ($y=9.26x + 0.01$, $n=31$, $P=0.03$, $R^2=0.28$). The correlations between milk components and the time the mother spend ashore and at sea are shown in Table 16.

Interactions

Interactions were tested against milk components and the only interaction that had a significant effect on milk composition was Year*month on milk lipid concentration and energy content. Overall there were no significant differences between January and February in the same year in the milk lipid concentration; however, the only significant difference was observed in year 2003 and 2005. In 2003, lactating females produced a milk in February (milk lipids= 24.93 ± 7.65 g/kgSD, $n=14$, $P<0.05$; energy content: 11.26 ± 2.83 kJ/g, $n=14$, $P<0.05$) that had significantly higher lipid concentration and energy content than that in January (milk lipid: 18.04 ± 6.96 g/kgSD, $n=25$; energy content: 8.82 ± 2.59 kJ/g, $n=25$). Equally, in 2005, females produced milk in February (milk lipids: 31.89 ± 8.30 g/kgSD, $n=14$, $P<0.05$; energy content: 14.33 ± 3.02 kJ/g, $n=14$, $P<0.05$) with significantly higher concentration of lipid and energy content than in January (milk lipid: 25.10 ± 5.60 g/kgSD, $n=17$; energy content: 11.76 ± 2.19 kJ/g, $n=17$).

Table 16. Correlation coefficients¹ between maternal body condition index (BCI; kg/cm), maternal body weight (kg), pup age (days), maternal attendance pattern (time ashore and time at sea, hrs), and the concentration of milk components (lipid g/kg, protein in whole milk g/kg, protein in skim milk g/kg, total solids g/kg, energy content kJ/g) of New Zealand sea lions, *Phocarctos hookeri*, in early lactation at Enderby Island, Auckland islands from 1997, 1999 to 2003 and 2005. Level of significance **P<0.01, ***P<0.001 after Bonferroni correction was applied.

| | Milk composition traits | | | | |
|------------------------------|-------------------------|-------------------|------------------------|------------------------|-----------------------------|
| | Lipid (g/kg) | Protein (g/kg) | Skim Protein (g/kg) | Total Solids (g/kg) | Energy content (kJ/g) |
| BCI (kg/cm) | 0.19* | 0.13 | 0.18* | 0.23** | 0.20** |
| Body weight (kg) | 0.07 | 0.18 | 0.18 | 0.15 | 0.10 |
| Lipid (g/kg) | | 0.11 | 0.46** | 0.90*** | 0.98*** |
| Protein whole milk (g/kg) | | | 0.92*** | 0.31 | 0.29 |
| Protein skim milk (g/kg) | | | | 0.64*** | 0.61*** |
| Total solids (g/kg) | | | | | 0.94*** |
| Pup age (days) | 0.30* | -0.07 | 0.07 | 0.28* | 0.30* |
| Time ashore (hrs) | 0.27 | 0.40 | 0.72 | 0.31 | 0.30 |
| Time at sea (hrs) | 0.17 | 0.50 | 0.70 | 0.22 | 0.17 |

¹ Correlation coefficients were adjusted for maternal age, year of sampling and random effect of animal.

Table 17. Least squares means (\pm SE) of maternal weight (kg) and length (cm), body condition (kg) and milk composition for each consecutive month in early lactation, for each age class (young mothers 3 to 10 years old; and old mother 11 to 26 years old) and offspring sex of New Zealand sea lions, *Phocarctos hookeri*, Enderby Island, Auckland islands.

| | Month | | | | | | Maternal age class | | | | | |
|---------------------------|--------------------|----------|-----|--------------------|----------|-----|--------------------|----------|-----|--------------------|----------|-----|
| | January | | | February | | | Young age | | | Old age | | |
| Maternal | Mean | \pm SE | n |
| Weight (kg) | 113.7 ^a | 1.63 | 231 | 116 ^b | 1.67 | 128 | 110.4 ^a | 1.24 | 233 | 116.7 ^b | 1.37 | 126 |
| Length (cm) | 168.4 | 8.90 | 231 | 168.8 | 8.91 | 126 | 176.6 ^a | 0.60 | 232 | 180.4 ^b | 0.68 | 125 |
| BCI (kg/cm) | 0.36 ^a | 1.27 | 231 | 2.61 ^b | 1.33 | 126 | -1.62 ^a | 0.90 | 232 | 1.97 ^b | 1.05 | 125 |
| Milk | | | | | | | | | | | | |
| Lipid (g/kg) | 21.69 ^a | 1.37 | 161 | 23.88 ^b | 1.15 | 96 | 22.26 | 0.74 | 166 | 22.89 | 0.93 | 91 |
| Protein whole milk (g/kg) | 9.40 | 0.34 | 161 | 9.02 | 0.34 | 96 | 9.28 | 0.23 | 166 | 9.22 | 0.29 | 91 |
| Protein skim milk (g/kg) | 11.98 | 0.53 | 161 | 11.95 | 0.53 | 96 | 12.23 | 0.45 | 166 | 12.24 | 0.55 | 91 |
| Energy (kJ/g) | 10.24 ^a | 0.45 | 161 | 11.24 ^b | 0.46 | 96 | 10.70 | 0.30 | 166 | 10.93 | 0.38 | 91 |

^{a, b} Means with different letters within each category month, maternal age class and sex of pup are significantly different $P < 0.05$.

Table 17. (Continued).

| | | | Offspring sex | | | | | |
|-----------------|-------|------|-------------------|----------|-----|--------------------|----------|-----|
| | | | Female | | | Male | | |
| Maternal | | | Mean | \pm SE | n | Mean | \pm SE | n |
| Weight (kg) | | | 115.5 | 1.91 | 132 | 114.3 | 1.85 | 188 |
| Length (cm) | | | 178 | 0.64 | 131 | 179.1 | 0.57 | 187 |
| BCI (kg/cm) | | | 1.83 ^a | 1.44 | 131 | -0.29 ^b | 1.40 | 187 |
| Milk | | | | | | | | |
| Lipid (g/kg) | | | 22.79 | 1.30 | 99 | 22.58 | 1.22 | 132 |
| Protein | whole | milk | 9.65 ^a | 0.37 | 99 | 9.00 ^b | 0.34 | 132 |
| (g/kg) | | | | | | | | |
| Protein | skim | milk | 12.58 | 0.59 | 99 | 11.77 | 0.55 | 132 |
| (g/kg) | | | | | | | | |
| Energy (kJ/g) | | | 10.98 | 0.51 | 99 | 10.73 | 0.48 | 132 |

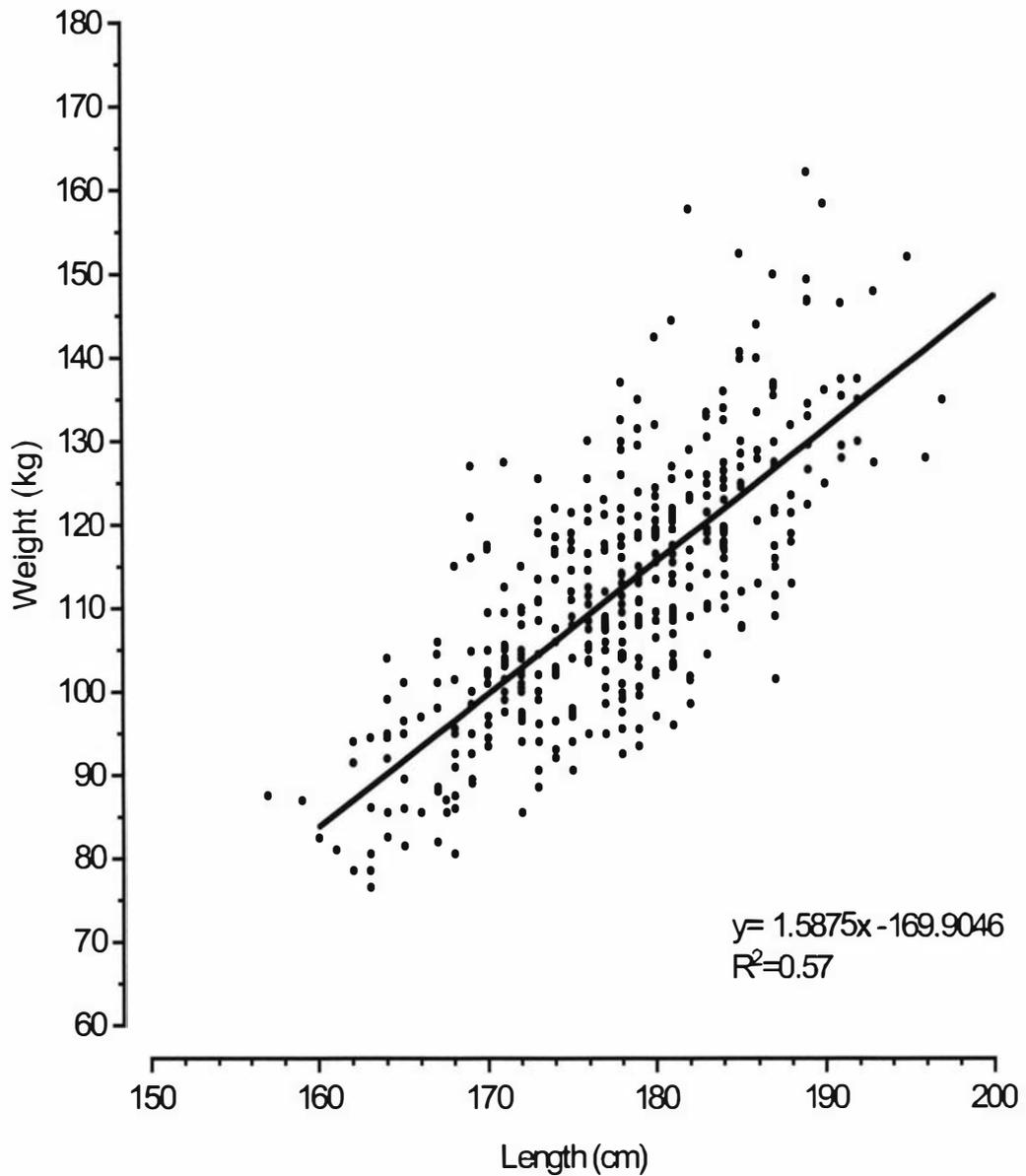


Figure 13. The relationship between maternal weight (kg) and maternal length (cm) for 402 adult lactating female New Zealand sea lions, *Phocarctos hookeri*, at Enderby Island, Auckland Islands.

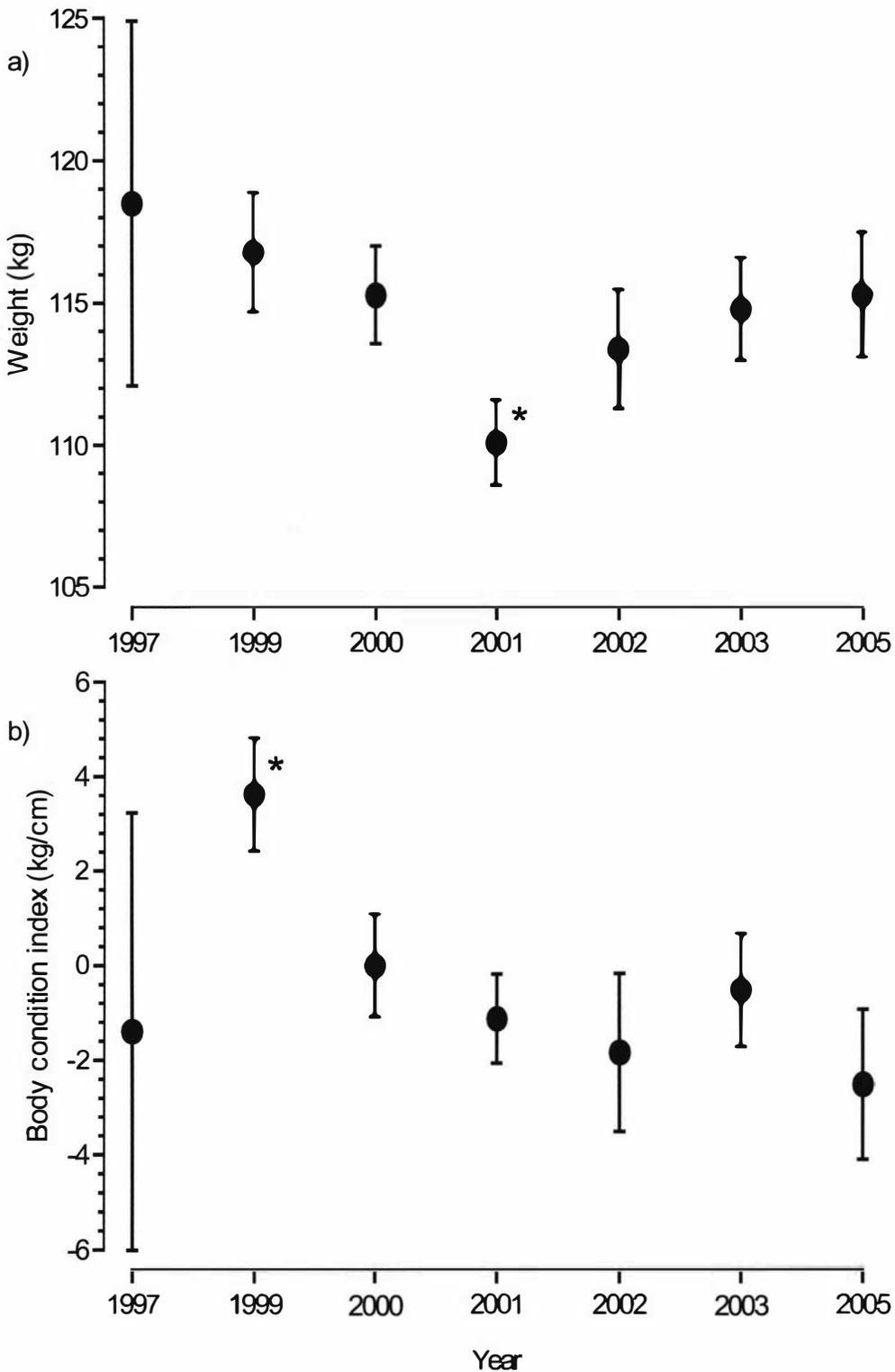


Figure 14. The a) body weight (kg) (1997 n=5, 1999 n=62, 2000 n=70, 2001 n=98, 2002 n=32, 2003 n=61, 2005 n=30) and b) body condition index (kg/cm) (1997 n=5, 1999 n=62, 2000 n=70, 2001 n=98, 2002 n=31, 2003 n=61, 2005 n=30) of lactating New Zealand sea lions, *Phocarctos hookeri*, in early lactation during seven years at Enderby Island, Auckland islands. Mean values are expressed with standard errors.

DISCUSSION

Milk composition

Variation in milk composition was related to maternal characteristics of the NZSL observed in this study. The concentrations in milk of lipid, energy and protein concentration in skim milk were significantly correlated with maternal BCI (Table 16). Hence lactating females in good body condition produced a milk more lipid and energy rich than females that were in poorer body condition, which is similar to the observations reported by Georges *et al.* (2001) for lactating subantarctic fur seals, and by Arnould and Hindell (1999) for lactating Australian fur seals, *Arctocephalus pusillus doriferus*. However, neither milk lipid concentration or energy content were related to maternal body weight in lactating NZSL (Table 16) which is consistent with data for Australian sea lions (Kretzmann *et al.*, 1991; Gales *et al.*, 1996) but not for Australian fur seals (Arnould and Hindell, 1999), or Antarctic fur seals (Arnould and Boyd, 1995a). Maternal body weight and protein concentration in whole milk and skim milk were significantly ($P < 0.05$) correlated (Table 16) which has not previously been reported in otariids. This coupled with the significant relationship between protein concentration in skim milk and BCI suggests that the protein concentration of the milk may be a useful indicator of the nutritional status of the lactating female. It is well known that a decrease in protein concentration of milk is an indication of suboptimal in dairy cows (Grainger *et al.*, 1982; Thomson *et al.*, 1997). Indeed the concentration of protein in skim milk has particular potential as an indicator of current nutritional status of the animal since it is independent of the large variations that can occur in fat concentration as a result of drawing on body lipid reserves.

Monthly variation /stage of lactation

The nature of the sampling regimen meant that stage of lactation and month of sampling are confounded. Thus changes in milk composition from January

to February may reflect variation in environmental factors, such as the availability of prey, or physiological or behavioural effects associated with the stage of lactation.

Maternal mass

Maternal mass increased significantly throughout early lactation (January to February). Similar results have been reported in northern fur seals, *Callorhinus ursinus* (Costa and Gentry, 1986), subantarctic fur seal (Georges and Guinet, 2000b), Australian fur seals (Arnould and Hindell, 1999) and in Antarctic fur seals (Arnould, 1997). Such changes may have been associated with an increase in the number of foraging trips.

Milk

This study demonstrated that milk lipid and energy content of NZSL increased significantly during early lactation (January and February). Because lactating females were captured in January and then again in February, and milk lipid concentration was higher in February this could be accounted for as an effect of stage of lactation (Table 17). Increase in lipid concentration and energy content during lactation has been reported in several species of otariids (Ponce De Leon, 1984; Costa and Gentry, 1986; Trillmich and Lechner, 1986; Oftedal *et al.*, 1987; Arnould and Boyd, 1995a; Gales *et al.*, 1996; Arnould and Hindell, 1999; Georges *et al.*, 2001). The protein concentration in milk and in skim milk in NZSL remained fairly constant throughout the early lactation period and this was consistent with what have been reported in other otariid species (Arnould and Boyd, 1995a; Gales *et al.*, 1996). The present study was only able to obtain data from early lactation because of logistic limitations. Therefore data from the present study are not comparable to those from lactation studies in otariids that covered the entire lactation period; however, they can be compared to data in early lactation from other species (see Figure 12, Chapter 3).

The increase in milk lipid and energy content could be explained by the positive relationship between BCI and these milk components as reported in

Subantarctic fur seals (Georges *et al.*, 2001). Subantarctic fur seals after parturition and fasting during the perinatal period lose body condition (Georges and Guinet, 2000a). An increase of lipid concentration and energy content in milk was associated with a recovery of maternal body condition during successive foraging trips which was also reported in Galapagos fur seals, *Arctocephalus galapagoensis*, (Trillmich, 1986).

The results from this study indicate that maternal body condition increased within early lactation; however, variation in the duration and/or number of foraging trips was not included in the analysis. Thus Chilvers *et al.* (2007) reported that the duration of foraging trips decreased during the lactation period, although, females that gave birth to smaller pups foraged for longer. Therefore, it could be suggested that females adjust their foraging trip durations in response to the offspring energy demands and at the same time they are able to recover their body condition by making short foraging trips. NZSL mothers may be in concordance with the optimal foraging theory which states that there is an optimal cycle duration governing the mothers' return to a fixed feeding site that maximizes the rate of delivery of food to the offspring (Pyke, 1984).

Maternal age classes

The observation that younger NZSL females were lighter and consequently had lower body condition is similar to Antarctic fur seals females (Lunn and Boyd, 1993). Most growth in NZSL females occurs up to age 10 after which annual growth rates for body length are less than 1% (Childerhouse unpublished data) and consequently younger females put energy resources to growth while also bearing the energetic cost of lactation. These additional costs reduce body condition and the milk quality produced by young lactating NZSL. Although the differences were not significant the younger mothers produced milk with lower lipid concentration and energy content than older females. Georges *et al.* (2001) suggested that for subantarctic fur seals individual foraging skill and body condition had more

influence on milk quality than maternal age. Maternal age in NZSL may play an important role in milk quality since older females were in better body condition, produced better milk quality and very likely were better at rearing a pup and foraging.

It is unclear if the apparent lower energy input to lactation by younger females had detrimental consequences for the survival and/or growth rates of their offspring. If NZSL behave in the same fashion as subantarctic fur seals, then younger females that gave birth to a male pup may have to invest a greater proportion of their resources into pre-natal growth of male pups than do older females (Georges *et al.*, 2001). The present study was unable to test this prediction since maternal body weight and length were not measured at parturition.

In summary, older females were larger and in better condition than younger females and therefore were better able to support the high energetic cost of lactation and produce a milk higher in lipids, and potentially increase their reproductive success. Better reproductive success in Antarctic fur seals was associated with maternal age and breeding experience (Lunn *et al.*, 1994).

Offspring sex and age

Offspring sex

Sexual dimorphism in otariids means that there is selection for larger body size in males (Ono and Bonness, 1996). As a consequence it is hypothesised that NZSL mothers may invest more in male pup than in a female pup and this was supported by the higher mass at birth in male pups (Chilvers *et al.*, 2007). The sexual dimorphism was also evident in this study since it was found that females nursing male pups a) produced milk with lower lipid concentration, energy content and protein concentration in whole milk and skim milk, b) were lighter, c) had lower body condition, d) gained less weight but also lost more weight between January and February than females nursing a female pup.

The result of this study indicated that NZSL females did not have the condition to withstand the cost of rearing a male pup which resulted in a lower milk quality. It may be possible that females produced more milk to compensate for the lower milk quality consumed by male pup. This seems unlikely since differences in milk consumption in Antarctic fur seals and Australian fur seals pups were not found and both consumed the same amount of milk (Arnould *et al.*, 1996; Arnould and Hindell, 2002). Although the differences in milk quality were negligible it may indicate that body condition and weight may limit the quality of the milk mother can produce. It could be postulated that the lower milk quality consumed by male pups did not influence their growth rates or that male pup consumed more milk to compensate for the lower milk quality. This further supported by the fact that male pups had faster growth rates than female pups (Chilvers *et al.*, 2007). In Antarctic fur seals, male pups directed more of their milk consumption towards lean tissue growth than females, which accumulate greater adipose stores (Arnould *et al.*, 1996). It may be possible that NZSL male pups followed this pattern and data on milk consumption are available to test this prediction (Riet Sapriza *et al.* unpublished data).

Rearing a male pup appears to be was more costly than rearing a female pup. This was evident in the lower body condition and lower weight of NZSL mother that nursed male pups. It must be taken into consideration that male pups demands higher energy because of larger size compared to female pups. In addition, NZSL mother nursing a male pup lost in average more weight between January and February, than mother that raised a female pup.

Offspring age

Data indicated that milk lipid and energy content increased with pup age whereas Kretzmann *et al.* (1991) failed to find a correlation in Australian sea lions. The present study was done during early lactation therefore changes in milk composition throughout the lactation period could only be speculated about. Increases and decreases in the concentration of milk lipid associated with temporal factors such pup age (stage lactation), the duration of preceding trip and duration of nursing bout ashore in otariids have been shown (Costa

and Gentry, 1986; Oftedal *et al.*, 1987; Costa, 1991; Higgins and Gass, 1993; Arnould and Boyd, 1995a; Gales *et al.*, 1996; Arnould and Hindell, 1999). These authors concluded that milk lipid concentration were related to foraging trip duration throughout lactation. Based on this, it could be concluded that a) milk lipid concentration throughout early lactation increased with pup age b) lactating females may transfer more milk but at low quality in response to long foraging trips in early lactation. This strategy of providing more milk at low quality rather than producing a higher energy rich milk has been reported in subantarctic fur seals (Georges and Guinet, 2000b; Guinet and Georges, 2000; Georges *et al.*, 2001). Furthermore, pups of lactating subantarctic fur seals making short and regular foraging trips grew faster and were heavier at weaning than pups of other seals (Georges and Guinet, 2000b). NZSL females conduct short foraging trips (1.7 to 2.7 days) alternated with short visits ashore (0.6-2.3 days) (Gales and Mattlin, 1997; Chilvers *et al.*, 2005), and hence may be adopting a similar strategy seen in subantarctic fur seals by transferring energy to their offspring at short but frequent intervals. Data on pup mass at weaning were not available to test whether this strategy is optimal for NZSL.

As in subantarctic fur seals (Georges *et al.*, 2001), NZSL protein in whole milk decreased with the age of the pup (Table 16). The results of this study are consistent with the hypothesis (Chilvers *et al.*, 2007) explaining the loss of weight of the pups during the early postpartum period, the time between the birth of the pup and the mother's first departure to forage. Thus the relatively low concentration of lipid in the milk of NZSL in early lactation (Chapter 3), and the decrease in the protein concentration in whole milk with pup age may limit pup growth since pup growth in otariids mainly consists of lean tissue deposition in the form of protein. Moreover, Arnould and Boyd (1995a) reported that during years of low food availability the concentration of protein in milk of Antarctic fur seals decreased with the age of the pup. Lower protein concentration in whole milk has been reported for NZSL in 2002 and 2003 and this may be an indication of underfeeding in lactating females

(Chapter 3). Whether malnutrition in NZSL is a response to local food shortage is not known but it is a hypothesis that needs to be tested.

Annual variation in maternal characteristic

Maternal Body Condition and body weight

This study demonstrated that maternal body condition and body weight varied between years in early lactation. The use of a body condition index has been validated for otariids, since a positive correlation with sternal blubber depth in adult Australian fur seals (Arnould and Warneke, 2002), and total body lipid in Antarctic fur seals (Costa *et al.*, 1989; Arnould, 1995) has been validated. Notwithstanding, it remains to be investigated whether this is true for NZSL.

In Cape fur seals, *Arctocephalus pusillus*, maternal body condition decreased through the first part of the reproductive cycle to a minimum at implantation but increased again through pregnancy (Guinet *et al.*, 1998). However, it is likely that a decline in the maternal body condition in NZSL may be related to low nutritional status and poor local food sources as shown in Antarctic fur seals (Lunn and Boyd, 1993). Low food availability resulted in low body condition in Cape fur seals females which led to higher number of abortions and pup growth rates which were low compared to other years (Guinet *et al.*, 1998). Information on annual catches (tonnes) for arrow squid species which are important prey for NZSL (Childerhouse *et al.*, 2001; Meynier *et al.*, 2006) and target of fisheries around the Auckland Islands give an indication of variable local food resources. The low the annual catches (tonnes) of arrow squid from 2000 to 2002 (Ministry of Fisheries, New Zealand, see Figure 33, Chapter 7) is consistent with lower maternal body condition (Figure 14b). Notwithstanding, the decline and low average maternal body condition in early lactation observed in this study suggest that a) the cost of pregnancy and the cost of parturition (perinatal period) was high or b) females were malnourished prior to and during early lactation. The present study indicated that females were able to replenish their body reserves as lactation progressed (Table 17). The cost of foetal growth and the cost of lactation should be addressed in NZSL.

Attendance pattern: time at sea and time ashore

The present study did not find a relationship between milk composition and preceding foraging trip duration (time at sea) which is consistent with Goldsworthy and Crowley (1999) (Antarctic fur seals) and Kretzmann *et al.* (1991) (Australian sea lions); while contrasting with findings in Antarctic fur seals and Australian fur seal, respectively (Arnould and Boyd, 1995b; Arnould and Hindell, 1999). The results from the latter study should be taken with caution since the sample size ($n=7$) was small. Also in the study by Arnould and Boyd (1995b), females were captured very shortly after they returned from sea. In the present investigation, and those of Kretzmann *et al.* (1991) and Goldsworthy and Crowley (1999), milk was collected over a greater range of time (3 to 56 hrs period, 0.3 to 30hrs period and 12 hrs period, respectively) after the arrival of the females on shore. The data in this study agree with Goldsworthy and Crowley's (1999) prediction that the longer periods of time that females were ashore prior to their capture may have masked the relationship between the duration of the preceding foraging trip and milk composition.

The result of the present study, despite the weak and non significant relationship, suggest that milk lipid decreased with time ashore, and this pattern has been demonstrated in a number of otariids (Costa, 1991). In northern fur seals (Costa and Gentry, 1986), Antarctic fur seals (Arnould and Boyd, 1995b) and Juan Fernandez fur seals, *Arctocephalus philippii*, (Ochoa-Acuna *et al.*, 1999) milk lipid concentration decreased significantly with time the female spent ashore, whereas in Australian sea lion the milk lipid concentration remained constant (Gales *et al.*, 1994). Kretzmann *et al.* (1991) did not find any relationship in the same species. In addition the lack of relationship between milk lipid and preceding foraging trip and the relatively low concentration of lipid in milk reported in Australian sea lions (Kretzmann *et al.*, 1991) and NZSL (the present Chapter and Chapter 3) suggest that a) NZSLs are consistent with the theory proposed by Trillmich and Lechner (1986) that otariids making shorter foraging trips produce a lower milk lipid

concentration than those species that make long foraging trips; and b) contrary to what was expected, lactating NZSL have adopted a maternal strategy closer to that of Australian sea lions than to their counterparts in higher latitudes.

Conclusion

It seems that NZSL females have an unusual strategy in which they produce low quality milk compared to other otariids but similar to sea lion species and may compensate this by having short foraging trips at sea (thus a short fast for the pup). The body condition, weight and age of the mother influenced the quality of the milk suggesting that maternal experience in nursing a pup and foraging may increase her own reproductive success. The inter-annual variation in maternal body condition may be due to the cost of pregnancy and parturition or variation in food resources between years which may affect the females' reproductive success. Testing predictions about changes in diet could be investigated by analysing the milk fatty acid composition in relation to the fatty acid composition of potential prey.

Acknowledgments

The research undertaken in this chapter was supervised by Pádraig J. Duignan (IVABS, MASSEY UNIVERSITY), Nicolas Lopez-Villalobos (IVABS, MASSEY UNIVERSITY), Louise B. Chilvers (Marine Conservation Unit, Department of Conservation), Ian S. Wilkinson (Department of Environment and Climate Change, Australia), Duncan D. S. Mackenzie (IFNHH, MASSEY UNIVERSITY) and Alastair MacGibbon (Fonterra Research Centre); and carried out in collaboration with Dan Costa (Department of Ecology and Evolutionary Biology, University of California, USA) and Nick Gales (Australian Antarctic Division, Australia). Milk samples, data about females and pup from 1997 were provided by Dan Costa and Nick Gales. Special thanks to Simon Childerhouse for providing the data for female's age's and

pup sexes and to D. Szteren for her comments on an earlier draft of this chapter. I would like to thank the Department of Conservation (DoC) Southland, and also A. Castinel and all DoC staff who assisted in the field. We are grateful to R. Richardson, Y. van der Does (Fonterra Research Centre) for their invaluable help and their technical support with laboratory analysis.

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CHAPTER 5

ABSORPTION AND DISTRIBUTION OF DIETARY FATTY ACIDS IN TISSUES OF LACTATING NEW ZEALAND SEA LIONS

ABSTRACT

The overall aim of this thesis was to investigate whether the analysis of lipid rich tissues (milk, blubber and serum) of otariids could be used to determine their diet. The aim of the study described in this chapter was to follow the movement of fatty acids (FAs) from the intestine to the tissues rich in lipids. A “feeding experiment” was conducted in which a “Cocktail of Natural Vegetable Oils” (CoNVO) with a distinct FA profile was fed by stomach tube to 24 lactating NZSLs and tissue samples collected at different time intervals (0 to 72hrs). The CoNVO was composed of 60% sunflower oil and 40% coconut oil and they were chosen because they are rich in FA not found in the milk of NZSL. Sunflower oil is mainly composed of oleic acid (C18:1) and linoleic acid (C18:2n-6) FA and coconut oil is composed mainly of lauric acid (C12:0), myristic acid (C14:0) and palmitic acid (C16:0). Samples were collected at different time intervals (4, 8, 12, 16, 20, 24, 30, 38, 48 and 72hrs) to study the temporal changes in FAs post-treatment. FA signature analysis was carried out on milk, serum and blubber collected before and after the CoNVO was administered. The data were analysed with discriminant (stepwise and canonical) analysis and classification analysis-regression trees (CART). Significant changes occurred in FA composition of milk and serum lipids after the CoNVO was administered. These included an increase in the weight% of C12:0, C14:0 and C18:2n-6. The times that these changes occurred were not consistent among the sea lions, which indicated individual variability in the rate of digestion and absorption of the CoNVO. Milk FA signatures were able to distinguish between sea lions pre-treatment and post-treatment. The findings of this study demonstrated that milk and blood serum FAs changes in response to the ingestion of the CoNVO, and that acute change in diet were rapidly reflected in the FA composition of milk and serum. Furthermore, the results of this study has demonstrated the potential of FA signatures in studying the diet of predators such as pinnipeds, but it has also shown that the transfer of FA from diet into milk is a complex process and that further work with otariids is needed in this area.

INTRODUCTION

It is accepted that the top predators play important roles in their ecosystems (Bowen, 1997) and therefore it is important to understand the roles of the pinnipeds within their marine ecosystem. To do this, detailed information on their diet is needed so that the impact on their prey species (trophic interactions), and the level of competition with fisheries can be assessed.

Traditional methods to determine diet in pinnipeds have been based on the recovery of hard parts that are resistant to digestion and that have been obtained by stomach lavage of captured animals (Antonelis *et al.*, 1987; Rodhouse *et al.*, 1992), from stomach contents of dead animals (Doidge and Croxall, 1985; Fisher and Stewart, 1997; Holst *et al.*, 2001; Bando *et al.*, 2005), from scats (Green *et al.*, 1990; Childerhouse *et al.*, 2001; Gudmundson *et al.*, 2006) and regurgitation (Kirkman *et al.*, 2000; Childerhouse *et al.*, 2001; Gudmundson *et al.*, 2006). As a means of diet reconstruction, this technique has a number of biases and limitations that have been discussed elsewhere (Jobling and Breiby, 1986; Jobling, 1987; Dellinger and Trillmich, 1988; Cottrell, 1996; Orr and Harvey, 2001; Trites and Joy, 2005). In particular samples may not necessarily represent recent feeding events because of the differential rates of erosion, retention and digestion of soft and hard parts by animals digestive systems (Murie and Lavigne, 1986; Harwood and Croxall, 1988; Pierce and Boyle, 1991; Gudmundson *et al.*, 2006).

Techniques involving the comparison of the fatty acid (FA) composition of the diet and body lipids provide complementary data and have proven to be reliable approaches for studying the diet of pinnipeds (Iverson *et al.*, 1997a; Iverson, 2001; Lea *et al.*, 2002; Bradshaw *et al.*, 2003; Iverson *et al.*, 2003; Budge *et al.*, 2006; Beck *et al.*, 2007). These techniques are based on two assumptions, (1) that various prey species have specific FA signatures that

can be used as biomarkers (Ackman, 1982; Ackman, 1989); and (2) that FAs of chain length of 14 carbons or longer are deposited in the predator tissues from their prey with little modification. If these assumptions are valid, the ratios of individual FAs within a predator's blubber and milk can be used to infer the composition of the diet and the foraging ecology of wild and captive pinnipeds (Iverson *et al.*, 1997b; Smith *et al.*, 1997; Brown *et al.*, 1999; Kirsch *et al.*, 2000; Cooper *et al.*, 2001; Iverson, 2001; Lea *et al.*, 2002; Bradshaw *et al.*, 2003).

Lactating pinnipeds either depend on their body reserves (capital breeders), or food resources that are available (income breeders) or both to support the nutrient demands of lactation. Thus diet and nutrient reserves (blubber) of female pinnipeds play important roles in lactation performance and support the principle of using fatty acid signature analysis (FASA) of prey and predators to study the ecology of the predators. Furthermore, in most mammals, including pinnipeds (phocids seals and some otariids), dietary FAs that are not catabolised are not modified but are incorporated directly into depot fat and milk (Iverson, 1993; Jensen *et al.*, 1996; Thiemann *et al.*, 2001; Cooper *et al.*, 2006). However, the deposition of FA derived from endogenous biosynthesis, as well as that derived from the diet, must be taken into account when making inferences about the diet from FASA (Iverson, 2001).

It has been hypothesised that milk FAs secreted during foraging trips are derived primarily from immediate dietary intake whereas blubber FAs represent the dietary history of the individual (Iverson *et al.*, 1997a) and the amount of endogenous FA synthesis. For instance, in capital breeders such as most phocids that fast during lactation and those otariids that fast during the perinatal period, milk and/or blubber FA have been suggested to reflect the diet prior to the lactation period (Iverson, 1993; Iverson *et al.*, 1995). Whereas, it has been proposed that income breeders, such as most otariids species and some small phocids species that have foraging trips during the lactation period, have milk FA that should reflect the intake during the preceding feeding periods (Brown *et al.*, 1999; Lea *et al.*, 2002; Staniland and Pond, 2004). The extent to which a recent meal and over what period that

they affect milk FA have not been measured. In addition, Staniland and Pond (2004) suggested that the potential contribution of FAs from sources (such as adipose tissue) other than from diet may explain the difference between milk FA and prey FA. This may complicate the way in which FA profiles of the predators can be used to infer their diet. These potential effects have been addressed only recently in pinnipeds by Staniland and Pond (2004) who reported the results of a feeding trial with captive female Antarctic fur seals (*Arctocephalus gazella*). The diet of the seals was changed to alter its FA signature and the effect on the FA signature of the milk measured. Although, following the switch in diet, the milk FAs reflected those in the diet, there were other differences in the milk FA between individual fur seals. It is likely that there were also changes in synthesis of FA in the mammary gland and mobilization of body reserves of lipid that may have contributed to the changes in the composition of the milk FA.

It has been suggested that NZSL are under nutritional stress during summer due to the low nutritional value of cephalopods, which at this time are apparently one of the main prey for lactating females (Bando *et al.*, 2005). This result was obtained from the analysis of stomach content of bycaught sea lion (Bando *et al.*, 2005); however, these results may be biased because stomach contents were obtained from animal bycatch by the squid fisheries and may not be a true representation of the prey taken by NZSL. Furthermore, the result from the analysis of hard part in faeces of NZSL indicated that two most numerically abundant prey species were octopus (*Enteroctopus zelandicus*) and opalfish (*Hemerocoetes* species) and they made up almost 50% of total prey items (Childerhouse *et al.*, 2001). In this connection, FASA of blubber indicated that the basal diet of NZSL is fish (Meynier *et al.*, 2006). While blubber gives a long term history of the diet, it is not as good an indicator of the diet over the short term as milk or blood. However, precise interpretation of the FASA is difficult because little is known about the dynamics of FA metabolism in NZSL. For example further data are needed on the rate and pattern of FA absorption from the intestine, the turnover rates of FA in the blubber and the relative uptake and synthesis of FA in the mammary gland. Previous studies with pinnipeds have addressed the effects of long

term (days-months) intake of artificial or natural diet on the composition of blubber and/or milk (Lea *et al.*, 2002; Staniland and Pond, 2005); however, only a few have investigated the effects of short-term (hrs-days) changes in diet (Staniland and Pond, 2004; Cooper *et al.*, 2005).

The objective of this study was to investigate in the NZSL the major effects of a single oral dose of a mixture of two vegetable oils (“Cocktail of Natural Vegetable Oils”, CoNVO), containing FAs not normally present in their lipids, on the FA composition of their milk, blubber and serum. The specific objective were to determine: a) the concentration of the unique FAs fed to NZSL in their milk, blubber and serum FA; b) the time course for the appearance of the unique FAs in the milk, blubber and serum; c) an estimate of the amount of dietary FAs appearing in the serum, milk and blubber; and d) whether the milk, serum, blubber FAs signatures of NZSLs fed with CoNVO differs significantly from control NZSL.

MATERIALS AND METHODS

Study site and Animals

This study was conducted at Sandy Bay, Enderby Island, the Auckland Islands (50° 30'S, 166° 17'E), during early lactation (January and February). Adult NZSL females (n=25) known to have a pup were selected for collection of samples from a pool of branded/tagged individuals of known age (Table 18). All the females were sampled for blood, blubber and milk and the samples for the analyses described in this paper were collected in the 2003/4 and 2004/5 austral summer seasons (hereafter referred to as 2004 and 2005, respectively). See MATERIALS AND METHODS section of Chapter 3 for details about capture protocol of NZSLs.

Experimental protocol

Each female was captured twice at different intervals (Table 19), and at each capture blood, blubber, and milk were collected. A number of female were captured and recaptured during their stay ashore while others went to sea and were recaptured on their return from the sea. All females were fitted with VHF transmitters (Sirtrack, Havelock North, New Zealand) that enabled them to be located and to record their time of arrival at, and departure from the colony.

At the first capture, females were given 500 ml of a “Cocktail of Natural Vegetable Oils” (CoNVO) consisting of a mixture of 60% sunflower oil and 40% coconut oil. The composition of CoNVO is presented in Figure 15 and Table 20. These are edible oils and were chosen because they are rich in FA not found in the milk of NZSL (Table 20) or present in only trace amounts (Weeks, 2002). They were also only found in low concentrations in organisms from the marine environment (Ackman, 1989). The CoNVO was administered to the NZSLs by gastric intubation modified from the methods described for northern elephant seal, *Mirounga angustirostris* (Antonelis *et al.*, 1987); Antarctic fur seals, *Arctocephalus gazella* (Arnould *et al.*, 1996; Arnould *et al.*, 2001); grey seals, *Halichoerus grypus* (Cooper *et al.*, 2001); northern fur seal, *Callorhinus ursinus* (Gentry and Holt, 1986; Donohue *et al.*, 2002); harp seal, *Phoca groenlandica* (Kirsch *et al.*, 2000; Storeheier and Nordoy, 2001); and southern elephant seal, *Mirounga leonina l.* (Rodhouse *et al.*, 1992).

During the administering of the oil, the NZSL was held in the weighing bed with her head elevated to ensure that the oil could run down into the stomach. This also decreased significantly the chances of regurgitation of the oil. A semi-flexible PVC veterinary stomach tube (diameter: inner 10 mm; outer 16 mm; length 257 cm) was inserted into the animal's mouth and gently pushed through the oesophagus into the stomach. The edges of the end of the PVC tube were rounded and the tube was coated with surgical lubricant (K-Y lubricating jelly, Johnson & Johnson Ltd, Maidenhead, U.K.) to facilitate the passage of the tube. To aid the administering of the oil into the stomach, the

sides at the end of the tube were perforated with two holes of less than 2mm in diameter and when the tube was in place the vegetable oil was poured slowly into a funnel attached to the outside end of the PVC tube. The NZSL was kept elevated for no longer than a few minutes thereafter she was monitored until she awakened and was fully aware before she was released.

a) *Milk samples*

See MATERIALS AND METHODS section of Chapter 2 for details about collection, handling and storage of milk samples in the field prior to analysis.

b) *Serum samples*

Approximately 15-20 ml of blood was collected from the gluteal vein using a 20 ml syringe and sterile hypodermic needle (20G, 1"). Blood was collected into 10 ml vacutainers (Becton Dickinson Vacutainer Systems, NJ, USA). Blood was centrifuged between 15-20 minutes in a centrifuge. Serum was subsampled into 2 ml cryovials and stored at -196°C in liquid nitrogen. On return from the field site, the milk samples were stored at -80°C until analysed.

c) *Blubber samples*

Blubber (~1 g) was biopsied from the females using standard aseptic surgical techniques. The posterior flank of the sea lion was scrubbed with surgical disinfectant (Biocil ©, a surgical scrub containing free iodine) and a 10 mm incision was made in the skin using a sterile # 15 scalpel. A core was taken through the full depth of the blubber layer (approx. 2 cm) excluding the skin. The blubber biopsy was transferred into a 2 ml cryovial and stored at -196°C in liquid nitrogen. During the anaesthesia, the wound was covered with cotton gauze with surgical disinfectant until no blood was observed escaping from the wound. On return from the field site, the blubber samples were stored at -80°C until analysed.

Laboratory analysis

Lipid extraction

Milk

Total lipids were extracted from the NZSL milk samples using an Accelerated Solvent Extractor (ASE-200, Dionex Corporation, Sunnyvale, CA, USA) with 11 ml stainless steel extraction cells and 40 ml glass receiving vials, and a mixture of solvents in the ratio of 3:2:1 petroleum-ether:acetone:isopropanol, as described by Richardson (2001) for liquid milk or cream. The five operator-adjustable instrumental parameters of the ASE-200 were adjusted to the settings for cream due to the high fat content of NZSL milk. The operational parameters were set as follows: 1) extraction temperature at 120 °C; 2) extraction pressure at 1500 psi; 3) static extraction time 2 min; 4) number of cycles 3 and; 5) flush volume at 100%.

Samples of NZSL milk (0.5-1.0g) were pipetted into an extraction cell containing 1.0-1.2 g of Hydromatrix on a tared balance (Richardson, 2001). The total amount of solvent recovered after the extraction was ~ 16 ml and the solvent was dried under a constant nitrogen flow in a heat block at 65 °C to 110 °C (for details see Richardson, 2001).

Validation of milk lipid extraction method

The ASE has not been used previously for the extraction of lipid from pinniped milk therefore this method was validated against the more commonly used lipid extraction "Folch" method for pinniped milk, which was described by Folch *et al.* (1957). Lipids were extracted from ten milk samples with ASE and with the standard method according to Folch *et al.* (1957) as modified by Iverson (1988) and analyzed with a temperature-programmed capillary gas liquid chromatography on a Shimadzu Chromatograph (as described below). The extraction mixture for the "Folch" method consisted of 8:4:3 chloroform:methanol:water mixed with 18 parts of 2:1 chloroform:methanol. To extract the lipid from NZSL milk, 4.5 parts of the extraction mixture was mixed with 1 part of sample and 1 part aqueous salt solution (0.9% NaCl) (Folch *et*

al. (1957) as modified by Iverson (2006)). The agreement between the ASE method and “Folch” method for lipid extraction was assessed with measures of statistical fitness (Table 24 in Appendix).

Serum

Total lipids were extracted from serum of lactating NZSL by mixing 0.5 ml of serum with 25 ml 2:1 chloroform: methanol (CHCl₃:MeOH) (Folch *et al.*, 1957). After phase separation the upper water-methanol layer was aspirated and the lower layer was washed with 2.0 ml of distilled water containing 0.9% NaCl (Wang and Peter, 1983). The chloroform layer was removed and evaporated under a stream of nitrogen and thereafter the extracted lipids were stored at -20 °C.

Blubber

Total lipids were extracted from the blubber of lactating NZSL by mixing 0.5g of blubber with 30 ml of a mixture of 2:2:1.4 chloroform:methanol: 1% NaCl (CHCl₃:MeOH: 1% NaCl) (Hanson and Olley, 1963). The upper layer was removed and the chloroform layer was evaporated under a stream of nitrogen and the lipids were stored at -20 °C.

FAME preparation

Fatty acid methyl esters (FAME) were prepared from 50mg of the pure extracted lipid from blubber and milk by direct transesterification with sodium methoxide in methanol (Christopherson and Glass, 1969; Richardson, 1989). Following transesterification, methyl esters were extracted into hexane, thereafter FAME neutralizing solution (KH₂PO₄ 10% NaCl 15% buffer solution) was added. The solution was vortex and centrifuged.

Micro-methylation of lipid was designed for methylating small amount of lipids extracts such as that obtained from the extraction of lipids from serum samples. For serum lipids the standard FAME mentioned above was scaled down for small amounts (ca 1 mg) in order to inject a similar quantity of FAME onto the analytical column. The final concentration of FAME was 5 mg/mL, which was further diluted with hexane (2:3 v/v) for on-column injection.

Gas-liquid chromatography

Equipment

Analysis of the FA methyl esters was performed using temperature-programmed capillary gas liquid chromatography on a Shimadzu model (Shimadzu Corporation, Kyoto, Japan) GC-17A version 3 Gas Chromatograph (GC) equipped with split-splitless and on-column/PTV injectors, and flame ionisation detection. Injection was performed using a Model AOC-20i automatic injector (Shimadzu Corporation, Kyoto, Japan). The GC-17A was linked to a computerized integration system (CLASS-VP version 7.3, Shimadzu Scientific Instruments, Inc., Columbia, MD) to identify the peaks by comparison with retention times from standards.

Chromatographic conditions

FAME solutions were injected onto a FFAP (free FA and phenols) capillary column (30 m x 0.32 mm i.d., 0.25 μm film thickness) (AT-1000, Alltech Associates Inc, Deerfield IL; or ZB-FFAP, Phenomenex, Torrance, CA). The carrier gas was hydrogen at 32 kPa ($u = 35 \text{ cm/s}$). The GC was equipped with a flame ionization detector (temperature 270°C). For split injection, the temperature of the injection port was maintained at 250°C, and 2.0 μL injected at a split ratio of 15:1. For on-column injection the injection port was maintained at 80 °C for 0.1 min after injecting 0.2 μL , then programmed at maximum linear rates to 220 °C which was held for 2 min. Following sample injection the temperature of the column oven was held at 50 °C for 1.5 min, then programmed to increase to 150 °C at the rate of 15 °C/min. and then to increase to 220 °C at the rate of 6 °C/min and held until the end of the run. The entire run was approximately 47 min.

Identification and quantitation of fatty acids

Identification of FA and isomers peaks were based on retention times of FAs in samples of known composition such as commercial cod liver oil (CLO)

(Ackman and Burgher, 1965), peanut oil, Menhaden fish oil FAME standard (Supelco 4-7116) and anhydrous milk fat (AMF). CLO was used as the standard to run daily to determine accurate retention times. Peak areas were measured with an electronic integrator and individual theoretical response factors, obtained from certified reference standards mentioned above, were used to verify the quantification (Bannon *et al.*, 1986). FAs were expressed as mass percent of total FA and were designated by the nomenclature structural system of carbon chain length:number of double bonds and location (n-x) of the double bond nearest to the terminal methyl group according to Morris (1961). Quantitation of FA was done using theoretical response factors calculated according to Ackman and Sipos (1964) and Bannon *et al.* (1986).

Calculations were made by the software CLASS-VP as followed:

$$\text{Weight \% of fatty acid} = \frac{\text{peak area}\%_i \times \text{correction factor}_i}{\sum_{i=1}^n (\text{peak area}\%_i \times \text{correction factor}_i)}$$

Quality control

On each run, commercial CLO was used as quality control samples; the CLO was positioned at the beginning, middle and at the end of the run. The consistency of the results was monitored day-to day by plotting control charts.

Data analysis

The experimental design was constrained by the limited number of sea lions that could be sampled and by welfare issues that meant that each sea lion could only be subjected to the collection regimen twice. Further, because the pattern of absorption of the exogenous oil was unknown it was necessary to spread the collection intervals over a wide period. Thus samples of serum, blubber and milk were collected at time 0 to establish baseline values for each animal and then another set of samples was collected at various intervals after the administering of the oil (Tables 18 and 19). The consequence of this

was that only one sample was represented at some of the intervals. Thus for statistical analysis the data were pooled into five intervals, 0, 12, 24, 48 and 72 hours relative to the time of administering of the oil (Table 19).

I. Temporal changes in fatty acid composition of the tissues

Descriptive statistics

A repeated-measures analysis of variance with the MIXED procedure of SAS (Statistical Analysis System, version 9.1; SAS Institute Inc., Cary, NC, USA), was performed on arcsine transformed data to test whether there were significant differences in the weight % of milk, serum and blubber FAs between time 0 (before the CoNVO was administered) and time intervals 12, 48hrs and 72hrs (after the CoNVO was administered). The model included the fixed effect of time. A compound symmetry error structure was determined as the most appropriate residual covariance structure for repeated measures over time within animals. Differences were considered significant at the 0.05 level.

The equivalency of oil (g/100) that was need to be secreted into the milk to change the concentration of C10:0, C12:0, C14:0, C16:0, C18;2n-6 in the milk at 0hrs by the amount observed at each of the time intervals after feeding was calculated by the following formulas:

$$A = \frac{(Fattyacid_{weight\%}^{time=0hrs}) * 100}{\sum_{weight\%}^{time=0hrs} Fattyacids}$$

where A is the fatty acid at baseline;

$$B = \frac{(Fattyacid_{weight\%}^{time=xhrs}) * 100}{\sum_{weight\%}^{time=xhrs} Fattyacids}$$

where time x hrs is any of the time intervals other than 0 hrs

$$oil = \frac{(Fattyacid_{weight\%}^{oil}) * 100}{\sum_{weight\%}^{oil} Fattyacids}$$

$$oilE = \left(\frac{(B - A)}{(oil - A)} \right) * 100$$

where oi/E is the oil equivalent g/100 ml.

Multivariate analysis

FAs common to milk, blubber and serum samples were considered for multivariate data analysis using the GLM procedure of SAS (Statistical Analysis System, version 9.1; SAS Institute Inc., Cary, NC, USA). The absolute difference between the CoNVO and the milk, blubber and serum samples in the levels of each FA were calculated. The raw data, presented as weight percent composition, were arcsine transformed prior to analyses.

The data were pooled to test whether the FA composition of the milk, blubber and serum varied after the CoNVO was administered. Only those FAs that were common in all samples in the milk, serum and blubber were included. The FA C8:0 was not included into the analysis since it is rarely seen in the milk, serum and blubber of NZSL.

Discriminant analysis

Milk, Serum and Blubber

The STEPDISC (Stepwise Discriminant Analysis, SDA) procedure of SAS (Statistical Analysis System, version 9.1; SAS Institute Inc., Cary, NC, USA); was used to identify the milk FAs that discriminated between samples from different collections times. FAs were chosen to enter or leave the model based on their contribution to the overall discriminatory power as measured by Wilks' λ test. A moderate significance level of 0.15 was chosen for selection (Costanza and Afifi, 1979).

The CANDISC (Canonical Discriminant Analysis, CDA) procedure of SAS (Statistical Analysis System, version 9.1; SAS Institute Inc., Cary, NC, USA); was used to analyse differences among collection times as applied by Staniland and Pond (2004). To test for significant differences between tissues (milk, serum and blubber) collection times, Pillai's trace approximation of the F statistic was used.

Classification and Regression Tree (CART) analysis

Classification and Regression Trees (CART) analysis, was used according to Iverson *et al.* (1997a; 1997b) and Smith *et al.* (1997). CART was performed on the arcsine-transformed data using Partition analysis in JMP software (SAS Institute Inc., Cary, NC, USA). The regression trees were constructed using the collection times (interval times) of a sample (milk, serum and blubber) as the response and the FAs as the predictors. The stopping rule was conservative with a minimum node size of four.

II. Pre and Post-treatment sea lions

Discriminant analysis

Milk, Serum and Blubber

The effect of treatment was analysed by comparing samples collected from the sea lions before administering the CoNVO (pre-treatment sea lions) with those collected after giving the CoNVO (post-treatment sea lions).

The milk, serum and blubber FA signatures of the two sea lions groups were compared using the STEPDISC (SDA) and CANDISC (CDA) procedures of SAS (Statistical Analysis System, version 9.1; SAS Institute Inc., Cary, NC, USA).

RESULTS

Females' body mass (kg), length (cm), offspring sex, age at first capture and interval to second capture are shown in Table 18. The average maternal body mass was 119.6 ± 12.77 kg (SD), body length was 179.88 ± 6.08 cm and the average maternal age was 12.66 ± 1.62 years.

The number of samples collected at each time interval and number of samples in each of the pooled time intervals are presented in Table 19.

The major FAs used in the analysis are shown in Table 20. The FA profile of the CoNVO is shown in Figure 15. The FA profile (mean percentage mass values for each FA) for milk, blubber and serum in samples at baseline i.e. collected prior to the administering of the CoNVO, are shown in Figure 15.

Table 18. Identification number (ID), mass, body length and age of the 25 branded female NZ sea lions and the sex of their pup, at first capture and the interval to second capture.

| ID | Mass (kg) | Length (cm) | Age (years) | Pup sex | Interval (hrs) |
|-------|-----------|-------------|-------------|---------|----------------|
| 1437 | 108.5 | 172.0 | 11 | F | 8 |
| 1472 | 105.0 | 171.0 | 13 | F | 4 |
| 1424 | 136.0 | 184.0 | 12 | M | 16 |
| 1419 | 108.5 | 185.0 | 12 | M | 72 |
| 1492 | 129.5 | 180.0 | 12 | F | 8 |
| 1472 | 105.0 | 171.0 | 13 | F | 72 |
| 1448 | 118.5 | 180.0 | 12 | F | 12 |
| 1445 | 121.5 | 172.0 | 13 | M | 12 |
| 1469 | 131.5 | 190.0 | NA | M | 4 |
| 1433 | 125.0 | 182.0 | 13 | F | 48 |
| 1367 | 130.5 | 179.0 | 10 | F | 16 |
| 1404 | 116.0 | 173.0 | NA | M | 48 |
| 1410 | 138.5 | 182.0 | 14 | M | 24 |
| 1481 | 148.5 | 193.0 | 17 | F | 24 |
| 1370 | 118.5 | 179.0 | NA | F | 20 |
| 1388 | 106.0 | 177.0 | 13 | M | 20 |
| 1393* | 135.5 | 187.0 | 12 | M | 72 |
| 1452* | 127.5 | 171.0 | 14 | M | 72 |
| 1470* | 119.0 | 183.0 | 15 | M | 24 |
| 1411* | 125.0 | 183.0 | NA | M | 72 |
| 1471* | 100.5 | 179.0 | 14 | M | 48 |
| 1437* | 110.0 | 184.0 | 12 | M | 30 |
| 1399* | 108.0 | 177.0 | 13 | M | 24 |
| 1491* | 114.5 | NA | 10 | M | 24 |
| 1367* | 104.5 | 183.0 | 11 | M | 38 |

*Females captured in summer 2005. NA: not available. There was not a year effect both years pooled together.

Table 19. Actual intervals (hrs) at which females (n=25) were captured and the number of samples of milk, blubber and serum collected together with the pooled time intervals used for statistical analysis.

| Time intervals (hrs) | | Number of samples | | |
|----------------------|-----------------------|-------------------|---------|-------|
| Actual Interval | Pooled Time Intervals | Milk | Blubber | Serum |
| 0 | 0 | 16 | 18 | 22 |
| 4 | 12 | 1 | 1 | 2 |
| 8 | 12 | 3 | 1 | 3 |
| 12 | 12 | 1 | 1 | 2 |
| 16 | 24 | 1 | 2 | 2 |
| 20 | 24 | 1 | 2 | 2 |
| 24 | 24 | 4 | 5 | 3 |
| 30 | 48 | 1 | - | - |
| 38 | 48 | 1 | 1 | 1 |
| 48 | 48 | 2 | 4 | 3 |
| 72 | 72 | 5 | 4 | 5 |
| Total | - | 36 | 39 | 45 |

I. Temporal changes: fatty acid composition of milk, serum and blubber

Descriptive statistics

Milk

Administering the CoNVO significantly ($P < 0.05$) increased the concentration of milk C12:0, from 0.11% at time 0 to 0.70% at 12hrs, to a peak of 2.26% at 24 hrs and remained significantly elevated, at 48 hrs at 1.56% and at 72hrs at 0.68% (Figure 16a). C14:0 concentration also increased significantly ($P < 0.05$) from 5.06% at time 0 to 7.53% at 48hrs (Figure 16a). The concentrations of C18:1n-7 decreased significantly ($P < 0.05$) from 3.95% at time 0 to 3.08% at 48hrs, and 3.27% at 72hrs (Figure 17a). In response to the ingestion of the CoNVO, C18:2n-6 concentration significantly increased from 1.08% at time 0 to a peak of 6.69% at 24hrs, and remained significantly elevated in relation to time 0 concentrations, 4.8% at 48hrs (Figure 17a). The FA composition of the milk samples before the CoNVO was administered was $29.13 \pm 4.72\%$ SAFA, $42.49 \pm 5.97\%$ MUFA, and $25.37 \pm 8.82\%$ PUFA (Table 20 and Figure 18a). A significant decrease was observed in MUFA at 48 hrs to 36.12% (Figure 18a). Various FAs that were unique to the CoNVO appeared in the milk after

feeding the CoNVO. The amount of oil that would be needed to supply sufficient unique FA to reach its observed concentration in 100g of milk fat was calculated as an 'equivalency of oil'. The calculated equivalencies of oil (g/100) for specific milk FAs are shown in Figure 19.

Serum

C12:0 concentration in serum increased rapidly on the ingestion of CoNVO with a significant ($P<0.05$) increase from 0.14% at time 0 to 0.82% at 12hrs, (Figure 16b). A similar response was observed in C14:0 with a significant ($P<0.05$) increase from 1.25% at time 0 to 3.7% at 72hrs (Figure 16b).

Significant changes were also observed in the concentration of the long-chain polyunsaturated FA, C18:2n-6 after the ingestion of the CoNVO (Figure 17b). C18:2n-6 increased significantly ($P<0.05$) from 0.80% at time 0 to 3.72% at 12hrs, and decreased to 1.78% at 24hrs (Figure 17b).

The FA composition of the serum samples at baseline (before the CoNVO was administered) was $29.87 \pm 6.36\%$ SAFA, $25.58 \pm 8.81\%$ MUFA, and $23.02 \pm 9.48\%$ PUFA (Table 20 and Figure 18b). Significant increases were observed in MUFA and PUFA from 24.68% and 22.26% at baseline, respectively, to 34.97% and 32.33%, respectively, at 72 hrs (Figure 18b).

Blubber

The baseline amounts of the FAs specifically tracked in the blubber were as follow: C10:0, $0.10 \pm 0.06\%$; C12:0, $0.06 \pm 0.03\%$; C14:0, $3.78 \pm 0.82\%$; C18:0, $2.08 \pm 0.35\%$; C18:3n-3, $0.43 \pm 0.13\%$; C18:1n-9, $29.07 \pm 3.63\%$; C18:1n-7, $4.09 \pm 0.36\%$; C18:2n-6, $1.54 \pm 0.19\%$ (Table 20 and Figure 15).

After the ingestion of the CoNVO no significant changes were observed in the FA composition of the blubber (Figures 16c and 17c).

No significant temporal changes were observed after the CoNVO was administered in SAFA, MUFA and PUFA composition of the blubber samples.

Multivariate statistics

Discriminant analysis

Milk

The FAs used in the SDA and CDA were as follow: C12:0, C14:0, C15:0 iso Br, C15:0, C16:0, C16:1n-7, C16:2n-4, C17:0, C18:0, C18:1n-9, C18:1n-7, C18:2n-6, C20:1n-9, C20:5n-3, C22:1n-9, C21:5n-3, C22:6n-3 and the ratio of essential $\omega_3 : \omega_6$.

The SDA indicated that the ratio of essential $\omega_3 : \omega_6$, C14:0, C22:6n-3, C20:1n-9, C16:2n-4, C18:0 and C12:0 were needed to discriminate between the milk collection times and these were significantly different individually at various intervals. The parameters with the greatest power to discriminate between milk collection times were the ratio of essential $\omega_3 : \omega_6$ (Partial $R^2=0.66$, $F=13.9$, $P<0.001$, Wilks' $\lambda =0.34$) and C14:0 (Partial $R^2=0.47$, $F=6.1$, $P>0.05$, Wilks' $\lambda =0.18$). The former component had a lower ratio in the CoNVO than in milk while the later component had higher concentration in the CoNVO than in the milk samples (Table 20).

The CDA indicated that there were significant differences between the milk collection times (Pillai's trace=2.71, $F_{(72,25)}=1.76$, $P<0.05$). The canonical scores were plotted (Figure 20) and illustrate a clear separation of the samples collected at different intervals after the administering of the CoNVO.

Serum

The serum FAs used in the SDA and CDA were as follow: C10:0, C12:0, C14:0, C15:0, C16:0 iso Br, C16:0, C16:1n-7, C16:2n-4, C17:0, C18:0, C18:1n-9, C18:1n-7, C18:2n-6, C18:3n-3, C20:1n-9, C20:2n-6, C20:4n-6, C20:4n-3, C20:5n-3, C22:6n-3 and the ratio of essential $\omega_3 : \omega_6$.

SDA identified C18:2n-6 (Partial- $R^2=0.47$, $F=8.49$, $P<0.001$, Wilks' $\lambda =0.53$) and C20:5n-3 (Partial- $R^2=0.29$, $F=3.90$, $P<0.001$, Wilks' $\lambda =0.38$) as having significant power to discriminate between collection times. However, the CDA indicated that the serum FAs signatures were unable to distinguish between the collection times (Pillai's trace = 2.30, $F_{(88, 84)}=1.29$, $P>0.05$) (Figure 21).

Blubber

The blubber FAs used in the SDA and CDA were as follow: C14:0, C14:1, C15:0 iso Br, C16:0, C16:1n-7, C16:2n-4, C17:0 iso, C17:0 ante, C17:0, C16:3n-4, C18:0, C18:1, C18:1n-9, C18:1n-7, C18:2n-6, C18:3n-3, C18:4n-3, C20:0, C20:1n-11, C20:1n-9, C20:2n-6, C20:3n-6, C20:4n-6, C20:3n-3, C20:4n-3, C20:5n-3, C22:1n-9, C21:5n-3, C22:5n-3 and C22:6n-3.

Time of collection or time intervals could be discriminate by the following blubber FAs, according to the SDA, C14:1, C17:0 ante, C18:1 and C14:0. The FA with the largest power to discriminate between time intervals were C14:1 (Partial- $R^2=0.35$, $F=4.55$, $P<0.01$, Wilks' $\lambda = 0.65$), C17:0 ante (Partial- $R^2=0.22$, $F=2.26$, $P<0.05$, Wilks' $\lambda =0.51$), C18:1 (Partial- $R^2=0.21$, $F=2.12$, $P>0.05$, Wilks' $\lambda =0.40$) and C14:0 (Partial- $R^2=0.24$, $F=2.46$, $P>0.05$, Wilks' $\lambda =0.31$). The results of CDA indicated that when the above listed FAs were used to discriminate between blubber time intervals there was no significant differences (Pillai's trace = 3.36, $F_{(120, 32)}=1.41$, $P>0.05$) (Figure 22).

Classification and Regression Tree (CART) analysis

Milk

The whole milk FA signature was used in the CART analysis. CART created a pruned tree from three FAs giving five terminal nodes when milk samples were classified by their time interval (collection times) (Figure 23). All 16 of the time interval 0 hr (control) samples were placed in terminal node 1, (14 samples) and terminal node 2 (2 samples) and were separated from the remaining samples by lower values of C18:2n-6 and C12:0, respectively. Terminal node 2 also contained one sample each from 12 hrs and 24hrs (Figure 23). The remaining samples from 12, 14, 48 and 72 were allocated fairly uniformly across terminal nodes 3, 4 and 5. Terminal nodes 3 and 5 were separated by higher values of C18:2n-6 and C15:0 (Figure 23). Whereas most samples ($n=5$) from time interval 24 hrs terminated in terminal node 3 separated by higher concentrations of C18:2n-6 (Figure 23).

Serum

The whole serum FA signature was used in the CART analysis. CART created a pruned tree from three FAs giving four terminal nodes when serum samples

were classified by their time interval (collection times) (Figure 24). Terminal node 1, consisting of 19 out of the 22 samples collected at time interval 0 hrs together with one sample each from 12, 24 and 48 hrs, were separated by lower concentrations of C18:2n-6, which was high in the CoNVO administered to the sea lions (Table 20 and Figure 23). Terminal nodes 2, 3 and 4 were separated by higher concentrations of C18:2n-6. Terminal node 2 consisted of samples from time interval 24hrs and 12hrs and was separated by lower values of C20:5n-3 which was not found in the CoNVO (Figure 23). Terminal node 4 was separated from terminal node 3 by higher concentrations of C20:2n-6 (Figure 23).

II. Pre and Post-treatment sea lions

Discriminant analysis

Milk

The FAs used in the SDA were as follow: C12:0, C14:0, C15:0 iso Br, C15:0, C16:0, C16:1n-7, C16:2n-4, C17:0, C18:0, C18:1n-9, C18:1n-7, C18:2n-6, C20:1n-9, C20:5n-3, C22:1n-9, C21:5n-3, C22:6n-3 and the ratio of essential ω_3 : ω_6 .

According to the SDA, from these FAs, C18:2n-6 and C14:0 were needed to discriminate between the pre-treatment sea lions from the post-treatment sea lions. The FAs with the largest power to discriminate between the control sea lion group from the experimental sea lion group were C18:2n-6 (Partial $R^2=0.32$, $F=20.3$, $P<0.001$, Wilks' $\lambda =0.67$) and C14:0 (Partial $R^2=0.05$, $F=2.37$, $P<0.05$, Wilks' $\lambda =0.63$), these FAs had a weight percentage much higher in the CoNVO than in the milk samples (Table 20). A significant differences was found between the milk FAs signature of the experimental sea lion group and of the control sea lion group (Pillai's trace = 0.59, $F_{(26, 17)}=2.23$, $P<0.05$).

Serum

The FAs used in the SDA and CDA were as follow: C14:0, C15:0, C16:0 iso Br, C16:0, C16:1n-7, C16:2n-4, C17:0, C18:0, C18:1n-9, C18:1n-7, C18:2n-6, C20:2n-6, C20:4n-6, C20:4n-3, C20:5n-3 and C22:6n-3.

According to the SDA, from these FAs, C14:0 and C18:2n-6 were needed to discriminate between the control sea lion group from the experimental sea lion group. The FA with the largest power to discriminate between the control sea lion group from the experimental sea lion group was C18:2n-6 (Partial- $R^2=0.33$, $F=20.33$, $P>0.001$, Wilks' $\lambda =0.67$), this FAs had a weight percentage much higher in the CoNVO than in the serum samples (Table 20). There was not a significant differences between the serum FAs signature of the experimental sea lion group and the milk FA signature of the control sea lion group (CDA: Pillai's trace=0.59, $F_{(26, 17)}=2.23$, $P>0.05$).

Blubber

The blubber FAs used in the SDA and CDA were as follow: C14:0, C14:1, C15:0 iso Br, C16:0, C16:1n-7, C16:2n-4, C17:0 iso, C17:0 ante, C17:0, C16:3n-4, C18:0, C18:1, C18:1n-9, C18:1n-7, C18:2n-6, C18:3n-3, C18:4n-3, C20:0, C20:1n-11, C20:1n-9, C20:2n-6, C20:3n-6, C20:4n-6, C20:3n-3, C20:4n-3, C20:5n-3, C22:1n-9, C21:5n-3, C22:5n-3 and C22:6n-3. SDA found three FAs that could discriminate between experimental sea lions and the control sea lions, there FAs were: C16:0 (Partial- $R^2=0.06$, $F=2.32$, $P<0.05$, Wilks' $\lambda =0.94$), C15:0 iso Br (Partial- $R^2=0.13$, $F=5.17$, $P<0.05$, Wilks' $\lambda =0.82$) and C22:5n-3 (DPA) (Partial- $R^2=0.08$, $F=3.22$, $P>0.05$, Wilks' $\lambda =0.75$). The former FA was found in similar concentration in the blubber and CoNVO whilst the later two FAs were not present in the CoNVO sample (Table 20). The CDA did not find significant differences between the blubber FAs signature of the experimental sea lion group and the milk FA signature of the control sea lion group (Pillai's trace=0.73, $F_{(30, 8)}=0.73$, $P>0.05$).

Table 20. Fatty acid composition (mass %) of New Zealand sea lion milk, blubber, serum and a cocktail of natural vegetable oils (CoNVO) at time 0. Values are means with standard errors. Fatty acids in bold indicate that a significant difference occurred after the CoNVO was administered.

| Fatty acids | n | Blubber | Serum | Milk | CoNVO |
|-------------------|---|------------------|-------------------|-------------------|-------|
| | | 18 | 25 | 16 | |
| Σ SAFA | | 16.75 \pm 2.02 | 29.87 \pm 6.36 | 29.13 \pm 4.72 | 45.5 |
| Σ MUFA | | 54.86 \pm 4.55 | 25.58 \pm 8.81* | 42.49 \pm 5.97* | 20.1 |
| Σ PUFA | | 25.35 \pm 4.35 | 23.02 \pm 9.48 | 25.37 \pm 8.82 | 34.1 |
| C08:0 | | - | - | 0.2 | 3.5 |
| C10:0 | | 0.10 \pm 0.06 | 0.08 \pm 0.48 | 0.10 \pm 0.07 | 2.60 |
| C12:0 | | 0.06 \pm 0.03 | 0.13 \pm 0.11 | 0.11 \pm 0.05* | 19.0 |
| C13:0 | | 0.00 \pm 0.00 | 0.05 \pm 0.03 | 0.02 \pm 0.01 | - |
| C14:0 | | 3.78 \pm 0.82 | 1.30 \pm 0.82* | 5.06 \pm 1.55* | 6.90 |
| C14:1 | | 0.31 \pm 0.11 | 0.08 \pm 0.05 | 0.17 \pm 0.06* | - |
| C15:0 iso Br | | 0.13 \pm 0.03 | 0.15 \pm 0.16 | 0.16 \pm 0.03 | - |
| C15:0 ante-iso Br | | 0.04 \pm 0.01 | 0.08 \pm 0.08 | 0.06 \pm 0.04 | - |
| C15:0 | | 0.40 \pm 0.08 | 0.19 \pm 0.16* | 0.44 \pm 0.08 | - |
| C16:0 Br | | 0.08 \pm 0.03 | 0.14 \pm 0.10 | 0.06 \pm 0.02 | - |
| C16:0 | | 9.52 \pm 1.05 | 14.80 \pm 3.35 | 19.79 \pm 3.38 | 10.50 |
| C16:1n-7 | | 5.66 \pm 1.04 | 2.30 \pm 1.13 | 5.72 \pm 1.44 | 0.1 |
| C17:0 iso Br | | 0.19 \pm 0.05 | 0.29 \pm 0.11 | 0.22 \pm 0.05* | - |
| C17:0 ante-iso Br | | 0.09 \pm 0.02 | 0.09 \pm 0.04 | 0.14 \pm 0.04 | - |

Table 20. (Continued).

| Fatty acids | n | Blubber | Serum | Milk | CoNVO |
|--|---|-------------|-------------|--------------|-------|
| | | 18 | 25 | 16 | |
| C16:2n-4? | | 0.93 ±0.12 | 2.69 ±2.12 | 0.81 ±0.11* | - |
| C17:0 | | 0.33 ±0.08 | 1.95 ±2.76 | 0.40 ±0.09* | - |
| C16:3n-4? | | 0.51 ±0.10 | 0.29 ±0.19 | 0.50 ±0.11 | - |
| C16:4n-1? | | 0.013 -- | 0.16 ±0.27 | 0.24±0.18 | - |
| C18:0 | | 2.08 ±0.35 | 10.79 ±4.47 | 2.76 ±0.61 | 2.20 |
| C18:1n-9 | | 29.07 ±3.63 | 15.95 ±6.20 | 23.22 ±6.24 | 19.50 |
| C18:1n-7 | | 4.09 ±0.36 | 3.50 ±1.12 | 3.94 ±0.57 | 0.40 |
| Other C18:1? | | 0.47 ±0.09 | 0.31 ±0.11 | 0.44 ±0.09* | - |
| C18:2n-6 (Linoleic) | | 1.54 ±0.19 | 0.79 ±0.40* | 1.05 ±0.26* | 33.7 |
| C18:3n-3 (Linolenic) | | 0.43 ±0.13 | 0.20 ±0.16 | 0.44 ±0.13 | 0.40 |
| C18:4n-3 | | 0.38 ±0.16 | 0.18 ±0.25 | 0.70 ±0.50* | - |
| C20:0 | | 0.11 ±0.03 | 0.04 ±.001 | 0.05 ±0.01 | 0.30 |
| C20:1n-11 | | 1.28 ±0.19 | 0.72 ±0.95 | 0.50 ±0.15* | - |
| C20:1n-9 | | 11.74 ±2.61 | 2.74 ±1.51 | 6.77 ±1.44* | 0.10 |
| C20:2n-6 | | 0.43 ±0.07 | 0.30 ±0.14 | 0.30 ±0.10 | - |
| C20:3n-6 | | 0.11 ±0.02 | 0.17 ±0.07 | 0.10 ±0.04 | - |
| C20:4n-6 | | 0.71 ±0.16 | 5.27 ±3.35 | 0.94 ±0.31 | - |
| C20:3n-3 | | 0.20 ±0.04 | 0.10 ±0.06 | 0.19 ±0.05 | - |
| C20:4n-3 | | 1.10 ±0.24 | 0.46 ±0.37 | 1.29 ±0.46 | - |
| C20:5n-3 (EPA) | | 1.83 ±0.49 | 4.24 ±2.57 | 5.90 ±2.95 | - |
| C22:1n-13&n-11 | | 1.34 ±0.52 | 0.17 ±0.10 | 1.01 ±0.41 | - |
| C22:1n-9 | | 0.72 ±0.24 | 0.24 ±0.12 | 0.83 ±0.41* | - |
| C21:5n-3?? | | 0.27 ±0.09 | 0.21 ±0.15 | 0.40 ±0.18 | - |
| C22:0 | | 0.00 ±0.00 | 0.40 ±0.05 | 0.56 ±0.45 | 0.10 |
| C22:4n-6 ? | | 0.25 ±0.06 | 0.16 ±0.11 | 0.18 ±0.06 | - |
| C22:5n-6 ? | | 0.30 ±0.05 | 0.19 ±0.11 | 0.19 ±0.08 | - |
| C22:5n-3 (DPA) | | 3.84 ±0.62 | 2.00 ±1.01 | 2.39 ±0.98 | - |
| C24:0 | | 0.37 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.1 |
| C22:6n-3 (DHA) | | 12.54 ±2.63 | 6.37 ±4.09 | 10.61 ±3.87 | - |
| Total Σ FA | | 96.88 ±0.16 | 78.85 ±2.90 | 97.05 ±0.40 | 99.7 |
| Σ n-3 | | 21.55 ±0.81 | 12.94 ±1.68 | 23.29 ±1.50 | 0.40 |
| Σ n-6 | | 1.82 ±0.06 | 6.37 ±0.80 | 2.36 ±0.34 | 33.7 |
| Ratio ω3:ω6 | | 11.99 ±0.38 | 2.25 ±0.53 | 10.13 ±0.49* | 0.01 |

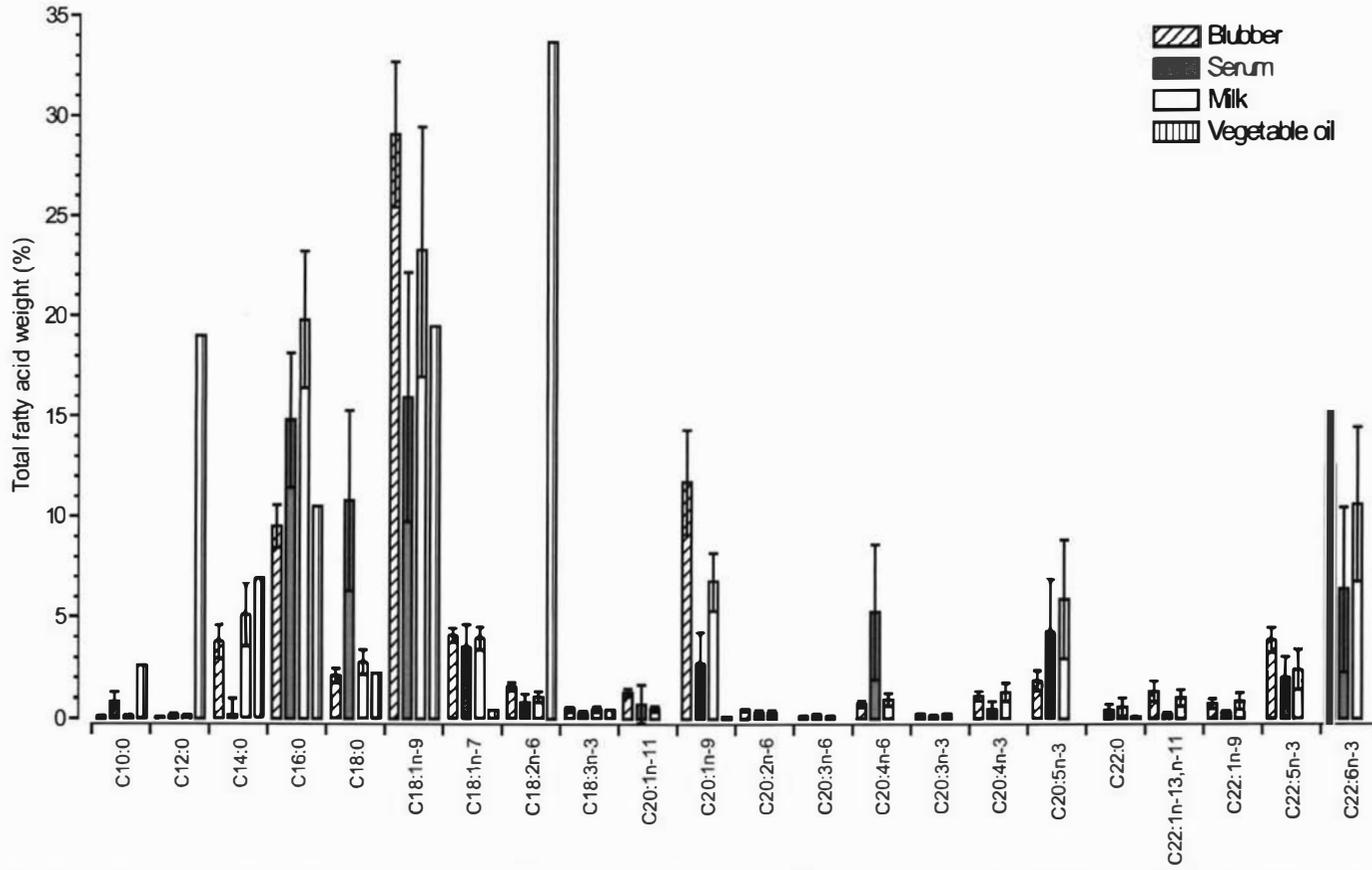


Figure 15. Most abundant fatty acids and their baseline composition (mass %) of a cocktail of natural vegetable oil composed of 60% sunflower oil and 40% coconut oil administered to lactating New Zealand sea lion; lipid extracted from milk (n=16), serum (n=22) and blubber (n=18) from lactating New Zealand sea lions, *Phocartos hookeri*, at Enderby Island, Auckland Islands. Values are means \pm SE.

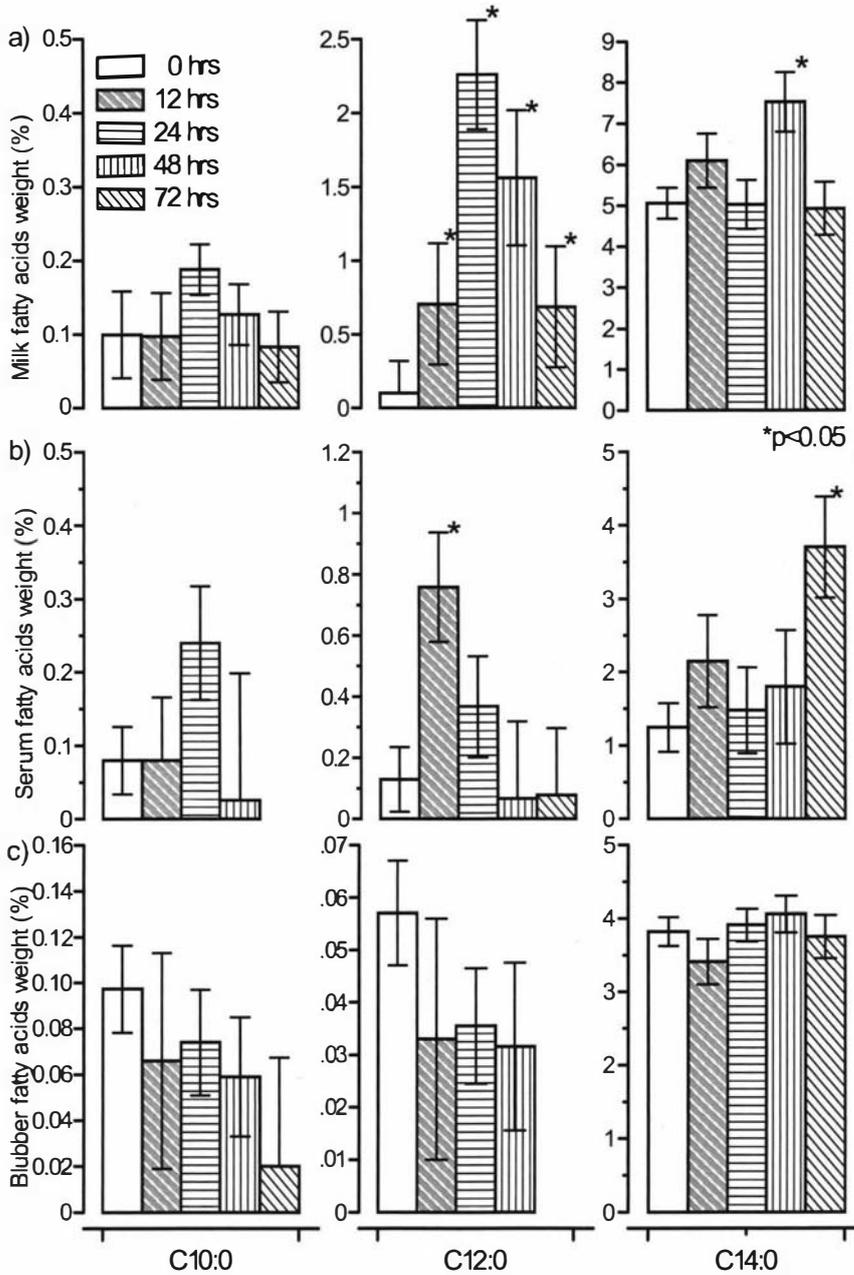


Figure 16. Temporal changes in the concentration of C10:0, C12:0 and C14:0 fatty acids (mass %) in a) milk , b) serum and c) blubber from 4 hrs to 72 hrs after a cocktail of natural vegetable oil was administered at 0 hrs to lactating New Zealand sea lions, *Phocarctos hookeri*, at Enderby Island, Auckland Islands. Values are means ±SE. * P>0.05.

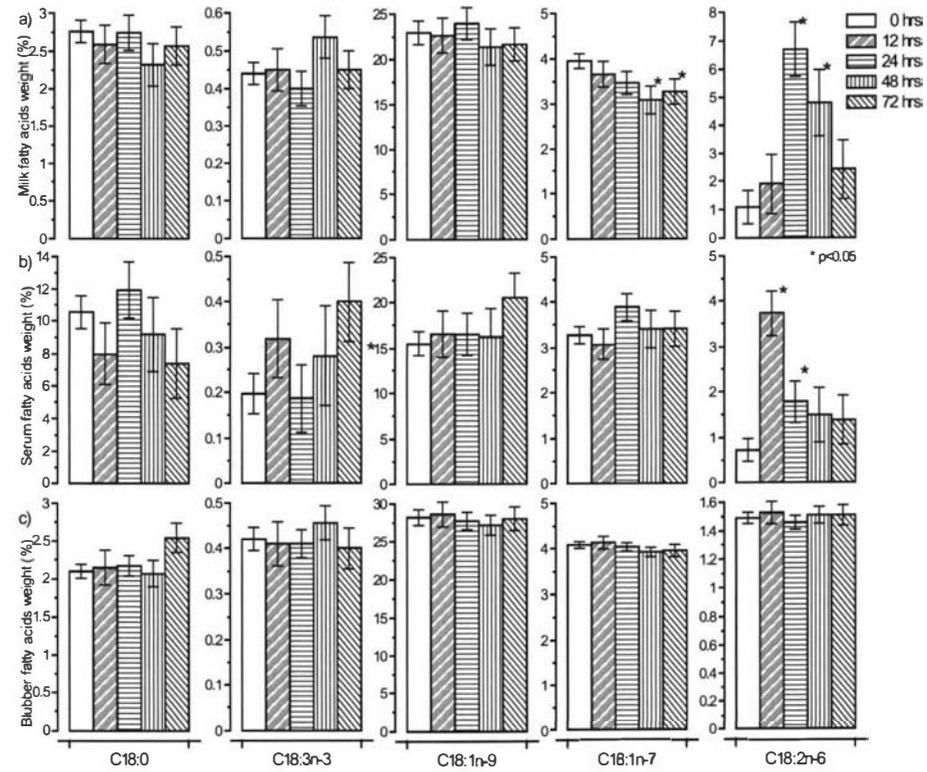


Figure 17. Temporal changes in the concentration of selected fatty acids mass (%) in a) milk, b) serum and c) blubber from 4 hrs to 72 hrs after a cocktail of natural vegetable oil was administered at 0 hrs to lactating New Zealand sea lions, *Phocarctos hookeri*, at Enderby Island, Auckland Islands. Values are means \pm SE. *P<0.05.

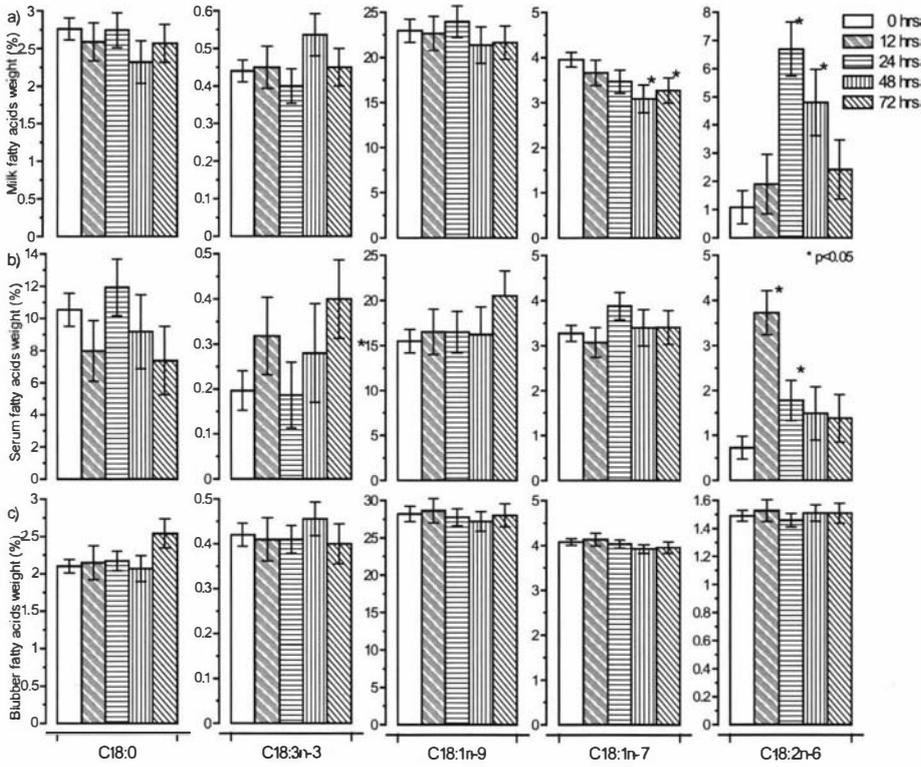


Figure 18. Temporal changes in the concentration of selected fatty acids mass (%) in a) milk, b) serum and c) blubber from 4 hrs to 72 hrs after a cocktail of natural vegetable oil was administered at 0 hrs to lactating New Zealand sea lions, *Phocarctos hookeri*, at Enderby Island, Auckland Islands. Values are means \pm SE. * $P < 0.05$.

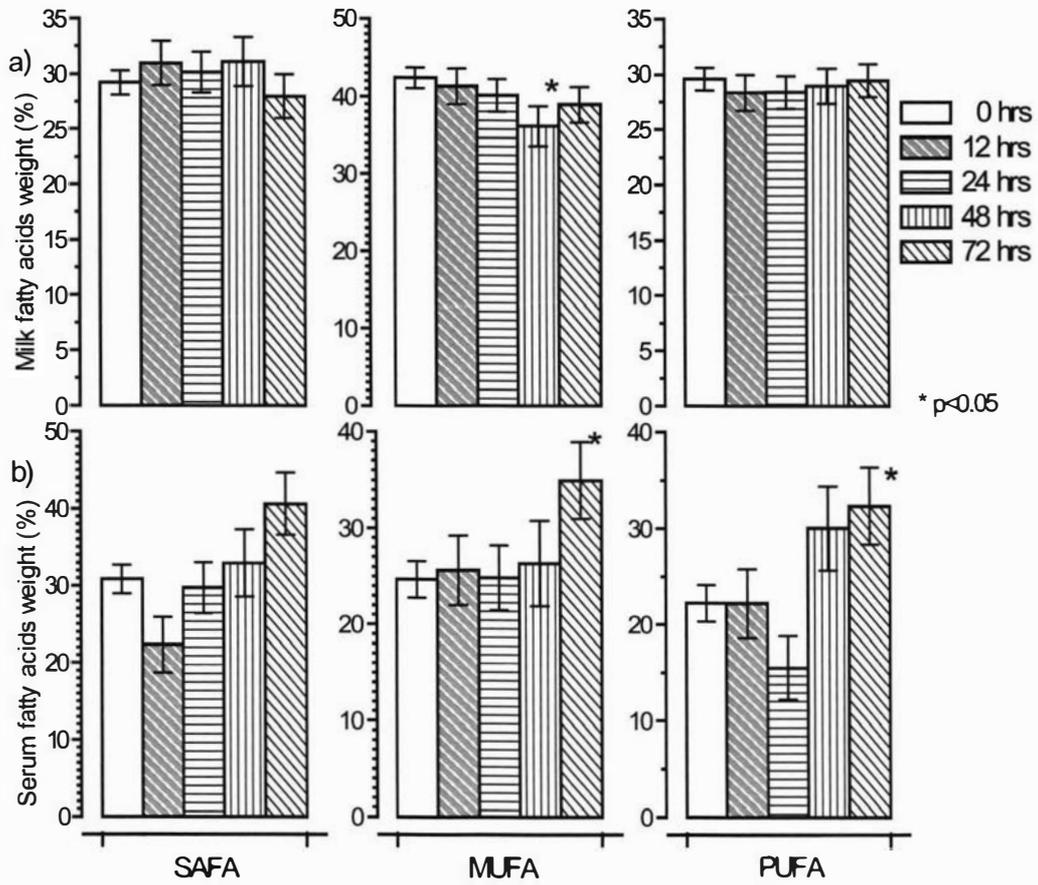


Figure 19. Temporal changes in the concentration (mass %) of saturated fatty acids (SAFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) in a) milk and b) serum 4 to 72 hrs after a cocktail of natural vegetable oil was administered at 0 hrs to lactating New Zealand sea lions, *Phocartos hookeri*, at Enderby Island, Auckland Islands. Values are means ±SE. * P<0.05

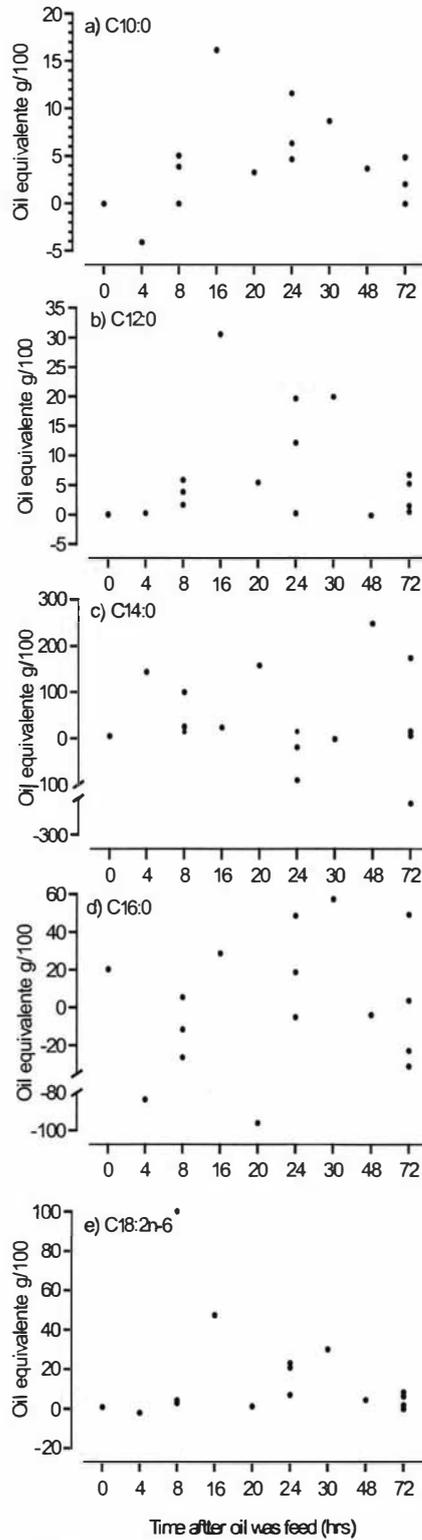


Figure 20. The calculated equivalency of oil (g/100) that was needed to be secreted into the milk to change the concentration of (a) C10:0, (b) C12:0, (c) C14:0, (d) C16:0 and (e) C18:2n-6 fatty acids in the milk fat from that measured at 0 hrs to that observed at each of the intervals from 4 to 72 hrs after a cocktail of natural vegetable oil was administered at 0 hrs to lactating New Zealand sea lions, *Phocarctos hookeri*, at Enderby Island, Auckland Islands. Each point represents the value for an individual sea lion.

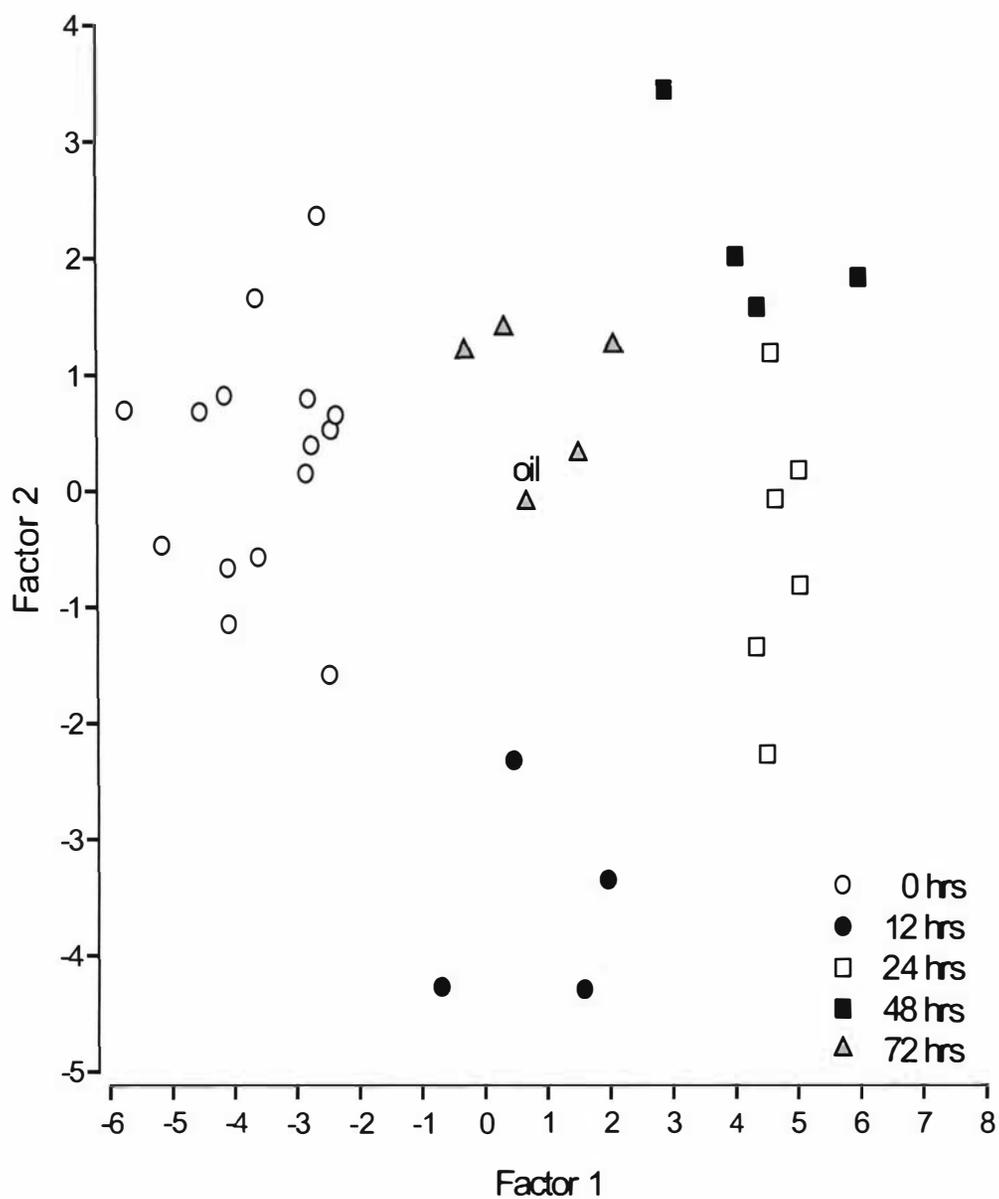


Figure 21. Plot of canonical scores of fatty acid data showing the relationship between the fatty acid composition of milk samples collected from New Zealand sea lions, *Phocarctos hookeri*, at the Auckland Islands and a mixture of natural vegetable oils (oil) administered at time 0. Legends represent the pooled time intervals at which the samples were collected before (0 hrs) and from 4 hrs to 72 hrs after the oil was administered.

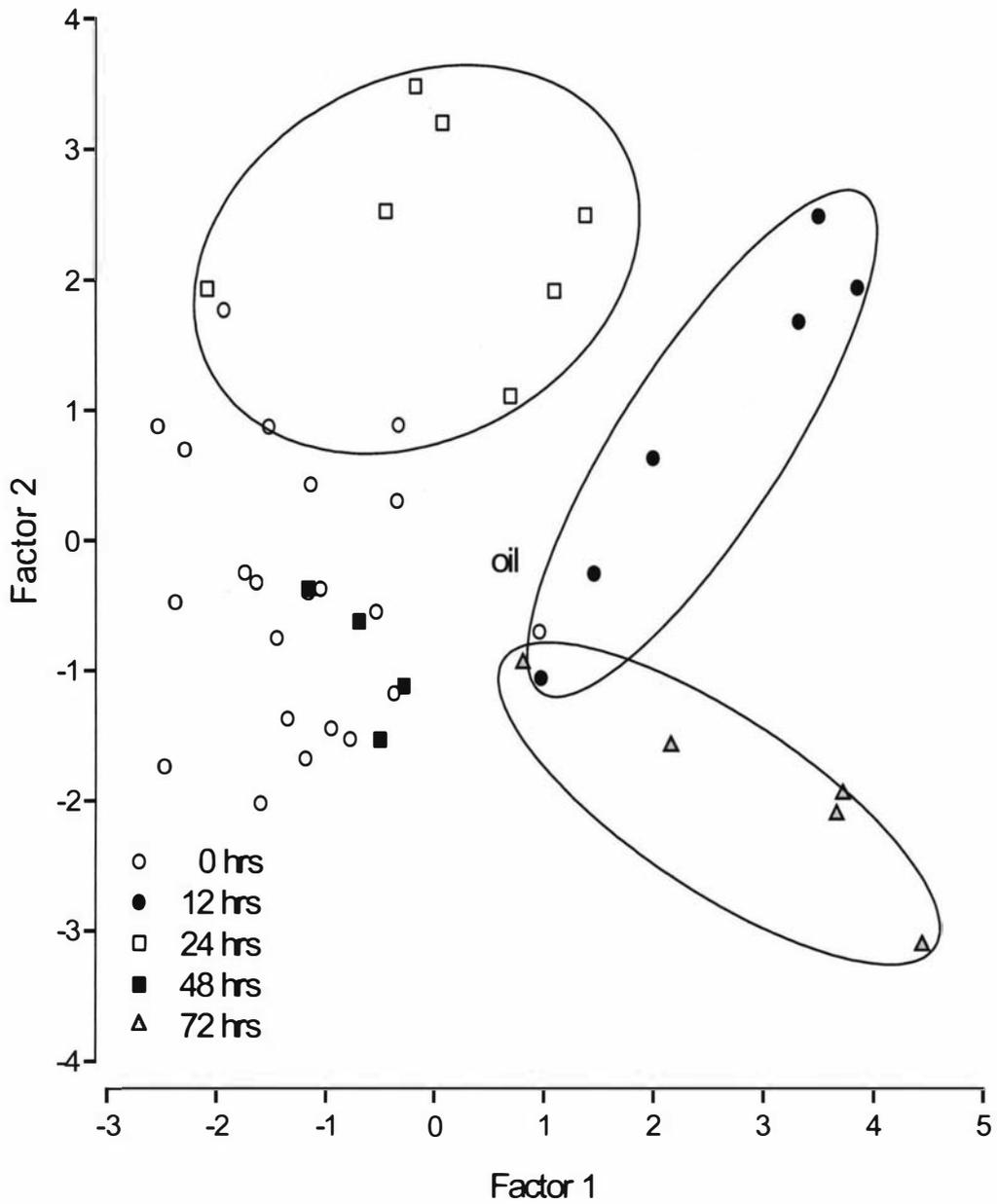


Figure 22. Plot of canonical scores of fatty acid data showing the relationship between the fatty acid composition of serum samples collected from New Zealand sea lions, *Phocarctos hookeri*, at the Auckland Islands and a mixture of natural vegetable oils (oil) administered at time 0 hr. Legends represent the pooled time intervals at which the samples were collected before (0 hrs) and 4 to 72 hr after the oil was administered.

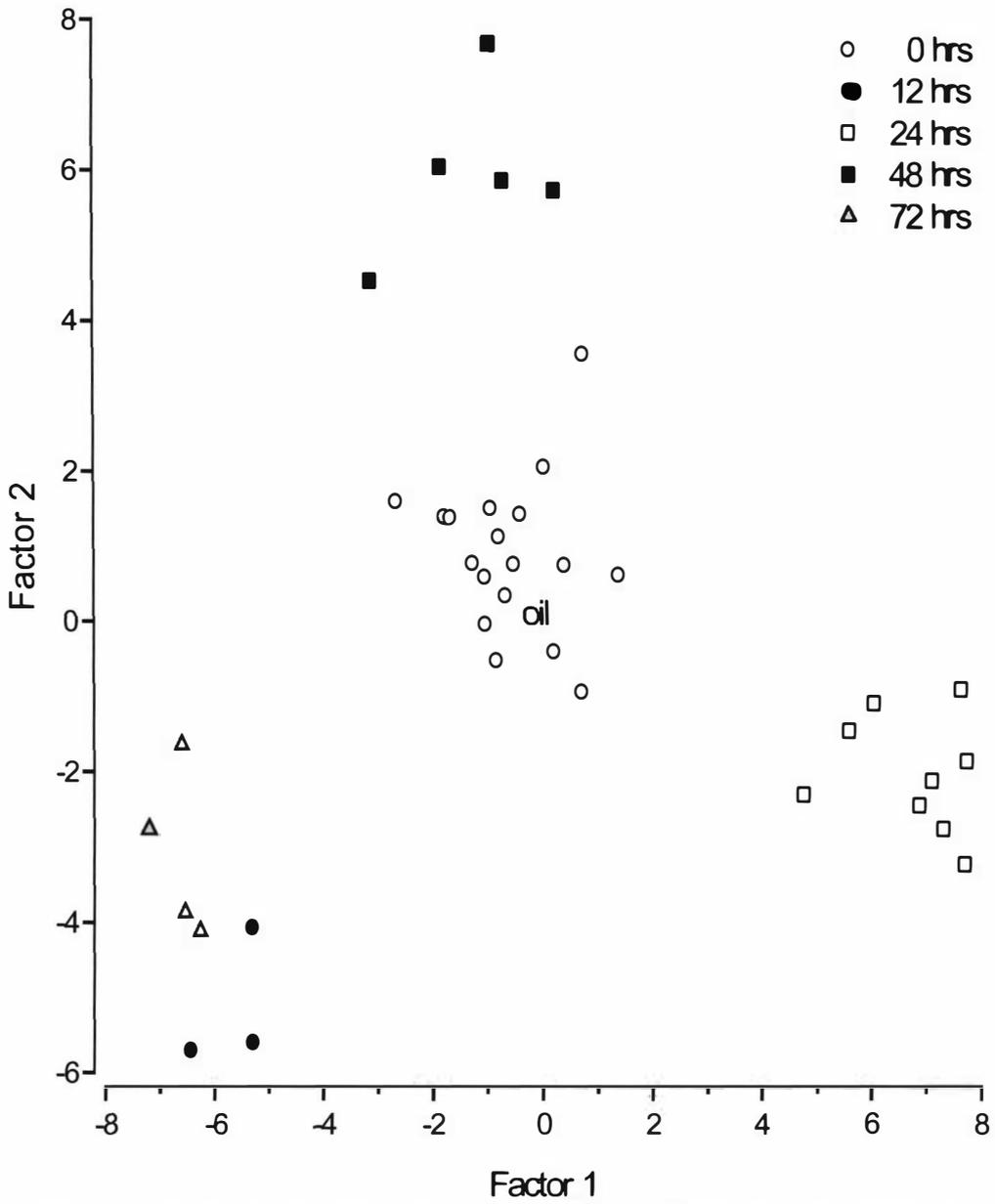


Figure 23. Canonical scores plot of the fatty acids data showing the relationship between blubber samples of New Zealand sea lions, *Phocarcctos hookeri*, collected at the Auckland Islands. Legends represent the time intervals at which sea lion were captured before (0 hrs) and after (4 hrs to 72 hrs) a mixture of vegetable was administered.

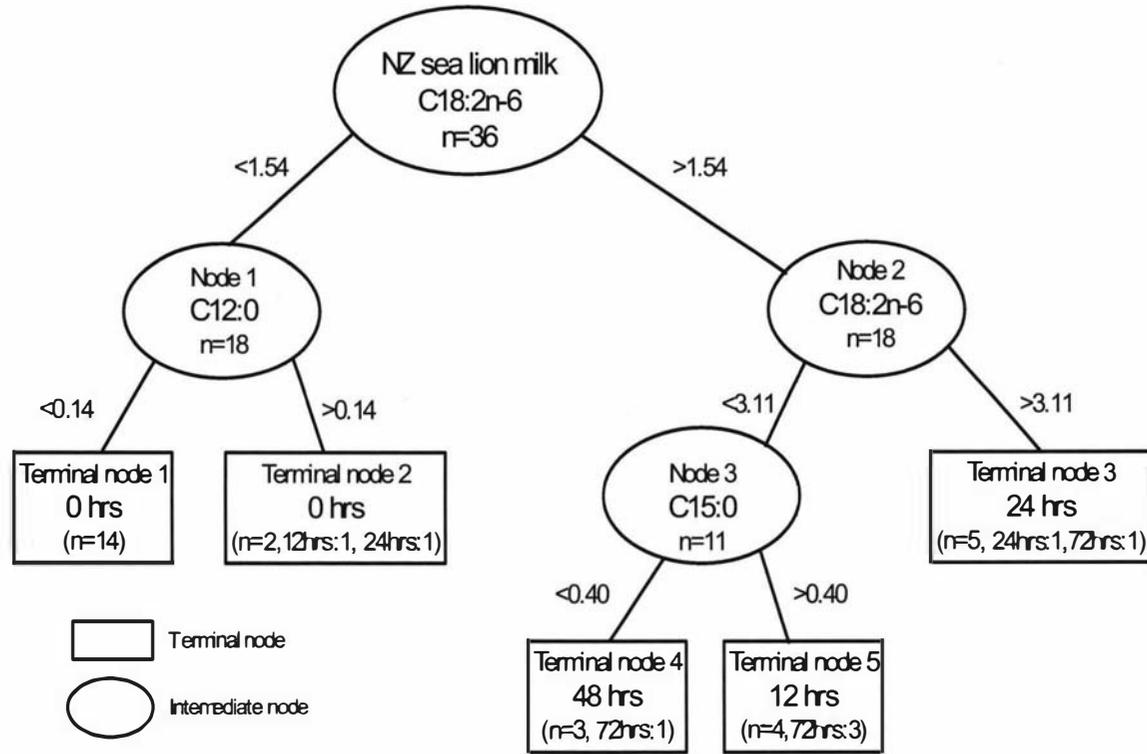


Figure 24. Classification tree, determined by CART ($R^2=0.65$), of milk samples classified by the pooled time interval (0, 12, 24, 48, 72 hrs) that they were collected after a cocktail of natural vegetable oil was administered to lactating New Zealand sea lions, *Phocarctos hookeri*, at the Auckland Islands. Each node is labelled with the fatty acid used by CART algorithm to create the split. Values on the branches (arrows) show the concentration (% mass) of the fatty acid at which the split was made. Bracketed numbers at the terminal nodes indicate the numbers of samples from each time interval (0, 12, 24, 48, 72 hrs).

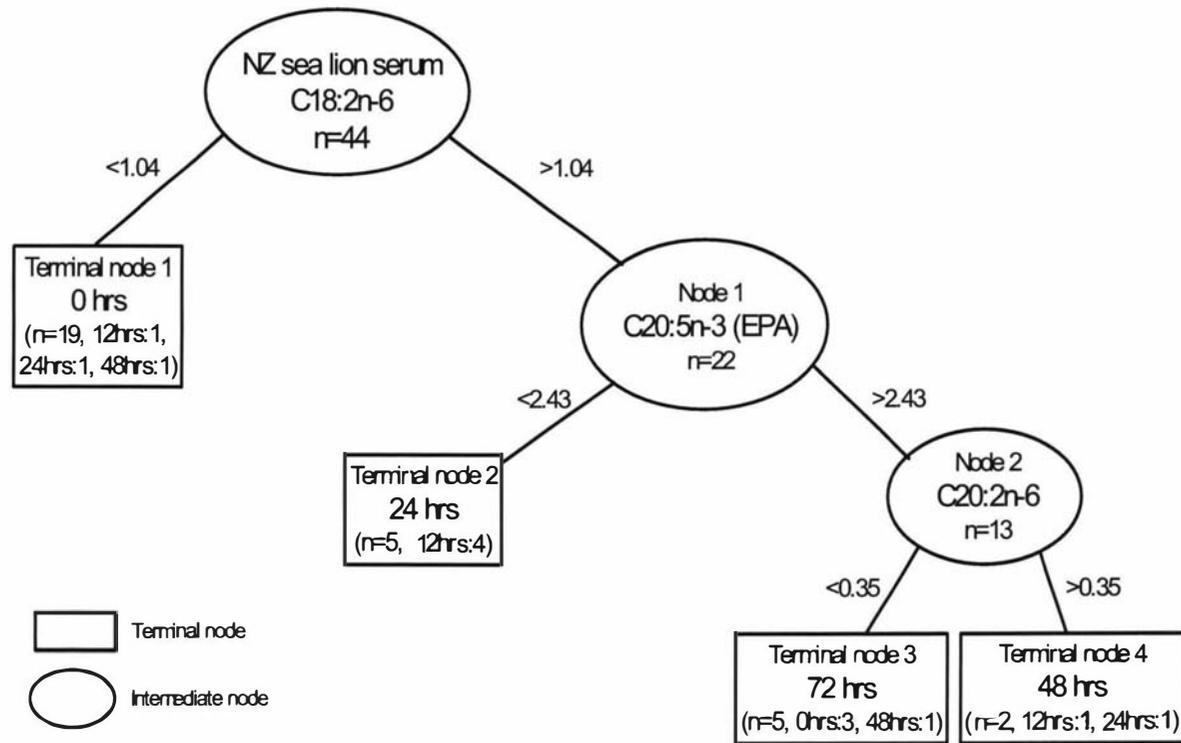


Figure 25. Classification tree, determined by CART ($R^2=0.48$), of serum samples classified by the pooled time interval (0, 12, 24, 48, 72 hrs) that they were collected after a cocktail of natural vegetable oil (CoNVO) was administered to lactating New Zealand sea lions, *Phocarctos hookeri*, at the Auckland Islands. Each node is labelled with the fatty acid used by CART algorithm to create the split. Values on the branches (arrows) show the concentration (% mass) of the fatty acid at which the split was made. Bracketed numbers at the terminal nodes indicate the numbers of samples from each time interval (0, 12, 24, 48 and 72 hrs).

DISCUSSION

The primary objective of the present study was to test the hypothesis that FAs in the diet of NZSL are absorbed from the intestine and incorporated into the milk fat unchanged. This was tested by administering the CoNVO containing a FA profile dissimilar to that found in the lipids of NZSL or their diet. In particular the concentrations of C12:0 and C18:2n-6 were present in high concentration in the CoNVO, 19% and 33.7% respectively, but less than 0.2% and 2% respectively in lipids in NZSL milk, serum or blubber (Table 20).

Temporal changes in lipid composition

Serum

Absorption of FA from the gut may first be detected by measuring their appearance in the blood. As long chain FAs, including C18:2n-6, are absorbed they are incorporated into TAGs (triacylglycerol) in the intestinal epithelial cells. The TAGs are released from the epithelial cells packaged into chylomicrons, which eventually enter the systemic circulation via the thoracic lymphatic duct. Thus the FAs in the TAG of the chylomicrons are ideal for inferring the lipid composition of the diet (Cooper *et al.*, 2005). The concentration of C18:2n-6 in the serum lipids peaked at 12hrs then decreased at 24hrs but remained elevated for up to 72hrs (Figure 17b). These data indicate that there was a rapid absorption and incorporation of C18:2n-6 into serum lipid. A peak in the rate of absorption within 12hrs of ingestion of the oil is consistent with the observation that the amount of chylomicrons in blood of seal, rats and humans tends to peak between 3hrs and 6hrs post-feeding and does not remain elevated (Harris *et al.*, 1988; Gibney and Daly, 1994; Sakr *et al.*, 1997; Summers *et al.*, 2000; Cooper *et al.*, 2005).

In addition to the significant increase in proportion of C18:2n-6 in the blood lipids there was also a peak of C12:0 at 12hrs and a much later but significant increase in C14:0 at 72hrs (Figure 17b). It is generally considered that short- and medium chain FAs (carbon chain lengths ≤ 14) are not

incorporated into chylomicrons but are instead absorbed as non esterified fatty acids (NEFA) and transported via the portal vein to the liver where they are oxidised (Nelson, 1992). The findings of the present study; however, suggest that the pattern of C12:0 absorption parallels that of C18:2n-6 since 12hrs after the ingestion of CoNVO its concentration was significantly increased in the serum (Figure 17b). This may suggest that this FA was not completely oxidized by the liver after absorption and that it is released into the circulation as NEFA or that it is also esterified in the intestinal epithelial cells and enters the blood as TAG. Thus C12:0 on the 2 position of the triacylglyceride in the oil may be absorbed as the 2-monoacylglyceride, which is then re-esterified and incorporated into the chylomicrons.

The significant increase in the proportion of C14:0 in the plasma lipids at 72hrs is not readily explained. It could originate from the diet (Iverson, 1993) or it and C12:0 may arise as products of peroxisomal β -oxidation of long chain FA (Wakil *et al.*, 1983). However, under the conditions of the experiment, the CoNVO would seem the most likely origin. The proportions of C12:0, C14:0 and C18:2n-6 were much higher in the CoNVO than in the serum lipid while those of C16:0, C18:0, C18:1n-9, C18:3n-3, were similar in serum lipids at baseline as in the CoNVO (Table 20). Consequently it was not possible to determine if the latter FAs in the CoNVO were incorporated into the serum lipids.

Based on the results of the present study it can be assumed that the mammary glands of the NZSL treated with CoNVO would be perfused with blood containing significant concentrations of C12:0 and C18:2n-6 within 12/24 hrs of ingestion of the CoNVO.

Milk

The most prominent changes in the composition of the milk fat were the increases in concentration of C12:0, C14:0 and C18:2n-6. However; the pattern of incorporation differed for each of the FAs. There was a rapid increase in the concentration of C12:0 at 12 hrs ($P < 0.05$), a further rise to a peak at 24hrs followed by a gradual fall but remaining elevated for up to

72hrs. While C14:0 peaked at 48hrs (Figure 16a). The concentration of C18:2n-6 peaked at 24hrs and was still elevated significantly at 48hrs ($P < 0.05$) (Figure 17a).

Thus there are differences in the times of both absorption and incorporation of C12:0, C14:0 and C18:2n-6 into milk lipids. The difference in the timing probably reflects the way that these FAs are absorbed from the gut, taken up by the mammary gland and incorporated into the milk fat. However, there are alternative explanations for the increases in the concentrations of C12:0 and C14:0 in the milk fat.

Saturated FAs such as C12:0 can be synthesised *de novo* in the mammary gland by chain elongation from acetyl CoA or by peroxisomal β -oxidation (Wakil *et al.*, 1983; Nelson, 1992). Thus the concentrations of C12:0 and C14:0 in human milk were highly correlated with their immediate precursor/s, caprylic acid C8:0, capric acid C10:0 for C12:0, and C12:0 as a precursor for C14:0 (Finley *et al.*, 1985). Thus the significant increase in the concentrations of C12:0 in the milk fat could be associated with the increase in their precursor/s due to the ingestion of the CoNVO (Figure 16a). C8:0 and C10:0 were found in higher concentrations in the CoNVO than in the milk fat (Table 20). Alternatively the ingestion of CoNVO may have inhibited the mechanism for elongating the FA during biosynthesis (Volpe and Vagelos, 1973; Wakil *et al.*, 1983).

Because mammals cannot synthesise C18:2n-6 (Iverson, 1993) the significant increase in this component in the milk of NZSLs must have resulted from its ingestion in the CoNVO (Figure 17a). Increases in the concentrations of C18:2n-6 in breast-milk have been associated with the consumption of this component (Potter and Nestel, 1976; Vuori *et al.*, 1982; Finley *et al.*, 1985). Further, when C18:2n-6 concentration increased in breast-milk, C14:0 and C18:0 among other FAs decreased (Finley *et al.*, 1985). This may explain the delay in the increase in C14:0 concentration until 48hrs which coincides with the decrease in C18:2n-6 following its peak at 24hrs (Figures 16a and 17a).

Throughout the experiment there was a gradual decrease in the concentration of C18:1n-7 which reached statistical significance ($P < 0.05$) at 48hrs and 72hrs. This could be due to an inhibition of its uptake from the serum, or inhibition of its incorporation into milk fat or inhibition of its synthesis in the mammary gland caused by some unidentified factor associated with the ingestion of CoNVO. Whatever the explanation, it indicates that changes in diet may lead to decreases as well as increases in the concentrations of individual FAs in the milk fat.

The results from the present experiment indicate that the time course for digestion of dietary fat and incorporation into milk commences within 12 - 24hrs after ingestion and continues for up to 72hrs. This is in agreement with other studies that have demonstrated that the ingestion of dietary FAs was rapidly reflected in the FA composition of the milk of lactating rats, humans and sows (Insull *et al.*, 1959; Finley *et al.*, 1985; Hachey *et al.*, 1987; Fritsche *et al.*, 1993; Jensen *et al.*, 1996; Francois *et al.*, 1998).

The results from this Chapter, show that milk FA signatures are responsive to short term changes in diet, which challenges the suggestion that milk FA profiles reflect dietary intake over at least 19 days and that FA profiles are therefore not particularly responsive to short term changes in prey consumption (Lea *et al.*, 2002). This may be explained by the rapid transfer of dietary FAs from blood to milk which is facilitated during lactation by the increased activity of the lipoprotein lipase in the mammary tissue, which releases the FAs from the TAG of the chylomicrons and very low density lipoproteins (Hamosh *et al.*, 1970).

The present investigation indicated that a change in the composition of the diet is rapidly reflected in a change in the composition of the milk fat and this was consistent with the results of Staniland and Pond (2004). They conducted an experiment with lactating Antarctic fur seals fed an artificial diet. They were able to discriminate between experimentally fed fur seals and naturally fed fur seals based on their milk FA signatures. The findings of the present study are

in agreement with the conclusion of Staniland and Pond (2004) that milk FAs reflect the diet consumed in the preceding foraging trip.

There are a number of factors that may account for the variability of the results in the present study. Significant individual variability in the rate of passage and recovery rates of hard parts of the digesta has been shown in a feeding trial with Antarctic fur seals (Staniland, 2002). Consequently different individuals may digest and process food at different rates and this may be reflected in the milk FA signatures in the short term. Thus Staniland and Pond (2004) found great individual variability in FA signatures of Antarctic fur seal fed the same diet. They attributed the observed individual variability to the complexity of metabolic pathway of lipids from diet to the milk since milk FAs are derived from a combination of somatic reserves, synthesis, prior feeding and recent feeding. They could control for the latter factors since the fur seal were fed with an experimental diet, but in this study neither of these factors could be controlled.

Individual variation in the rate of digestion of CoNVO may be due to whether the mothers went on a foraging trip after their first capture and had thus had a recent meal just prior to second capture or whether they stayed on land and were fasting for a considerable period of time (2-3 days) prior to the second capture. The first capture was 3 hrs after their return from the foraging trip (see MATERIALS AND METHODS section, Chapter 4) so the oil was administered onto a full stomach. For those females that returned to the sea after the first capture the CoNVO may have been mixing in the alimentary tract in association with other food components. Whereas, the stomach of the animals that remained on land would have been gradually emptying over the period between captures. It is possible that these variations in alimentation may have influenced the rate of passage, digestion and absorption of the CoNVO. Notwithstanding, the rate of digesta in pinnipeds have been shown to be rapid compared to other carnivores or omnivores (Helm, 1984; Krockenberger and Bryden, 1994).

Blubber

This study failed to find significant changes in the blubber FAs that could be associated with the ingestion of CoNVO. It is possible that the time between ingestion of the CoNVO and time of collection of the last sample was too short to allow deposition of absorbed FAs in the blubber. However, this seems unlikely in that there were significant increases in concentration of C12:0 and C18:2n-6 within 12 hrs of administering the oil. During lactation, the activity of lipoprotein lipase is increased in mammary tissue, whilst decreased in adipose tissue, apparently to channel lipid from the adipose tissue to the mammary gland for milk secretion (Hamosh *et al.*, 1970). This may be true for pinnipeds as well. Therefore it has been suggested that there is little deposition of lipid in the blubber of otariids during lactation (Iverson, 1993). Alternatively, the lipid pool in the blubber of lactating NZSL is so very large relative to the amount of CoNVO administered that any lipid from the CoNVO incorporated into the blubber was diluted to below levels of detection. In addition, little is known about the pattern of lipid deposition and mobilisation in the blubber of otariids. In phocids the mobilisation of blubber lipids appears to occur uniformly around the body (Nordy and Blix, 1985; Slip *et al.*, 1992; Beck and Smith, 1995; Cooper, 2004). Recent work has suggested that in otariids, contrary to phocids, the deposition and mobilisation of blubber lipids occurs heterogeneously around the body (Arnould *et al.*, 2005). Data on the regional pattern of blubber lipid are needed to adequately sample and infer diet from blubber lipids.

Pre and Post-treatment sea lions

Milk

It was possible to discriminate between post-treatment sea lions (sea lions administered with CoNVO) from pre-treatment sea lion group based on their milk FA signatures but not based on their serum and blubber FA signatures. The important discriminatory FAs, C12:0 and C14:0, that were higher in the CoNVO were higher in the post-treatment sea lions, but were lower in the pre-treatment sea lions. CART was able to separate milk FAs signatures of post-treatment sea lions from pre-treatment sea lions (Figure 23). These findings

agree with those of Staniland and Pond (2004) and highlight the potential of milk FASA to distinguish between seal lions that are fed different diets even though there are other sources of lipids that are incorporated into the milk.

Conclusion

The main findings of this study were that a) C12:0 and C18:2n-6 from the CoNVO were absorbed from the gut and secreted unchanged in the milk (Figure 16a and 17a) and b) that the concentration of these two FAs peaked in the serum 12 hr and in the milk 24 hr after administering of the CoNVO (Figure 16b and 17b). From these result I concluded, that milk and serum FA signatures are sensitive to small and acute changes in diet, and that the present study was able to determine the period of feeding (ingestion to incorporation) that specific CoNVO FAs (biomarkers) in milk represent. Significant temporal changes were observed in particular FAs in milk and serum in response to the ingestion of CoNVO. The results of this study demonstrated that it was possible to use milk FAs to distinguish between sea lions fed a CoNVO and those that were not. Furthermore, the present study have shown that factors such as *de novo* synthesis, deposition and mobilization of lipids are complex and little is known about these in otariids. The findings of this research indicated that milk FA signatures can be used to infer diet.

Acknowledgments

The research undertaken in this chapter was supervised by Pádraig J. Duignan (IVABS, MASSEY UNIVERSITY), Nicolas Lopez-Villalobos (IVABS, MASSEY UNIVERSITY), Louise B. Chilvers (Marine Conservation Unit, Department of Conservation), Duncan D. S. Mackenzie (IFNHH, MASSEY UNIVERSITY) and Alastair MacGibbon (Fonterra Research Centre). I would like to thank A. Castinel and all DoC staff who assisted in the field. I am very grateful to M. Reynolds, R. Richardson, and Y. van der Does (Fonterra

Research Centre) and M. Zou (IFNHH) for their invaluable help and their technical support with laboratory analysis. I am thankful to M. Reynolds for his comments and corrections on an earlier draft of this chapter and his invaluable assistance during the fatty acids analysis.

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CHAPTER 6

TEMPORAL AND SPATIAL MILK FATTY ACID SIGNATURE ANALYSIS OF LACTATING NEW ZEALAND SEA LIONS

ABSTRACT

Fatty acids signature analysis (FASA), through the use of specific fatty acids and/or entire profile of fatty acids, has largely been used to study the foraging behaviour, trophic relationships and the diet composition in marine mammals. The main principle of FASA is that a unique array of fatty acids are transferred from prey to predator with little or no modification. Thus the fatty acids profiles of prey and predator (blubber, blood, milk) can be compared and matched. The present study examined the milk fatty acid composition of New Zealand sea lions (NZSLs), *Phocarctos hookeri*, to distinguish if differences occur between years and breeding colonies and enable comparison with fatty acids profile of prey. Milk samples were collected from NZSLs in 1997 and in three consecutive years, from 2003 to 2005 (n=52) and from two different breeding colonies of the Auckland Islands. Milk samples from 2005 were collected from two breeding sites: Dundas Island and Sandy Bay, Enderby Island. Lipids were extracted and fatty acids signatures determined by temperature-programmed capillary gas liquid chromatography. Fatty acids signatures of five potential prey species collected near the Auckland Islands were included in this study. Discriminant function analysis (DFA) indicated that milk fatty acids signatures were significantly different between years. DFA as well as classification and regression trees (CART) indicated that the fatty acid signatures in the year 1997 were significantly different from those in the following years. DFA and CART failed to distinguish milk fatty acids signatures between years 2003, 2004 and 2005. Fatty acid signatures at the two breeding sites could not be clearly differentiated, thus on the basis of milk fatty acid composition they may not be regarded as two distinct foraging groups. This study demonstrated a predominance of teleost fish in the diet of lactating NZSLs and this was consistent with the previous diet studies based on traditional methods.

INTRODUCTION

Trophic interactions between top predators (e.g. pinnipeds: seal, fur seal and sea lions) and their prey are integral objectives of marine ecosystem research (Bowen, 1997). Trophic interactions between fisheries and marine mammals are also important components for marine ecosystem management as in most cases these interactions are detrimental for marine mammals (Gales *et al.*, 2003). To understand these trophic interactions details of the diet of the mammals are needed. In the present study differences in milk fatty acid composition of the NZSL between years were measured and the relationship between these differences and changes in diet were investigated.

Most investigations of pinniped diet have used traditional methods based on the recovery of the hard parts of the prey either from scats, regurgitates or stomach contents, but it has been shown that these methods have a number of important biases (Jobling and Breiby, 1986; Jobling, 1987; Dellinger and Trillmich, 1988; Cottrell, 1996; Orr and Harvey, 2001; Trites and Joy, 2005). An alternative method that has found favour more recently is to measure the fatty acid composition of the tissue of the predators to identify the prey eaten. Marine prey species have a unique array or signature of fatty acids and it is assumed that fatty acids in the prey are digested and incorporated into the predator tissue with little modification. Hence, by comparing the fatty acid signature of the predator tissues that are rich in lipids i.e. blubber, to the fatty acid signature of the prey the diet can be deduced (Iverson, 1993). Similarly, in lactating pinnipeds the source of milk lipid comes primarily from the diet and adipose tissue, therefore milk fatty acids should reflect a combination of both the current diet and prior dietary history (Green *et al.*, 1993; Iverson, 1993; Iverson *et al.*, 1995; Iverson *et al.*, 1997a; Staniland and Pond, 2004; Staniland and Pond, 2005). The relative contributions of diet and the adipose tissue will depend in part on the foraging and nursing strategies of the lactating female. Thus during lactation, female otariids alternate between suckling bouts and foraging trips, while the phocids fast during lactation. Consequently in lactating otariids, fatty acids incorporated into the milk following foraging trips should reflect the recent diet

since the dietary FAs absorbed by the intestine and released into the circulation as triacylglycerols will be directed to the mammary gland by lipoprotein lipase (Iverson, 1993, 1995; Staniland and Pond, 2004). During lactation the activity of the lipoprotein lipase is greatly increased in the mammary gland, which consequently competes effectively for circulating triacylglycerols and hence the dietary FAs are rapidly transferred from the blood to milk (Hamosh *et al.*, 1970).

The potential of fatty acid signature analysis (FASA) has been demonstrated in several studies (Budge *et al.*, 2006). However, the influence of various factors such as the turnover rates, *de novo* biosynthesis and metabolism of fatty acids, still need to be addressed to improve the reliability of the technique (See Chapter 6, Staniland and Pond, 2004). FASA of milk has been shown to detect significant inter-seasonal (Iverson *et al.*, 1997a; Lea *et al.*, 2002) and within intra- seasonal (Staniland and Pond, 2005) shifts in the diet of the Antarctic fur seal, *Arctocephalus gazella*. Milk FASA revealed interspecies differences in the diets of an otariid and a phocid (Brown *et al.*, 1999), and inter-site dietary differences in the gray seal, *Halichoerus grypus* (Walton *et al.*, 2000). Furthermore, changes in composition of Antarctic fur seal milk and particularly the proportion of different fatty acids in the milk were explained by differences in diet between and within years (Iverson *et al.*, 1997a; Lea *et al.*, 2002).

Nevertheless, there is a need to obtain baseline data on the variation in the fatty acid composition of the milk of the NZSL and whether it is related to the available food resources. The importance of squid in the diet of lactating NZSL is still not quantified and therefore it is unclear if changes in squid availability due to fisheries or seasonal conditions would affect the reproductive success of NZSL females. Implications for the management of the squid fisheries may arise if a correlation existed between the annual catch of squid, variability of food source, and the population viability of NZSL in the Auckland Islands.

In this study, the FA signature of milk samples was measured as a means of assessing changes in diet of lactating NZSL in early lactation at the Auckland Islands.

The objectives of this study were:

- a) to determine if there were;
 - i) temporal (inter-annual) and
 - ii) spatial (inter-breeding site) differences in milk FA;
- b) to attempt to explain any differences by comparison with available fatty acid profiles of prey.

MATERIALS AND METHODS

Study site and Animals

Adult female NZSL known to have pupped were selected for collection of milk samples from a pool of branded/tagged individuals of known age. All females were sampled for milk and the samples for the analyses described in this study were collected in the 1996/7, 2002/3, 2003/4 and 2004/5 austral summer seasons (hereafter referred to as 1997, 2003, 2004 and 2005, respectively). The milk samples collected in 1997 (n=18), 2003 (n=16), 2004 (n=9) were from Sandy Bay females, whereas in 2005 milk samples were collected in Sandy Bay (n=7) (50° 30' S, 166° 17') and Dundas Island (n=10) (located 8 km south of Enderby Island) (Table 21, see Figure 5 in Chapter 1, GENERAL INTRODUCTION section).

Capture of sea lions and sampling, handling and storage of milk samples were conducted as *per* MATERIALS AND METHODS section in Chapter 3.

Laboratory analysis

Lipid extraction, FAME preparation, Gas-liquid chromatography: equipment and chromatographic conditions; identification and quantification of fatty acids;

and quality control were carried out *as per* MATERIALS AND METHODS section in Chapter 5.

Data analysis

Only fatty acids common to all the samples were used in the data analysis (Table 21). The raw data, presented as weight percent composition, were arcsine transformed prior to analyses

Inter-breeding sites differences in milk fatty acids

To test whether there were significant differences in the FA signatures between milk samples collected in Sandy Bay and Dundas Islands breeding sites in 2005, samples were grouped according to the site of their collection.

Discriminant analysis

The STEPDISC (Stepwise Discriminant Analysis, SDA) procedure of SAS (Statistical Analysis System, version 9.1; SAS Institute Inc., Cary, NC, USA); was used to identify the milk FAs that would discriminate between milk samples collected from Sandy Bay and Dundas Island breeding sites. The procedure performs a SDA to select a subset of the numeric variable (fatty acids) for use in discriminating among the classes. FAs were chosen to enter or leave the model based on their contribution to the overall discriminatory power as measured by Wilks' λ test. A moderate significance level of 0.15 was chosen for selection (Costanza and Afifi, 1979).

The CANDISC (Canonical Discriminant Analysis, CDA) procedure of SAS (Statistical Analysis System, version 9.1; SAS Institute Inc., Cary, NC, USA) was used to analyse differences in FA signature between the breeding sites. The CDA uses linear combinations of the variables (FAs) and these canonical scores account for the group structure (sampling year or sampling breeding site) and then the mean values of the linear combinations are compared using an approximation of the F statistic. For each canonical score, eigenvalues, which represent the ratio of the between-group variation to the pooled within-

group variation, were calculated. To test for significant differences between breeding sampling sites and years of sampling, Pillai's trace approximation of the F statistic was used. In order for the CDA to be significantly reliable requires that the total number of observations (milk samples) be greater than the number of variables (fatty acids). Otherwise the test of significance may lose reliability. For this reason the number of variables was reduced by a *Stepwise variable selection* method in the discriminant analysis procedure of JMP software (SAS Institute Inc., Cary, NC, USA). The percentage and number of milk samples that were misclassified by the discriminant analysis was obtained using JMP software (SAS Institute Inc., Cary, NC, USA). The FAs with the most power, based on their large F ratios or small p-values, were identified and entered into the model. The procedure was stopped when the largest number of FAs possible was retained, and this was the number of observations (milk samples) minus 2.

Classification and Regression Tree (CART) analysis

To test whether there were differences in the milk FA signatures of lactating NZSL among breeding colonies Dundas Island and Sandy Bay, Milk FA data were grouped according to the site of collection and analysed using CART (Classification and regression trees) with the partition procedure of JMP software (SAS Institute Inc., Cary, NC, USA). CART is a non parametric multivariate technique, that has been described for FASA (1997a; Iverson *et al.*, 1997b; Smith *et al.*, 1997).

Inter-annual differences in milk fatty acids

MUFA –PUFA correlation

Estimates of correlation coefficients between MUFA and PUFA after the arcsine-transformed were obtained for each year (1997, 2003, 2004 and 2005) using the CORR procedure of SAS (Statistical Analysis System, version 9.1; SAS Institute Inc., Cary, NC, USA). To test significant differences between correlation coefficients a Fisher-test was conducted (Snedecor and Cochran, 1980). The correlation coefficient (r) is transformed into the Fisher statistic μ_z as:

$$\mu_z = \frac{1}{2} \ln \left[\frac{1+r}{1-r} \right]$$

with variance $= (m-3)^{-1}$, and where m is the number of pair samples of MUFA and PUFA. The confidence interval for μ_z is given by

$$L = \mu_z - Z_{\alpha/2} \cdot \frac{1}{\sqrt{m-3}} \quad \text{and}$$

$$U = \mu_z + Z_{\alpha/2} \cdot \frac{1}{\sqrt{m-3}}$$

The value $Z_{\alpha/2}$ was obtained from the standard normal table for a 95% confidence interval.

Discriminant analysis

SDA was used to determine if the milk FAs could discriminate between samples collected in different years. CDA used to analyse differences among years.

Classification and Regression Tree (CART) analysis

To test whether there were differences in the milk FA signatures of lactating NZSL between years, milk FA data were grouped according to the year of collection and analysed using CART (Classification and regression trees) with the Partition procedure of JMP software (SAS Institute Inc., Cary, NC, USA).

Milk and prey species fatty acid data

FA profiles of milk samples were compared with FA profiles from five prey species (Meynier unpublished data). These prey species are known to occur in the diet of NZSL (Childerhouse *et al.*, 2001; Bando *et al.*, 2005; Meynier *et al.*, 2006a; Meynier *et al.*, 2006b).

Discriminant analysis

SDA was used to identify the FAs used to discriminate between milk FA signature and prey FA signature. The differences among milk samples by year and prey were analysed with CDA. The percentage and number of milk samples that were misclassified by the discriminant analysis was obtained from JMP software (SAS Institute Inc., Cary, NC, USA).

Classification and Regression Tree (CART) analysis

To be able to assess whether there was a shift in the diet of lactating NZSL among years, the data on prey species FA signatures were incorporated into the original CART analysis. Only the means of the prey FAs were available for analysis therefore trees were not constructed with the prey data as CART analysis could not use data from a single observation. Therefore, to include the data of FAs signatures from the prey species into the tree constructed from NZSL milk data, the prey data were dropped through trees at the root node to determine in which part of the tree they terminated as done in Brown *et al.* (1999). Prey species were dropped through trees manually by using the weight % of the FAs designated in the tree's root nodes. Thus whether the prey passed to the right or left of the tree was determined by whether the weight % of that particular FA in the prey was greater (<) or less (>) than the cut off weight % for the node. This was done until the prey species were all allocated to a terminal node.

RESULTS

New Zealand sea lion milk fatty acids

Forty-three different fatty acids were identified and quantified in the milk lipid of NZSL (Table 21). In all of the 1997, 2003, 2004 and 2005 samples, 41 of the 43 fatty acids accounted for more than 0.05% by mass of the total fatty acids (Table 21). The dominant fatty acids (>2%) were C14:0, C16:0, C18:0, C16:1n-7, C18:1n-9, C18:1n-7, C20:1n-9, C20:5n-3, C22:5n-3 and C22:6n-3. These 10 fatty acids accounted for 84.7% (1997), 87.1% (2003), 86.5% (2004) and 84.7% (2005) of the total fatty acids.

Saturated fatty acids (SAFA) accounted for 26.98 \pm 1.60% (1997), 26.94 \pm 1.53% (2003), 25.01 \pm 1.75% (2004), 28.64 \pm 1.04% (2005), whereas monounsaturated fatty acids (MUFA) accounted for 39.73 \pm 2.02% (1997), 43.03 \pm 1.94% (2003), 42.30 \pm 2.21% (2004) and 40.95 \pm 1.31% (2005), and polyunsaturated fatty acids (PUFA) accounted for 30.37 \pm 2.34% (1997), 27.43 \pm 2.24% (2003), 30.22 \pm 2.56% (2004) and 26.63 \pm 1.52% (2005) of the total fatty acids (Table 1 and Figure 25).

Prey species fatty acids

Data on the FA profile of prey species were obtained by Meynier *et al.* (unpublished data) where twenty-nine FAs were identified in five potential prey species. These included red cod (*Pseudophycis bachus*), javelin (*Lepidorhynchus denticulatus*), opalfish (*Hemerocoetes* spp.), arrow squid (*Nototodarus sloanii*) and hoki (*Macruronis novaezelandiae*) (Table 21, Figures 26 and 27). The mean values and standard deviations for the FA in each prey species provided a broad indication of the potential dietary intake of FA. Differences in FA compositions between prey species in many components were apparent (Table 21, Figures 26 and 27). Complete FA data of prey species was not available for the present study so it was not possible to test for significant differences between FAs. Notwithstanding, some FAs have the potential to be significantly different between prey species. These FAs were C14:0, C16:1n-7, C18:1n-9, C20:1n-9, C18:4n-3, C20:5n-3, C20:1n-13/11 and C22:6n-3 (DHA) (Table 21, Figures 26 and 27) with some difference between fishes and arrow squid.

Table 21. Fatty acid (FA) composition (% contribution by weight of total FA) of New Zealand sea lion milk in early lactation for 1997, 2003, 2004 and 2005 and of major prey species collected at Campbell plateau summer and autumn 2005/2006. Saturated FA (SAFA), monounsaturated FA (MUFA), polyunsaturated FA (PUFA). Values are means \pm standard errors.

| Fatty acids | n | Milk FA profile | | | | Prey species FA profile ⁺ | | | | |
|---------------------------|-------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|--------------------------------------|----------------------------|----------------------------|-----------------------------|-------------------------|
| | | 1997 12 | 2003 14 | 2004 9 | 2005 17 | Red cod ¹ 10 | Javelin ² 10 | Opalfish ³ 3 | A. squid ⁴ 10 | Hoki ^b 11 |
| C10:0 | - | | 0.05 \pm 0.03 | 0.14 \pm 0.04 ^a | 0.04 \pm 0.02 ^a | - | - | - | - | - |
| C12:0 | 0.18 \pm 0.04 | 0.16 \pm 0.03 | 0.14 \pm 0.03 | 0.13 \pm 0.02 | - | - | - | - | - | |
| C14:0 | 4.26 \pm 0.51 | 4.24 \pm 0.49 | 3.47 \pm 0.52 | 5.35 \pm 0.33 | 5.4 \pm 0.42 | 4.4 \pm 0.32 | 6.0 \pm 0.29 | 8.00 \pm 0.46 | 5.4 \pm 0.30 | |
| C15:0 iso Br | 0.17 \pm 0.02 | 0.18 \pm 0.02 | 0.15 \pm 0.02 | 0.16 \pm 0.01 | - | - | - | - | - | |
| C15:0 ante-iso Br | 0.05 \pm 0.02 | 0.04 \pm 0.01 | 0.05 \pm 0.02 | 0.05 \pm 0.01 | - | - | - | - | - | |
| C15:0 | 0.48 \pm 0.03 | 0.53 \pm 0.03 | 0.47 \pm 0.03 | 0.42 \pm 0.02 | 0.5 \pm 0.04 | 0.6 \pm 0.03 | 0.5 \pm 0.06 | 0.70 \pm 0.04 | 0.60 \pm 0.03 | |
| C16:0 Br | 0.08 \pm 0.01 | 0.09 \pm 0.01 | 0.07 \pm 0.01 | 0.07 \pm 0.01 | - | - | - | - | - | |
| C16:0 | 18.12 \pm 1.11 | 18.20 \pm 1.06 | 17.19 \pm 1.21 | 19.02 \pm 0.72 | 21.9 \pm 1.61 | 22.6 \pm 0.69 | 22.6 \pm 1.58 | 24.9 \pm 0.91 | 23.3 \pm 0.66 | |
| C17:0 | 0.48 \pm 0.05 | 0.51 \pm 0.04 | 0.39 \pm 0.03 | 0.43 \pm 0.03 | 0.7 \pm 0.08 | 0.6 \pm 0.04 | 0.9 \pm 0.20 | 1.70 \pm 0.10 | 0.9 \pm 0.05 | |
| C17:0 iso Br | 0.25 \pm 0.03 | 0.28 \pm 0.03 | 0.23 \pm 0.03 | 0.24 \pm 0.02 | - | - | - | - | - | |
| C17:0 ante-iso Br | 0.19 \pm 0.02 | 0.12 \pm 0.02 | 0.13 \pm 0.02 | 0.14 \pm 0.01 | - | - | - | - | - | |
| C18:0 | 2.77 \pm 0.19 | 2.66 \pm 0.18 | 2.53 \pm 0.21 | 2.72 \pm 0.12 | 4.1 \pm 0.29 | 2.4 \pm 0.12 | 4.7 \pm 0.10 | 3.70 \pm 0.12 | 3.2 \pm 0.14 | |
| C20:0 | 0.09 \pm 0.01 | 0.09 \pm 0.01 | 0.07 \pm 0.01 | 0.06 \pm 0.01 | - | - | - | - | - | |
| SAFA total | 26.98 \pm 1.60 | 26.94 \pm 1.53 | 25.01 \pm 1.75 | 28.64 \pm 1.04 | 32.6 | 30.6 | 34.7 | 26.3 | 33.4 | |
| C14:1 | 0.19 \pm 0.03 | 0.18 \pm 0.03 | 0.14 \pm 0.02 | 0.18 \pm 0.02 | 0.2 \pm 0.02 | 0.1 \pm 0.01 | 0.2 \pm 0.03 | 0.20 \pm 0.04 | 0.1 \pm 0.02 | |
| C15:1 | - | - | - | - | 0.1 \pm 0.02 | 0.2 \pm 0.02 | 0.1 \pm 0.04 | 0.30 \pm 0.05 | 0.1 \pm 0.02 | |
| C16:1n-7 | 6.24 \pm 0.43 ^{ab} | 5.34 \pm 0.41 ^a | 4.90 \pm 0.25 ^b | 6.19 \pm 0.25 | 5.4 \pm 0.19 | 6.8 \pm 0.27 | 7.0 \pm 0.20 | 2.80 \pm 0.12 | 5.6 \pm 0.20 | |
| C18:1n-9 | 22.88 \pm 1.92 | 25.45 \pm 1.84 | 23.80 \pm 1.24 | 22.38 \pm 1.24 | 14.4 \pm 0.43 | 20.8 \pm 1.39 | 16.2 \pm 0.11 | 6.90 \pm 0.25 | 17.9 \pm 0.34 | |
| C18:1n-7 | 4.07 \pm 0.24 | 3.94 \pm 0.23 | 4.20 \pm 0.26 | 3.88 \pm 0.16 | 3.6 \pm 0.04 | 3.8 \pm 0.21 | 3.7 \pm 0.22 | 2.80 \pm 0.11 | 3.5 \pm 0.04 | |
| Other C18:1? | 0.48 \pm 0.03 ^a | 0.44 \pm 0.03 | 0.41 \pm 0.02 ^a | 0.47 \pm 0.02 | - | - | - | - | 0.5 | |
| C20:1n-11 | 0.45 \pm 0.07 | 0.52 \pm 0.07 | 0.40 \pm 0.05 | 0.60 \pm 0.04 | 0.3 \pm 0.03 | 1.1 \pm 0.12 | 0.3 \pm 0.06 | 0.40 \pm 0.10 | 0.4 \pm 0.02 | |
| C20:1n-9 | 4.35 \pm 0.68 ^a | 5.82 \pm 0.65 ^a | 6.52 \pm 0.75 | 6.02 \pm 0.44 | 6.2 \pm 0.66 | 9.6 \pm 0.49 | 7.1 \pm 0.85 | 4.00 \pm 0.33 | 8.9 \pm 0.31 | |
| Ratio 20:1n-9/n-11 | 0.11 \pm 0.01 ^a | 0.09 \pm 0.01 | 0.08 \pm 0.01 ^a | 0.10 \pm 0.01 | 0.048 | 0.11 | 0.04 | 0.1 | 0.04 | |
| C22:1n-13&n-11 | 1.34 \pm 0.30 | 0.28 \pm 0.38 | 1.13 \pm 0.38 | 0.49 \pm 0.19 | 1.2 \pm 0.13 | 1.8 \pm 0.22 | 1.4 \pm 0.27 | 0.60 \pm 0.13 | 2.6 \pm 0.13 | |
| C22:1n-9 | 0.50 \pm 0.19 | 0.69 \pm 0.17 | 0.56 \pm 0.10 | 0.99 \pm 0.10 | 0.6 \pm 0.06 | 0.7 \pm 0.10 | 0.5 \pm 0.10 | 0.20 \pm 0.03 | 0.8 \pm 0.06 | |
| MUFA total | 39.73 \pm 2.02 | 43.03 \pm 1.94 | 42.30 \pm 2.21 | 40.95 \pm 1.31 | 32.0 | 44.9 | 36.5 | 18.2 | 40.4 | |
| C16:2n-4? | 0.78 \pm 0.04 | 0.75 \pm 0.04 | 0.84 \pm 0.03 | 0.79 \pm 0.03 | - | - | - | - | - | |
| C16:3n-4? | 0.65 \pm 0.04 ^a | 0.63 \pm 0.03 | 0.60 \pm 0.02 | 0.51 \pm 0.02 ^a | 0.4 \pm 0.02 | 0.6 \pm 0.06 | 0.4 \pm 0.06 | 0.20 \pm 0.02 | 0.4 \pm 0.02 | |

Table 21. (Continued).

| Fatty acids | n | Milk FA profile | | | | Prey species FA profile [†] | | | | |
|-----------------------|---|--------------------------|-------------------------|-------------|--------------------------|--------------------------------------|----------------------------|----------------------------|-----------------------------|-------------------------|
| | | 1997 12 | 2003 14 | 2004 9 | 2005 17 | Red cod ¹ 10 | Javelin ² 10 | Opalfish ³ 3 | A. squid ⁴ 10 | Hoki ⁵ 11 |
| C16:4n-1? | | 0.11 ±0.06 | 0.03 ±0.07 | 0.08 ±0.09 | 0.21 ±0.04 | 0.1 ±0.02 | 0.1 ±0.01 | 0.2 ±0.03 | 0.30 ±0.03 | 0.1 ±0.02 |
| C18:4n-3 | | 0.80 ±0.17 | 0.71 ±0.16 | 0.58 ±0.19 | 0.80 ±0.11 | 1.7 ±0.28 | 0.9 ±0.10 | 1.3 ±0.07 | 3.30 ±0.25 | 0.8 ±0.06 |
| C18:3n-3 (Linolenic) | | 0.51 ±0.05 | 0.50 ±0.05 | 0.47 ±0.06 | 0.51 ±0.03 | 0.9 ±0.08 | 0.5 ±0.08 | 0.5 ±0.17 | 1.30 ±0.10 | 0.6 ±0.04 |
| C18:2n-6 (Linoleic) | | 1.31 ±0.10 | 1.34 ±0.10 | 1.31 ±0.11 | 1.19 ±0.07 | 1.2 ±0.04 | 1.1 ±0.03 | 1.0 ±0.08 | 1.30 ±0.09 | 1.2 ±0.04 |
| C20:2n-6 | | 0.32 ±0.03 | 0.26 ±0.03 | 0.29 ±0.05 | 0.32 ±0.03 | 0.3 ±0.02 | 0.2 ±0.01 | 0.3 ±0.02 | 0.20 ±0.03 | 0.2 ±0.01 |
| C20:3n-6 | | 0.12 ±0.02 | 0.11 ±0.02 | 0.10 ±0.02 | 0.12 ±0.01 | 0.1 ±0.003 | 0.1 ±0.01 | 0.1 ±0.01 | - | - |
| C20:4n-6 | | 1.36 ±0.13 | 1.14 ±0.12 | 1.23 ±0.14 | 0.93 ±0.08 | 0.90 ±0.18 | 0.5 ±0.03 | 0.8 ±0.11 | 0.90 ±0.05 | 0.5 ±0.04 |
| C20:3n-3 | | 0.21 ±0.02 | 0.18 ±0.02 | 0.22 ±0.02 | 0.19 ±0.01 | 0.2 ±0.02 | 0.2 ±0.02 | 0.2 ±0.01 | 0.20 ±0.03 | 0.1 ±0.01 |
| C20:4n-3 | | 1.19 ±0.14 | 1.28 ±0.14 | 1.36 ±0.16 | 1.28 ±0.09 | 1.6 ±0.21 | 1.1 ±0.12 | 1.3 ±0.05 | 0.70 ±0.05 | 1.5 ±0.08 |
| C20:5n-3 (EPA) | | 7.01 ±0.87 ^a | 5.20 ±0.84 ^a | 5.90 ±0.95 | 6.45 ±0.57 | 8.1 ±0.97 | 6.1 ±0.34 | 8.0 ±0.50 | 12.3 ±0.53 | 6.4 ±0.37 |
| C21:5n-3?? | | 0.33 ±0.13 | 0.43 ±0.12 | 0.55 ±0.13 | 0.75 ±0.08 | 0.3 ±0.05 | 0.2 ±0.02 | 0.2 ±0.03 | 0.30 ±0.03 | 0.2 ±0.02 |
| C22:0 | | 0.39 ±0.06 | 0.29 ±0.06 | 0.33 ±0.07 | 0.42 ±0.04 | - | - | - | - | - |
| C22:4n-6 ? | | 0.29 ±0.04 ^a | 0.21 ±0.04 ^a | 0.22 ±0.04 | 0.21 ±0.02 | - | - | - | - | - |
| C22:5n-6 ? | | 0.15 ±0.03 | 0.14 ±0.03 | 0.16 ±0.03 | 0.21 ±0.02 | 0.1 ±0.03 | 0.1 ±0.01 | - | 0.10 ±0.03 | - |
| C22:5n-3 (DPA) | | 3.01 ±0.27 | 2.67 ±0.26 | 3.12 ±0.30 | 2.78 ±0.18 | 1.7 ±0.26 | 0.9 ±0.11 | 1.1 ±0.07 | 0.60 ±0.06 | 1.4 ±0.06 |
| C22:6n-3 (DHA) | | 12.30 ±1.03 | 12.27 ±0.98 | 14.21 ±1.06 | 10.13 ±0.66 | 14.5 ±1.12 | 7.3 ±0.63 | 9.9 ±0.41 | 17.8 ±1.31 | 9.6 ±0.63 |
| PUFA total | | 30.37 ±2.34 | 27.43 ±2.24 | 30.22 ±2.56 | 26.63 ±1.52 | 32.1 | 19.9 | 25.3 | 39.5 | 23.0 |
| Total ∑ FA | | 97.12 ±0.36 ^a | 97.36 ±0.36 | 97.57 ±0.38 | 96.24 ±0.25 ^a | 97.94 | 97.06 | 97.76 | 97.3 | 96.97 |
| ∑ n-3 | | 25.30 ±2.08 | 23.16 ±1.99 | 26.03 ±2.27 | 22.92 ±1.35 | - | - | - | - | - |
| ∑ n-6 | | 3.40 ±0.27 | 3.09 ±0.26 | 3.01 ±0.29 | 2.71 ±0.18 | - | - | - | - | - |
| Ratio ω3:ω6 | | 7.41 ±0.72 | 7.11 ±0.69 | 8.56 ±0.78 | 8.89 ±0.47 | - | - | - | - | - |

[†]Source Meynier (unpublished data); ¹*Pseudophycis bachus*; ²*Lepidorhynchus denticulatus*; ³*Hemerocoetes* spp; ⁴*Nototodarus sloanii*; ⁵*Macruronus novaezelandiae*. FA in bold are significantly different (P>0.05), means with same letter (upper case) are significant different.

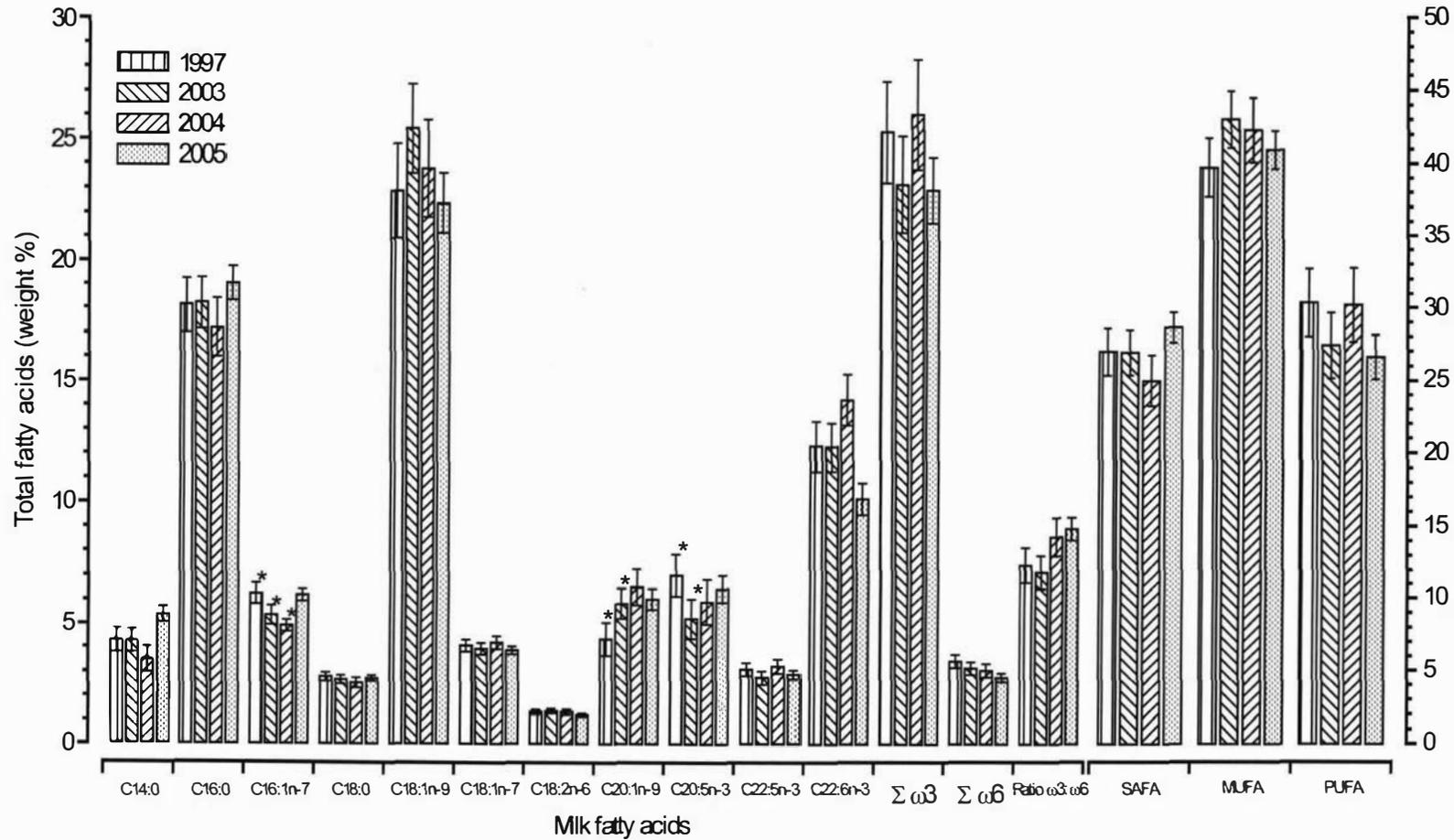


Figure 26. Fatty acid (FA) composition (weight percent of major (FA)), sum of Omega 3 fatty acids, Omega 6 fatty acids (and their ratio) and saturated fatty acids (SAFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in the milk fat of New Zealand sea lions, *Phocarcetos hookeri*, during early lactation in 1997 (n = 12), 2003 (n = 14), 2004 (n = 9) and 2005 (n = 17) summer seasons. Values are given as means \pm SEM. * $p < 0.05$.

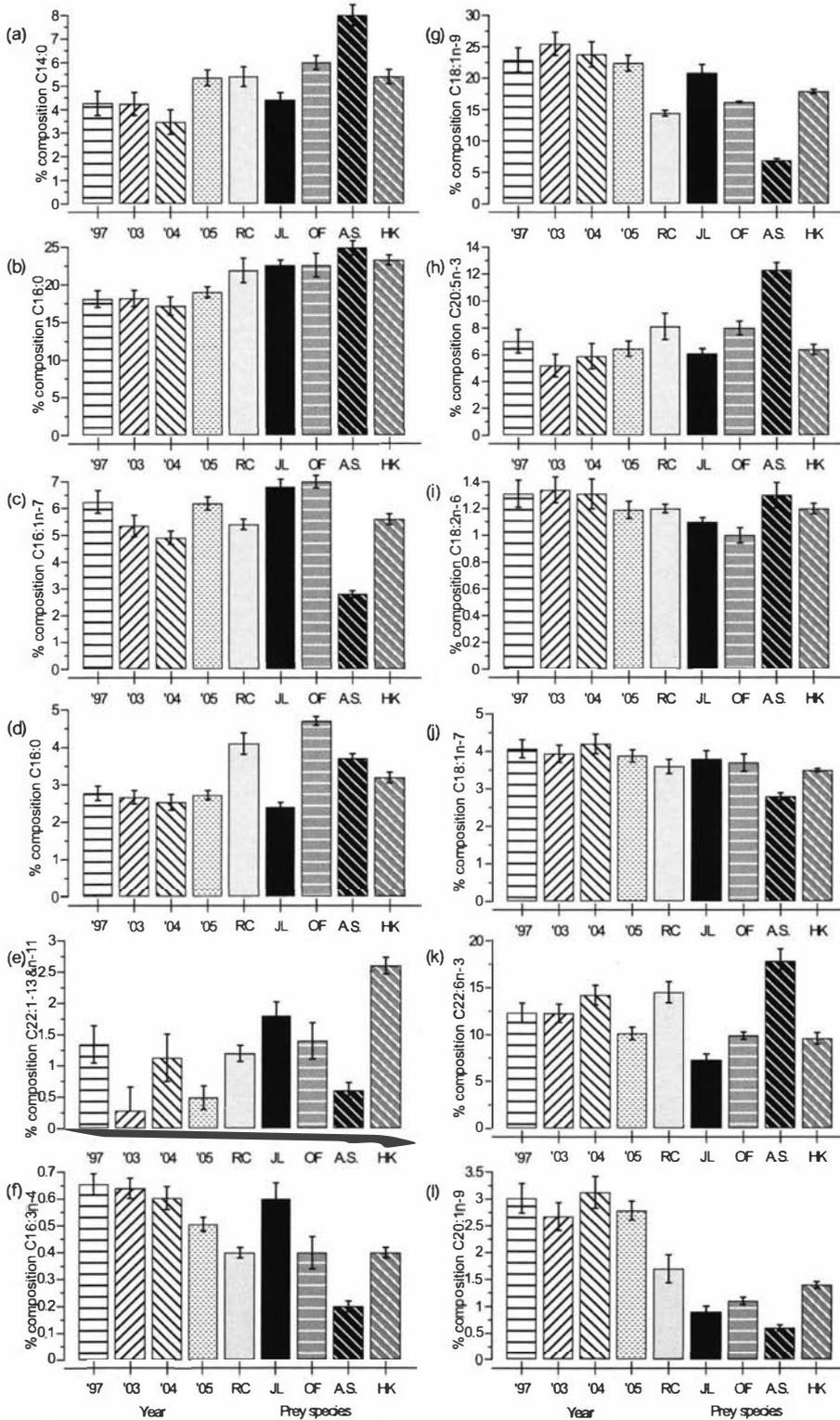


Figure 27. Means \pm SE of the weight percent of major fatty acids in milk of the New Zealand sea lion, *Phocartos hookeri*, collected in summer ('97:1997, n=12; '03:2003, n=14; '04:2004, n=9; and '05:2005, n=17) at the Auckland Islands and prey species collected at Campbell plateau in the summer and the autumn of 2005/2006 (Meynier unpublished data). Prey species: RC: Red cod (*Pseudophycis bachus*); JL: Javelin (*Lepidorhynchus denticulatus*); OF: Opalfish (*Hemerocoetes spp.*); AS: Arrow squid (*Nototodarus sloanii*); HK: Hoki (*Macruronis novaezelandiae*).

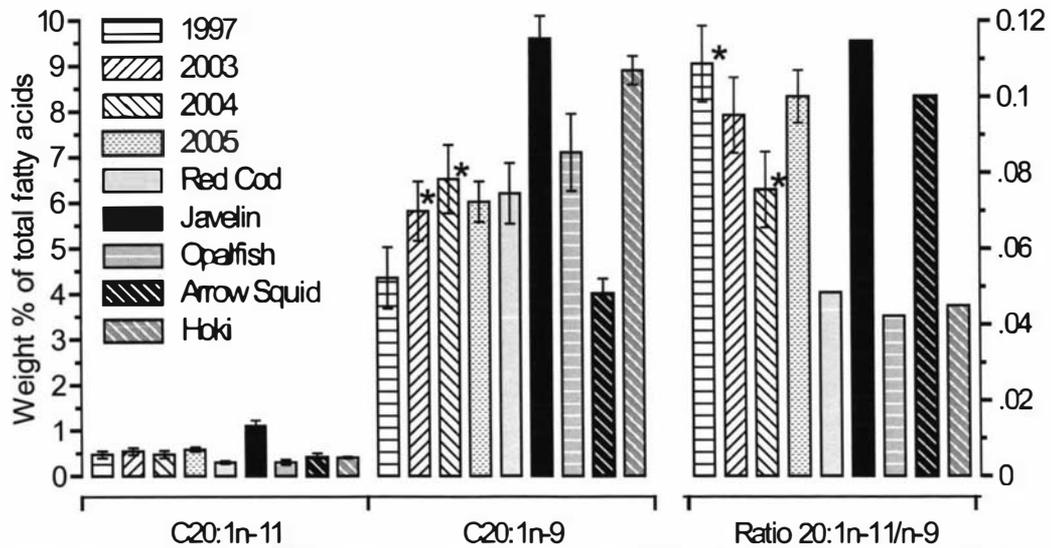


Figure 28. The weight percent of selected fatty acids and the ratio of C20:1n-11 : C20:1n-9 in milk (sampled in four summer seasons) of New Zealand sea lion, *Phocarctos hookeri*, and in prey species collected at Campbell plateau summer and autumn 2005/2006 (Meyner et al. unpublished data). Values are means \pm standard errors. See (Iverson, 1993; Iverson *et al.*, 1997b). *Significantly different $p < 0.05$.

Inter-breeding sites differences in milk FAs signatures

Discriminant analysis

a) When using the whole milk FA (21 FA) profile, SDA identified two parameters, the ratio $\omega 6 : \omega 3$ (Partial- $R^2 = 0.26$, $F = 5.37$, $p < 0.05$, Wilks' $\lambda = 0.74$) and C20:3n-3 (Partial- $R^2 = 0.17$, $F = 2.78$, $p < 0.05$, Wilks' $\lambda = 0.61$) that had the greatest power to discriminate between milk FAs from Sandy Bay and Dundas Islands breeding sites. When this model (the ratio $\omega 6 : \omega 3$ and C20:3n-3) was applied in DFA analysis in JMP the number of misclassified milk samples were 3 which represented 17.7%.

b) A SDA was conducted using the dietary indicator FAs derived from the prey namely C14:0, C16:3n-4, C18:2n-6, C18:3n-3, C18:4n-3, C20:1n-11, C20:1n-9, C20:4n-6, C20:4n-3, C22:6n-3 and the ratios (C20:1n-11: C20:1n-9 and $\omega 6 : \omega 3$). This SDA identified that the concentration of C18:2n-6 (Partial- $R^2 = 0.21$, $F = 3.99$, $p > 0.05$, Wilks' $\lambda = 0.79$) has the greatest power to discriminate between milk samples from the two breeding sites.

Classification and Regression Tree (CART) analysis

CART created a tree from two FAs giving 3 terminal nodes when classifying NZSL milk samples into breeding sites, Dundas Island or Sandy Bay (Figure 28). Fatty acid C16:0 was chosen to split the root node into the 1st terminal node (Dundas Island). This fatty acid split 4 Dundas Island samples from the remaining 6 Dundas Island samples and all 7 of those from Sandy Bay. Allocation on the basis of the concentration of C16:2n-4 discriminated between the two sites with misallocation of 2 out of 7 samples from Sandy Bay samples 1 out of 6 from Dundas Island samples (Figure 28).

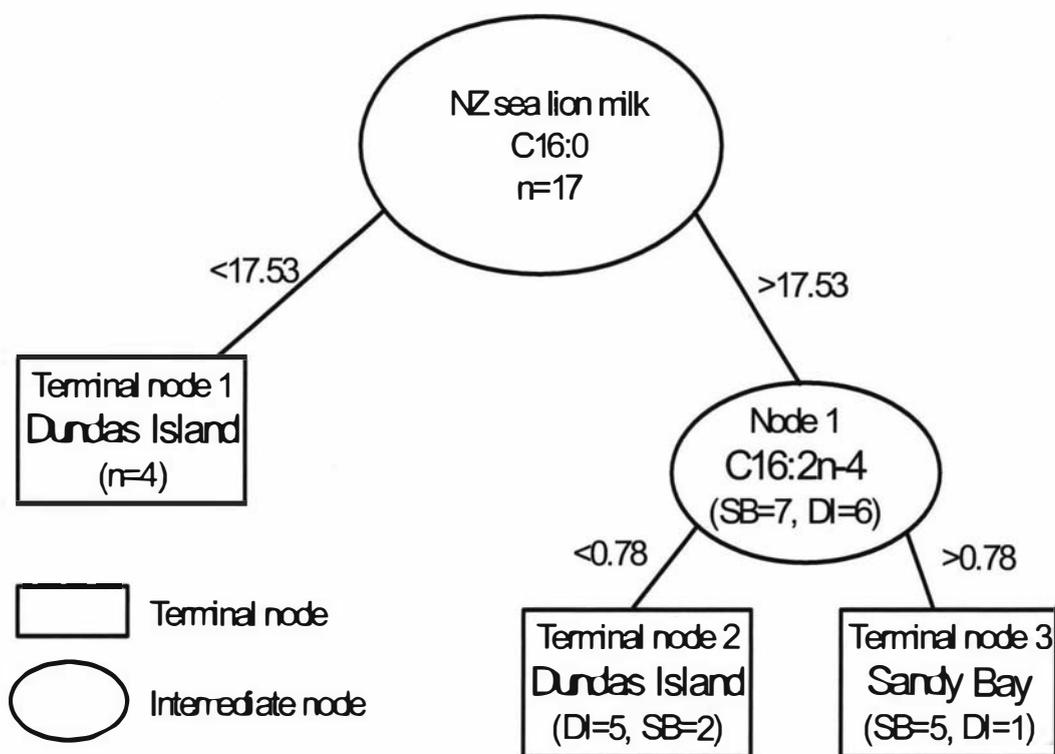


Figure 29. Classification tree, determined by CART, of the fatty acid composition of milk from New Zealand sea lions, *Phocartos hookeri*, collected at their breeding site on either Dundas Island or Sandy Bay at the Auckland Islands. Each node is labelled with the fatty acid used by CART algorithm to create the split. Values on the branches (arrows) show the level (% mass) of the fatty acid at which the split was made. Bracketed numbers at the terminal nodes indicate the numbers of samples from each breeding site.

Inter-annual differences in milk fatty acids

The FA profiles of NZSL milk in 1997 (n=12), 2003 (n=14), 2004 (n=9) and 2005 (n=17) were variable both within and between years (Table 21 and Figure 25). The differences in FA profile are illustrated by the 14 major fatty

acids (Figures 26 and 27). Significant differences between years in specific FA was observed (C10:0, C16:1n-7, C18:1, C20:1n-9, ratio C20:1n-9 : C20:1n-11, C16:3n-4, C20:5n-3, C22:4n-6 and in the total FA) and were shown in Table 21, Figures 26 and 27.

MUFA –PUFA correlation

The relationship between the concentrations of MUFA and PUFA for each year is shown in Figure 29 together with the pooled regression line (PUFA = -0.65*MUFA + 55.38, $r^2 = 0.76$). Within year regressions were not significantly different ($x^2 = 0.74 < x_{0.05,2}^2 = 7.815$).

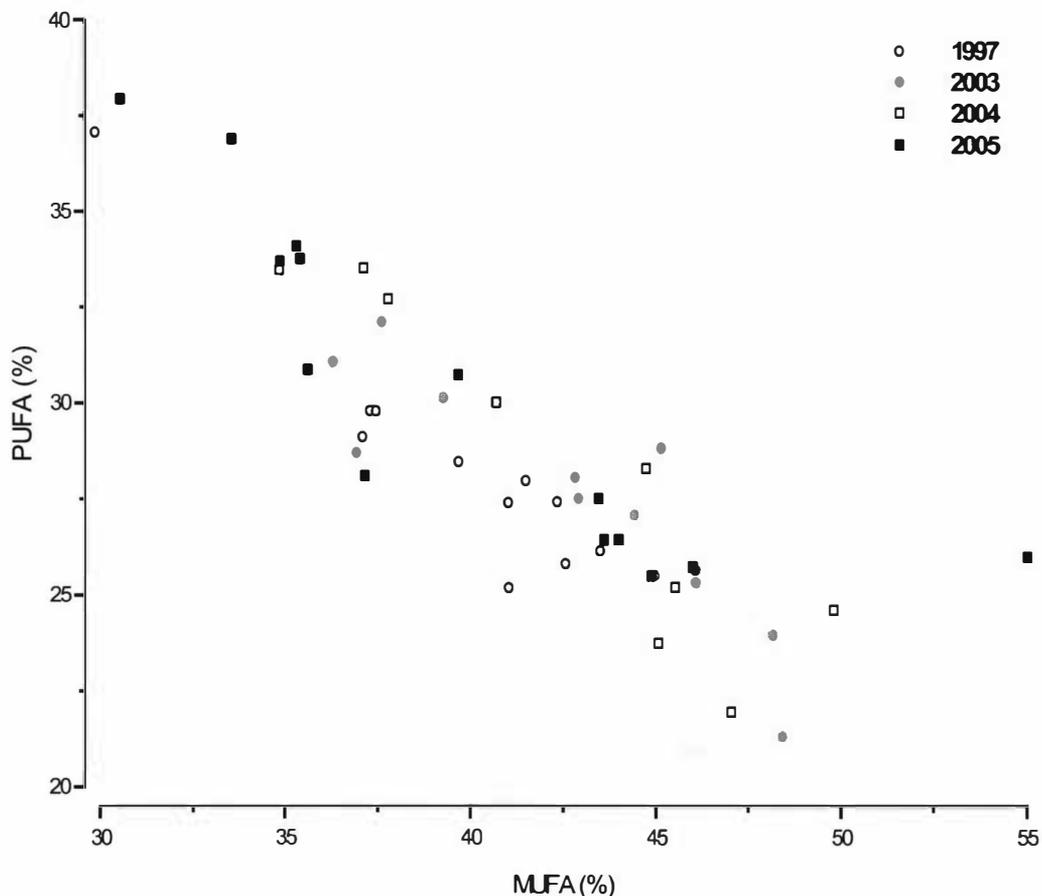


Figure 30. Relationship between monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) in the austral summers of 1997, 2003, 2004 and 2005 (PUFA = -0.65*MUFA + 55.38, $R^2 = 0.76$) milk samples collected from New Zealand sea lions, *Phocarctos hookeri*, in early lactation at the Auckland Islands.

Discriminant analysis

Two discriminant analyses (SDA and CDA) were conducted on each of three sets of data.

- a) One in which all the FAs that are present in the milk were included in the analyses.
- b) The FAs that were included were those that could have originated only from the diet. These components have been suggested to be dietary “indicator FAs” (Iverson, 1993)
- c) The FAs in the milk that were selected for the analysis were also the most abundant in the prey species (Table 21).

a) Whole milk FA profile (Figure 30)

Twenty-one FAs and two ratios (C14:0, C15:0 iso Br, C15:0, C16:0, C17:0 iso, C17:0, C16:1n-7, C16:2n-4, C16:3n-4, C18:0, C18:1n-9, C18:1n-7, C18:1, C18:2n-6, C18:3n-3, C18:4n-3, C20:1n-11, C20:1n-9, C20:4n-6, C20:4n-3, C20:5n-3, C22:5n-3, C22:6n-3, the ratios C20:1n-11: C20:1n-9 and $\omega 6 : \omega 3$) were used in the SDA and CDA (Table 21). SDA identified two parameters (C16:3n-4 see Figure 26f and ratio C20:1n-11: C20:1n-9 see Figure 27) that had the most power to discriminate between milk samples from different years. The ratio C20:1n-11: C20:1n-9 (Partial- $R^2=0.19$, $F=3.85$, $p<0.05$, Wilks' $\lambda = 0.81$) had a higher power to discriminate between years than C16:3n-4 (Partial- $R^2=0.20$, $F= 3.85$, $p<0.05$, Wilks' $\lambda = 0.65$). A plot of the canonical scores indicated a considerable overlap between 2004, 2005 but the data for 1997 and 2003 years are clearly separated (Figure 30).

According to the canonical discrimination analysis it is possible to distinguish between year of sampling (Pillai's Trace = 1.76, $F_{(81,72)} = 1.49$, $p<0.05$), and a canonical variable could be used to explain the between- to within-group variation (Eigenvalue= 3.01, $F_{(112,66)} = 1.65$, $p<0.05$). A total of 5 milk samples (9.6%) were misclassified (Figure 30).

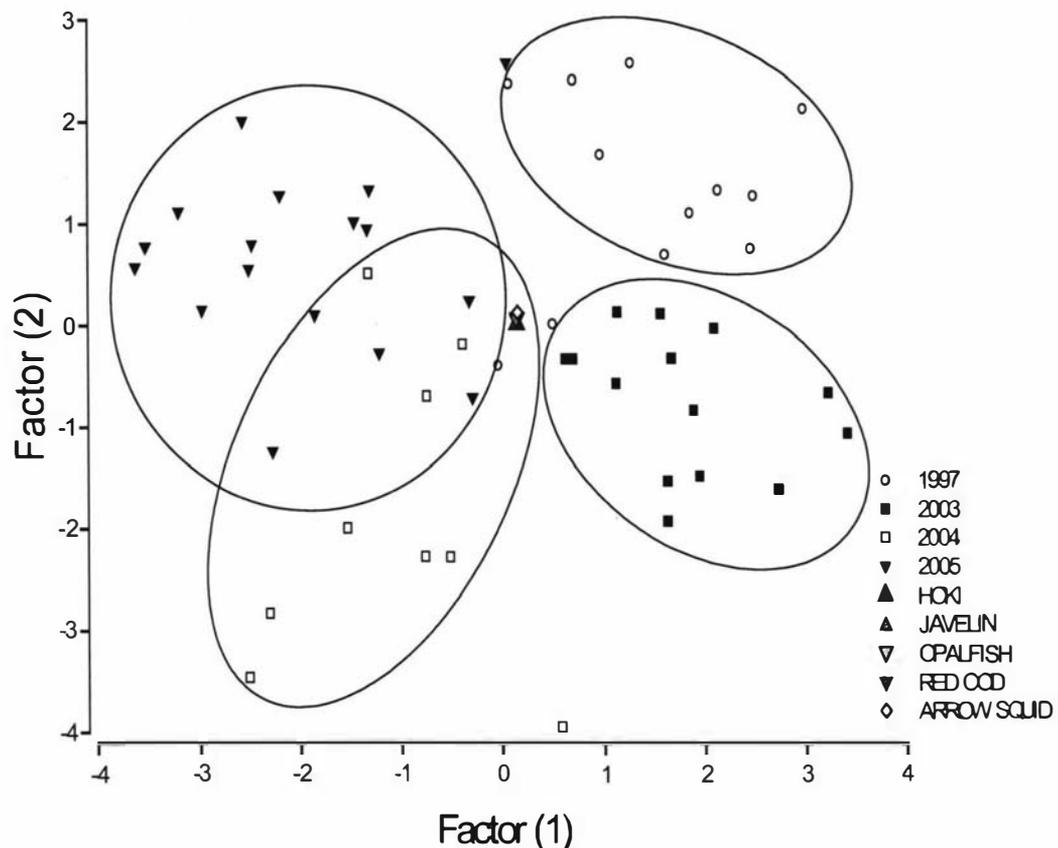


Figure 31. Plot of the canonical scores of the milk fatty acids in milk of New Zealand sea lion, *Phocarctos hookeri* milk fatty acids and prey species in milk samples collected in 1997, 2003, 2004, 2005 (lines are illustrative and delimit the samples by years) at the Auckland Islands and prey species collected at the Campbell plateau in the summer and autumn of 2005/2006 (Meynier unpublished data). Prey species were as follows. Prey species: RC: Red cod (*Pseudophycis bachus*); JL: Javelin (*Lepidorhynchus denticulatus*); OF: Opalfish (*Hemerocoetes* spp.); AS: Arrow squid (*Nototodarus sloanii*); HK: Hoki (*Macruronus novaezelandiae*).

b) FA dietary indicators (Figure 31a)

Twelve dietary indicators, C14:0, C16:3n-4, C18:2n-6, C18:3n-3, C18:4n-3, C20:1n-11, C20:1n-9, C20:4n-6, C20:4n-3, C22:6n-3 and the ratios (C20:1n-11: C20:1n-9 and $\omega 6 : \omega 3$) were used in the SDA. C22:5n-3 was not used because it is believed to be an intermediate of C20:5n-3 and C22:6n-3 FA (Ackman *et al.*, 1988). The SDA results indicated that the FAs with the greatest power to discriminate was ratio C20:1n-11: C20:1n-9 (Partial- $R^2=0.20$, $F=3.85$, $p<0.05$, Wilks' $\lambda = 0.92$), followed by the C16:3n-4 (Partial- $R^2=0.20$, $F=3.85$, $p<0.05$, Wilks' $\lambda = 0.65$). When the twelve FAs were used to

discriminate (CDA) between milk samples there were no significant differences (Pillai's Trace= 0.79, $F_{(117, 36)} = 1.15$, $p > 0.05$) (Figure 31a).

c) Main prey FAs (Figure 31b)

The analysis was performed with the most abundant FAs present in the lipid of prey species, and these FAs were as follow: C14:0, C16:0, C18:1n-9, C18:1n-7, C18:2n-6, C20:1n-9, C20:5n-3, C20:1n-11 and C22:6n-3 (Table 21).

The ratio C20:1n-11: C20:1n-9 was also included in the analysis, since it was found to have discriminatory power in the previous analyses above. Out of these eleven components, two parameters the ratio C20:1n-11: C20:1n-9C18:1n-9 (Partial- $R^2=0.19$, $F = 3.85$, $p < 0.05$, Wilks' $\lambda = 0.80$) and C14:0 had the largest power to distinguish between milk samples (Partial- $R^2=0.11$, $F=1.87$, $p > 0.05$, Wilks' $\lambda=0.72$). According to the canonical discrimination it was not possible to distinguish between milk samples by their year of collection when using the most main FAs in prey (Pillai's Trace= 0.76, $F_{(123, 30)} = 1.38$, $p > 0.05$). In the DFA (JMP software) indicated that 23 (44.2%) of 52 milk samples were misclassified (Figure 31b).

Milk and prey species fatty acids data

Discriminant analysis

An *a priori* test revealed CDA was not performing when all milk samples and all prey samples formed separate group, because the number of samples in the prey species group was very small ($n=5$). As a result the prey were not included in the discriminant analysis but were plotted in the canonical plots by calculating their position. This was done by e.g. CAN 1 for hoki = (arsine transformed value of C14:0 for Hoki) * (the canonical coefficient of C14:0) + (arsine transformed value of C16:0 for Hoki) * (the canonical coefficient of C16:0) +.....=

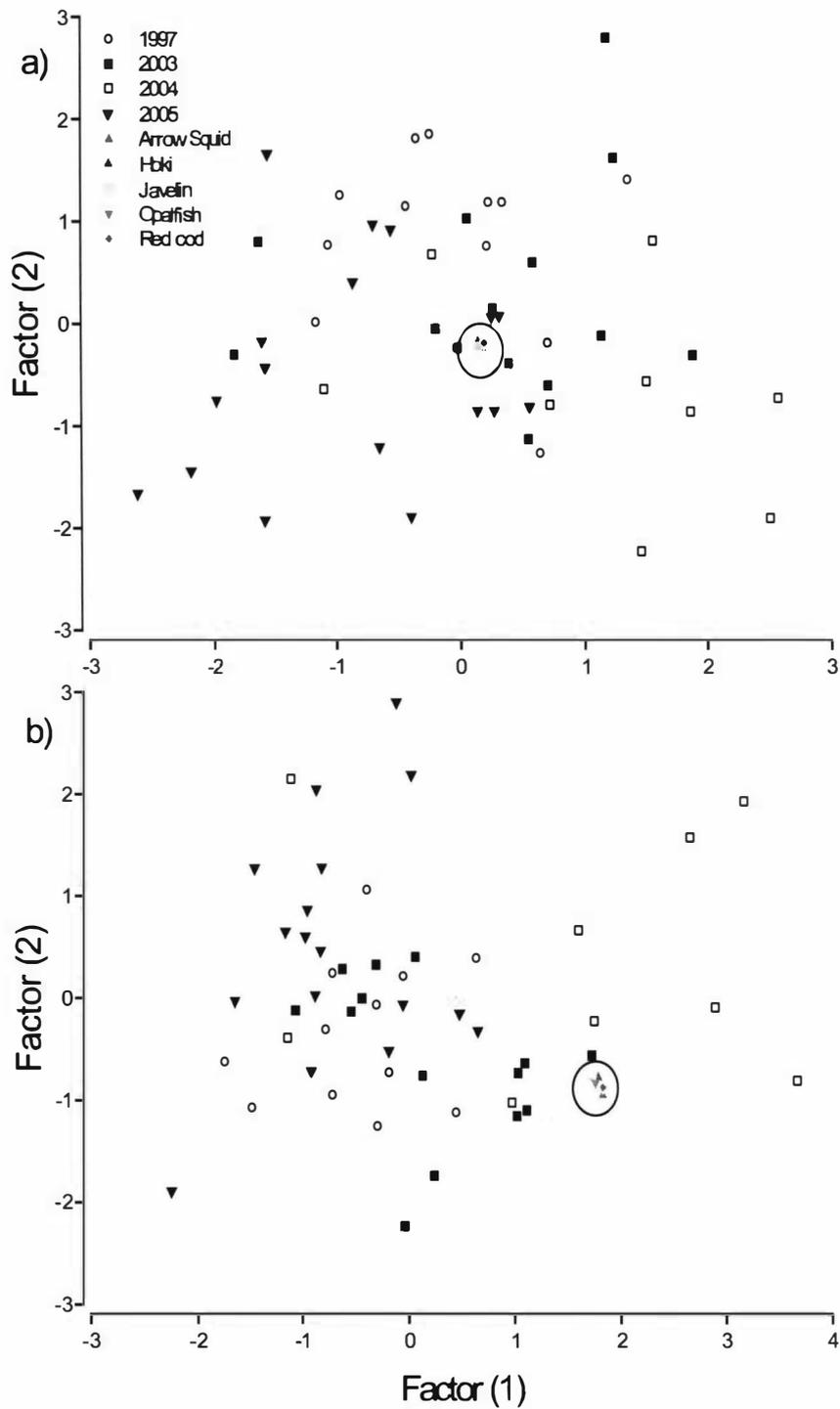


Figure 32. Plot of canonical scores of the fatty acids (FA) in milk of Zealand sea lions, *Phocarctos hookeri* milk fatty acids and FA in potential prey species. Milk samples collected in 1997, 2003, 2004, 2005 at the Auckland Islands and prey species collected at the Campbell plateau in the summer and autumn of 2005/2006 (Meynier unpublished data). a) only dietary “indicator FAs” and b) only main FAs in prey species were used. Prey species: RC: Red cod (*Pseudophycis bachus*); JL: Javelin (*Lepidorhynchus denticulatus*); OF: Opalfish (*Hemerocoetes* spp.); AS: Arrow squid (*Nototodarus sloanii*); HK: Hoki (*Macruronus novaezelandiae*).

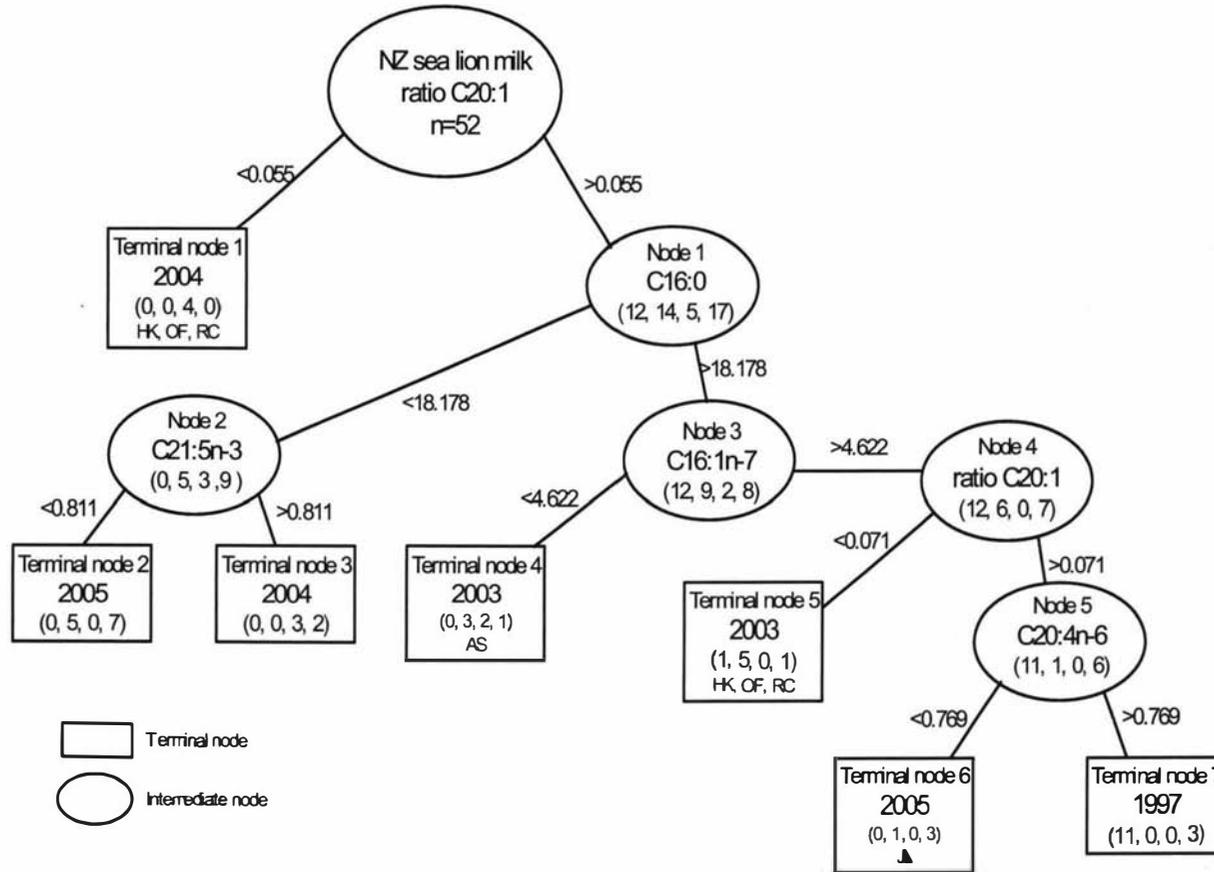


Figure 33. Classification tree, determined by CART, of the fatty acid (FA) composition of milk collected from the New Zealand sea lion, *Phocarctos hookeri*, in 1997, 2003, 2004 and 2005 at the Auckland Islands. Each node is labelled with the FA used by CART algorithm to create the split. Values on the branches (arrows) show the level (% mass) of the FA at which the split was made. Bracketed numbers at the terminal nodes indicate the numbers of samples from each year (1997, 2003, and 2004 2005) and letters at each the terminal indicate prey species (AS= arrow squid; HK=Hoki; JL=Javelin; OF=Opalfish; RC= Red cod).

Classification and Regression Tree (CART) analysis

CART created a pruned tree from six FAs giving seven terminal nodes when milk samples were classified by their year of collection (Figure 32). Fatty acid ratio C20:1n-11: C20:1n-9 was chosen by the tree algorithm to split the 1st node. This resulted in four of the 2004 milk samples being separated by lower values of the ratio C20:1 into terminal node 1. When prey species data were manually “dropped down” the tree Hoki, Red cod and Opal fish were classified with the 2004 samples (terminal node 1) (Figure 32). The 2nd split was based on C16:0 (node 1) which distributed the samples for 2003, 2004 and 2005 evenly across both nodes but allocated all the samples from 1997 to node 3 (Figure 32). This FA was present in higher concentrations in the five prey species than in the milk samples (Table 21). In the 3rd split at node 2 samples from 2003, 2004 and 2005 were only partially separated by C21:5n-3. The 4th split was based on C16:1n-7 and the low levels of this FA classified samples from 2003, 2004 and 2005 and Arrow squid into terminal node 4 but all of the samples from 1997 were allocated to Node 4. FA C16:1n-7 was present in lower levels in Arrow squid samples than in the milk samples and the remaining prey species (Table 21). The 5th split was based on the ratio C20:1n-11 : C20:1n-9 and the lower values of this parameter distributed 5 of the 6 remaining 2003 samples and only 1 of the 12 1997 samples into terminal node 5 together with Hoki and Opal fish. The values for the ratio C20:1n-11: C20:1n-9 was particularly low in Hoki, Red cod and Opal-fish in comparison with Javelin and Arrow squid (Table 21 and Figure 27). Although Hoki, Red cod and Opal-fish were initially classified into terminal node 1, for exploratory reasons, it was assumed that some prey samples may have a higher ratio of C20:1n-11 : C20:1n-9 (than 0.055%) and therefore these prey continued to be dropped down the tree to observe into which other terminal node they would be classified. The 6th split was based on C20:4n-6, which classified 3 of the remaining 6 2005 milk samples into terminal node 6 and the remaining 11 1997 milk samples into terminal node 7. The lower values of C20:4n-6 classified Javelin with 2005 milk samples (terminal node 6). This FA was present in lower proportions in prey species than in the average milk samples (Table 21).

DISCUSSION

The primary objective of this study was to examine the changes in the milk FA signature of lactating NZSL between years and the two main breeding sites and to assess whether these may be related to variations in the composition of the diet. The secondary objective was to carry out a preliminary investigation relating the variation in FA signatures to the general patterns of FA signatures of some potential prey species. The FA signatures of only five potential prey species were included in the analysis, and while these species make up the bulk of the dietary intake of the NZSL they do not represent the full range of potential species in the diet (Childerhouse *et al.*, 2001; Bando *et al.*, 2005; Meynier *et al.*, 2006a). Therefore the conclusions that can be drawn from this study about the composition of the diet of NZSL and how it varies between years are limited.

Inter-breeding sites differences in milk fatty acids

The present study does not indicate that the diets of the two groups of females breeding in Dundas Island and Sandy Bay were different. Discriminant analysis failed to discriminate between milk samples from NZSLs breeding on Sandy Bay and Dundas Island. This finding was supported by CART (see Figure 28). CART failed to group the milk samples by their sites of collection (breeding site). CART was able to group four samples from Dundas Island; however, most samples from Dundas Island and samples from Sandy Bay terminated in the same side of tree, indicating that females from both breeding site and similar milk FA signatures and thus very likely similar diets. Consequently in all subsequent analyses the data collected in 2005 from the two sites were pooled.

FA signatures are influenced by spatial or temporal heterogeneity in habitats and food webs (Iverson, 1993). For example, the analysis of FA in harbour seals and grey seals allowed the differentiation of the foraging spaces and habitat uses of these two species (Iverson *et al.*, 1997b; Walton *et al.*,

2000). Therefore studying the FA signatures in NZSL and potential prey could provide a similar understanding. The results of this study indicated that the habitat use and spatial scale of foraging are similar for lactating NZSLs breeding on Dundas Islands and Sandy Bay. This is supported by data on movements of satellite tagged lactating NZSLs from Dundas Island (Chilvers *et al.* unpublished data) and Sandy Bay (Chilvers *et al.*, 2005, 2006) that showed females from both breeding colonies overlap in their foraging range and diving behaviour.

Inter-annual differences in milk fatty acids

The main finding of this study is that milk FA signatures in 1997 were clearly different from the other years as shown by the CART and the discrimination analysis results (Figures 30 and 32). In addition the discriminant analysis indicated that milk samples collected in 2003 differed from those collected in all other years (Figure 30) even though the CART analysis failed to discriminate between the samples from 2003 and 2004 and 2005 (Figure 32).

Variations in environmental conditions between years are most likely responsible for these differences in FA signatures in the milk of the NZSL. A possible reason that may explain the inter-annual changes in the composition of milk FAs may be due to significant changes in maternal body condition and in milk lipid concentration at early lactation as has been reported (see Chapter 4 and 5). These data suggest that the lactating sea lions were malnourished in 1997, 2001, 2002 and 2005 (see Chapter 5) and may have to draw more on body reserves than in other years and therefore the differential mobilization of fat reserves for the production of milk may explain the observed changes in milk FA composition. It is well known that lipids mobilized from the blubber in fasting lactating phocids and otariids (peri-natal stage, period in which mother give birth and fast for several days) to produce milk are reflected in the milk FA signatures (Iverson *et al.*, 1995; Iverson *et al.*, 1997a; Grahl-Nielsen *et al.*, 2000). In lactating grey seals the FAs that were higher in the milk than in blubber were the polyunsaturated acids C22:6n-, C22:5n-3, C20:5n-3 and C18:4n-3, C20:1n-9 and C20:1n-11 and monosaturated C14:0, C16:0 and

C18:0 (Grahl-Nielsen *et al.*, 2000); however, little is known about how body reserves are mobilized when otariid mothers resume feeding after parturition and when food is scarce. Nevertheless, the data from 1997 and 2003 are separated from 2005 and 2004 data by their scores in Factor 1 (Figure 30), and the variable that contributed most to the canonical scores was C16:2n-4 (Factor 1). This medium chain occur in the blubber of NZSLs at higher concentration than in milk (see Chapter 5, Table 20) and is not reported in the potential prey species (Table 21) and therefore is it likely that the differences in the concentration of this FA arise from blubber mobilization or endogenous sources. The components that contributed most to the canonical score for Factor 2 in Figure 30 and that separated 1997 data from 2003 was the ratio C20:1n-9 and C20:1n-11. This component is likely to arise only of mostly from the diet (Iverson, 1993; Iverson *et al.*, 1997b).

Furthermore the differences in milk FAs signatures may be explained by differences in prey or diet consumed at early lactation throughout the years of this study. Temporal shift in diet has been demonstrated in Antarctic fur seals with milk FA signatures (Brown *et al.*, 1999; Lea *et al.*, 2002; Staniland and Pond, 2005). Changes in the composition of the milk fatty acids generally reflect changes in either the composition of the diet or of intake. Indeed changes in the concentrations of the isomers of C20:1, C20:1n-11 and C20:1n-9 and their ratio have been shown to reflect changes in diet in other marine species (Iverson *et al.*, 1997b). The significant differences (Table 21, Figures 25 and 27) found in the concentration of 20:1n-9 and in the values of the ratio of C20:1n-11: C20:1n-9 between 1997 and 2004 are also consistent with a shift in the composition of the diet (Figure 27). There are two possibilities to explain the inter-annual variation in these components. High concentration of C20:1n-9 in Antarctic FS milk has been explained by consumption of teleost fish which is have the characteristic of having high concentration of this component among other (Reinhardt and Van Vleet, 1986; Iverson *et al.*, 1997a; Lea *et al.*, 2002). Elevated concentration of C20:1n-9 in teleost fish comparison with milk was observed in the present study (Table 21, Figure 27). Further, the concentration of C20:1n-9 and C20:1n-11 in blubber

of some Atlantic phocids species have been directly associated with the composition of fish prey (Iverson, 1993; Iverson *et al.*, 1997b). C20:1n-9 and C20:1n-11 was found to have significantly higher concentration in the blubber and a similar ratio than in the milk of NZSLs (see Chapter 5, Table 20) which may suggest the mobilization and incorporation of these components into the milk. In lactating hooded seal the FA composition in blubber mirror that of milk and this is a evidence of mobilization and incorporation of blubber FA into milk (Iverson *et al.*, 1995). It must be taken into consideration that FA milk composition in NZSL's may be a reflection of the diet and in part a blubber mobilization, and this is most obvious when dietary intake is limited (Iverson, 1993).

Milk and prey species fatty acids data

The diet of NZSLs, according to the analysis of scats and regurgitates collected at the Auckland Islands, consist mainly of fish followed by cephalopods (Childerhouse *et al.*, 2001). Diet analysis of stomach content of NZSLs bycatch in the squid fisheries were consistent with this result and indicated that the diet consisted predominately of teleost fishes (opalfish and rattail, *Coelorinchus spp*) followed by arrow squid (Bando *et al.*, 2005; Meynier *et al.*, 2006a; Meynier *et al.*, 2006b). To further support the hypothesis that squid is an important component of the diet of lactating NZSLs, studies on the foraging pattern (telemetry data) have demonstrated overlap between foraging areas of lactating NZSLs and squid fisheries, and studies on the diving behaviour match the depth of distribution of arrow squid (Gales and Mattlin, 1997; Chilvers *et al.*, 2005, 2006). The position of the 5 potential prey species in the plots in Figures 30 and 31 indicate that all these species could be contributing to the diet of the NZSL sampled in this study. Thus the results presented here are consistent with those based on traditional methods, and indicate that teleost fish and arrow squid are an important part in the diet of lactating NZSLs. However, the data do not indicate that any one of the 5 prey was making a greater contribution to the diet than the others in any of the years. Indeed the lack of any apparent relationship between the FA signature of the individual prey and the different FA signatures of the milk in different

years suggest either that there are other prey that make a significant contribution to the diet or that the more research is required to refine the technique before it can be used as a reliable qualitative indicator of dietary intake.

Limitations of statistical analysis

The application of CART in the study of the foraging ecology of pinnipeds has been widely used (Iverson *et al.*, 1997a; Iverson *et al.*, 1997b; Brown *et al.*, 1999; Staniland and Pond, 2004); however, the validity of its use in this context has been debated vigorously (Iverson *et al.*, 1997a; Iverson *et al.*, 1997b; Grahl-Nielsen, 1999; Smith *et al.*, 1999; Grahl-Nielsen *et al.*, 2004). Similar debate has surrounded the use of discriminant function analysis and principal component analysis (Walton *et al.*, 2000; Lea *et al.*, 2002; Staniland, 2002; Bradshaw *et al.*, 2003; Grahl-Nielsen *et al.*, 2003; Grahl-Nielsen *et al.*, 2005). The main advantages of CART over the discriminant analysis are that it is a non-parametric method, and that a preselection of variables is not needed i.e. number of variables is not limited (Smith *et al.*, 1997, 1999). Furthermore, CART has the advantage to detect differences in complicated data sets such that of FAs signatures which may contain up to 70 FAs (Iverson *et al.*, 1997a; Smith *et al.*, 1997, 1999). Smith and co-workers (1997) found that the results from CART and discriminant analysis were comparable. In the present study discriminant analysis and CART have been used as complementary statistical analysis therefore the results in the present study were in broad agreement with Smith and co-workers (1997, 1999) conclusion in that principal component analysis and CART are different and complementary analyses for classifying and differentiating samples based on numerous variables. The use of CART in this context would have not been affected the conclusion of the present study.

Conclusion

The present study was able to detect that the milk FA signatures of lactating NZSLs was different in 1997 and 2003 due to probably a shift in diet or differential mobilization of adipose tissues or endogenous sources. This study was unable to explain the inter-annual difference due to diet. The main reason was because it was not possible to identify the components of prey species which fully contribute to the milk FAs signatures because complete data on prey FAs signatures is needed to adequately determine the diet and foraging pattern of NZSLs; however, a method (QFASA) is being developed that adequately quantified the contribution of prey to the diet of NZSL. Conclusion made from individual component (e.g. isomers, ratios, class of FAs) must be taken with cautions, because the FA of prey would never match exactly that of their predators (Iverson, 1993). It is well known that the FAs signatures of predator and prey will never match exactly (Iverson, 1993). New models are being developed, e.g. QFASA (Iverson *et al.*, 2004), that account for the difference between prey and predators FAs signatures among other important factors. It would be crucial to identify the preys and contribution to the diet, in particular of the commercially Arrow squid, since it may be possible that shifts in the diet of NZSLs associated with the abundance of this prey may around the Auckland Islands imply changes in the management of the squid fisheries.

Acknowledgments

The research undertaken in this chapter was supervised by Pádraig J. Duignan (IVABS, MASSEY UNIVERSITY), Nicolas Lopez-Villalobos (IVABS, MASSEY UNIVERSITY), Louise B. Chilvers (Marine Conservation Unit, Department of Conservation), Duncan D. S. Mackenzie (IFNHH, MASSEY UNIVERSITY) and Alastair MacGibbon (Fonterra Research Centre). The research undertaken in this chapter was carried out in collaboration with Dan Costa (Department of Ecology and Evolutionary Biology, University of California, USA) and Nick Gales (Australian Antarctic Division, Australia) who

provided the milk samples from 1997 and in collaboration with Laureline Meynier (IVABS) who provided the prey species fatty acids data. I would like to thank Aurelie Castinel and all DoC staff who assisted in the field. I am very grateful to Mark Reynolds, Russell Richardson, and Yvonne van der Does (Fonterra Research Centre) for their invaluable help and their technical support with laboratory analysis. I would like to thank Mark Reynolds and Laureline Meynier for their useful comments on earlier draft of this chapter. I am also thankful to Sara Iverson for providing the laboratory protocol (reference method) to extract the milk lipids.

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Chapter 7

GENERAL DISCUSSION

INTRODUCTION

The rationale for this research is the increasing concern that human activities, in particular fisheries, are significantly affecting the viability of pinniped populations (Northridge and Hofman, 1999; Gales *et al.*, 2003). For this thesis the species of concern is New Zealand sea lions. This concern is supported by disturbing trends such as the low weight and growth rates of the pups, high rates of disease and death, and a general decline in sea lion population numbers (Campbell *et al.*, 2006; Castinel, 2007; Chilvers *et al.*, 2007; Chilvers *et al.*, in press). These trends indicate a need for scientific research to help developed and verify conservation and management strategies for pinniped population in NZ and worldwide (Slooten and Dawson, 1995; Fraker and Mate, 1999; Maunder *et al.*, 2000; Furness, 2002; Gales *et al.*, 2003; Australian Government Department of Agriculture Fisheries and Forestry, 2006).

The aim of this research was to investigate the role of lactation in the ecology of the NZSL by analysing the milk composition and by identifying some of the factors that influence it. In addition, to be able to explain how milk composition changes a better understanding of the physiology of lactation in the NZSL was sought. The aim of this General Discussion is to integrate the main outcomes of the research in the context of the biology of the NZSL and in relation to conservation and management practices for the NZSL.

CONSTRAINTS ON THE RESEARCH

There are a number of factors that constrain the experimental design when working in an isolated environment with a small group of threatened animals. These are outlined briefly so that the discussion of the results is in an appropriate context. The milk samples, collected in January-February when the lactating females are most accessible, only cover the early part of a 9 – 10 month lactation period. By the end of the summer females with their offspring

move away from the colonies and are no longer accessible for capture or observation. In addition, because the NZSL is a threatened species, access to the lactating females is also restricted particularly if the experimental protocol requires anaesthetising the animal and collecting blood, blubber and milk samples as described in Chapter 5. In this case the experimental design adopted, while allowing valid statistical analysis, was restricted by the constraint of being able to capture individual females only twice. Similarly it was not permitted to collect milk samples from the same animals in consecutive years. Solutions to these constraints are discussed in future research section later in the chapter.

Lactation strategy in New Zealand Sea Lions

The “central place foraging” theory states that animals that have feeding grounds away from a central place should make fewer trips, and return with a greater amount of energy per trip than those animals feeding closer (Stephens and Krebs, 1986). If this is true, then NZSL should produce high energy-rich milk, since the total foraging distances are long and high relative to other otariids ((Costa and Gales, 2000; Chilvers *et al.*, 2005). The average duration of a foraging cycle was 2.9 days of which 1.7 days (57%) were spent at sea and 1.2 days (43%) ashore (Gales and Mattlin, 1997) which is characteristic of a central place foraging strategy. Thus the current sub-polar breeding range (latitude) and foraging strategy of the NZSL suggest that milk composition should be closer to those species producing milk with a higher nutrient and energy content. Yet the average concentration of fat (19.95 %) and protein (9.44%) in the milk (Table 11, Chapter 3) is much less than 39.4-45% fat and 11.9-18.1% protein (Table 4, Chapter 1), in the milk of species that have adopted a central place foraging strategy (Arnould and Boyd, 1995; Francis *et al.*, 1998; Ochoa-Acuna *et al.*, 1999; Georges *et al.*, 2001). Indeed milk composition of NZSL is much closer to that of the temperate Australian sea lions, Cape fur seals and tropical Galapagos fur seals (Figure 12, Chapter 3). It is unclear why these species produce milk with a low energy content, but it may be related to their lactation length and strategy, foraging patterns and

latitude (a proxy for oceanic productivity) or ultimately to the energy quality of their prey. Abundance and distribution of prey may influence their foraging pattern, while proximity and richness of prey may influence their lactation length and strategy (Francis *et al.*, 1998; Schulz and Bowen, 2005).

Indeed, the historical breeding range and distribution of NZSL includes the New Zealand mainland and overlaps in latitude with the historical breeding range of Australian sea lions (Childerhouse and Gales, 1998; Ling, 1999). Therefore the historical distribution and milk composition of NZSL (Chapter 3) strongly support the hypothesis that their natural lactation strategy is one in which a milk of low quality is produced with short foraging trips similar to the temperate species rather than species in lower latitudes. Further the current breeding range of the NZSL in the sub Antarctic may be at the extreme of habitats to which they are able to adapt.

The data on milk composition (Chapter 3) may indicate that NZSL evolved under conditions that favoured a foraging strategy that involved short trips to rich feeding grounds and that in recent times they have had to adapt and forage in a marginal habitat at a distance because the local feeding grounds have been depleted or are naturally low or a combination of both. Under these conditions the composition of the milk is not well suited to meeting the needs of pups as shown by their low growth rates compared to those of other sea lion species such as the Australian sea lion (Chilvers *et al.*, 2007). NZSL females dive to greater depths and forage over greater areas and distances than has been reported for the other sea lion species. It has been postulated that the Auckland Islands represent an area of marginal foraging environment for lactating NZSL (Gales and Mattlin, 1997; Chilvers *et al.*, 2005), that during early lactation, have adopted a diving-foraging behaviour that is near their maximum physiological limits (Gales and Mattlin, 1997; Costa and Gales, 2000; Chilvers *et al.*, 2006). This means that a disproportion amount of energy is spent by the females in foraging relative to the amount of nutrients they are able to deliver to their pups in a milk of low nutrient density. If this is true then reasons for the NZSLs adopting this strategy must be sought. One suggestion

that requires further research is that an ecological resource competition between fisheries and sea lion exist.

Management and Conservation: New Zealand Sea Lions at Auckland Islands

NZSLs are among the least abundant of otariids species. The population has been estimated between 12,000 and 13,000 (Gales and Fletcher, 1999; Wilkinson *et al.*, 2003; Campbell *et al.*, 2006) and recently it has been proposed that the population is in decline (Campbell *et al.*, 2006; Chilvers *et al.*, in press). Their localized distribution and restricted breeding grounds, 90 percent of the population breeds in the Auckland Islands, put this species in a threatened status. The main threats to the population include the direct interaction with the fisheries through bycatch and bacterial epidemics that cause great mortality in pups (Woodley and Lavigne, 1993; Duignan, 1999; Wilkinson *et al.*, 2003; Castinel, 2007). The findings of this thesis may have some implications for the management and conservation of the NZSL population and the arrow squid fisheries management around the Auckland Islands.

Milk quality as an indicator of the health of pinniped population and their environment.

The results presented in Chapter 3 revealed that NZSLs are producing a milk of lower quality compared to other otariids and furthermore that there were significant inter-annual differences in the milk quality. The findings in Chapter 4, suggest that the maternal age of the lactating NZSL and her body condition are factors in determining her milk quality. Thus older mothers produced milk with a higher concentration of fat but similar protein concentration than younger females (Chapter 4). In addition, mothers in poor body condition secreted milk of lower quality than those in good body condition and variation

in milk quality between years mirrored the variation in maternal body condition and body weight (Chapters 3 and 4).

The variation in milk composition between years indicates that in some years milk quality, which is already low in comparison with otariids but expected due to the lactation strategy that is adopted by NZSL and most sea lion species, may be further compromised by unfavourable environmental conditions. This leads to loss of body condition of the mother, she is unable to fully meet the needs of her pup and ultimately her reproductive success and consequently, the survival and viability of the population suffers. The present study also revealed that mothers nursing a male pup were in poorer body condition, lost more body weight and produced milk of lower solids content than those feeding a female pup. Apparently the male pup, which has a greater intrinsic potential for growth, is able to stimulate greater milk production but of lower quality in its mother and further threatening her wellbeing (Arnould *et al.*, 1996). The physiological mechanism that allows this to occur deserves further study.

Thus the data in this thesis suggest that in some years the NZSL were under nutritional stress that was most likely due to an inadequate access to food. The data in Chapter 6 indicated that the FA signatures of the milk fat differed significantly between years. The most likely explanation for this is a variation in food sources and possibly in quantity of food between years or differences in mobilization of adipose lipids.

An attempt to determine the nature of the variation in the food source was not successful in that none of the FA profiles of the five potential prey species, which are known to be components of the NZSL diet, better matched that of the milk fat in any one year (Chapter 6). This indicated that any variation in the diet of the NZSL between years was not due to a variation in the proportion of the five potential prey species studied. This may be because other prey species of appropriately different FA profile constituted a significant portion of the diet in some years, particularly 1997 and 2003.

Alternatively it may be that FA profiles of milk fat may not reflect that of the diet. However, the data presented in Chapter 5 indicate that fat in the diet is rapidly digested and the component FAs may be detected in the milk within 24 hours of ingestion. These data clearly indicate that FA signatures of the milk fat have the potential to accurately reflect those of a recent meal. Since the milk samples analysed in Chapter 5 were collected soon after the females had returned from a foraging trip the expectation is that the dietary fat was making a substantial contribution to the milk FA. A further complication arises if the contribution of FA from the blubber to the milk fat varied substantially between years. In this connection it should be noted that the data in Chapter 6 indicates that the FA profiles of the samples from 2003 differed significantly from those of other years and that in 2003 the protein concentration of the skim milk was particularly low (Chapter 3) indicating a severe nutritional stress. Thus maybe in 2003 foraging trips were less successful and FAs in the blubber were contributing more heavily to the milk fat than in other years. While such an analysis is highly speculative it does indicate the potential of FASA of milk fat for detecting changes in the composition of the diet.

The FA composition of the TAG (triacylglycerol) in the chylomicrons of the blood serum is expected to give the most immediate indication of the composition of dietary lipid (Cooper *et al.*, 2005). However, single blood samples reflect a very short period (Cooper *et al.*, 2005). The dynamic nature of milk synthesis and secretion integrates the flow of precursors for fat synthesis to the mammary gland over a period of several hours, thus providing a measure that is more likely to reflect the flow of nutrients from the gut more accurately.

It was not possible from the data presented in the thesis to attach particular significance to any potential prey species. However, nor did the data eliminate any of the five potential prey species studied as of particular importance to the NZSL. Thus the FA profiles of all five prey species could not be distinguished from those of the NZSL in two of the four years in which the FA profiles of the milk fat were measured. In the other two years the contribution of FA from

another species or from body reserves may have reduced the significance of the five prey species but not detracted from its importance.

While in the present thesis, the results indicated there was not direct relationship between diet and the milk quality, it is apparent that factors influencing the milk quality such as maternal body condition, age and weight (Chapter 4) are directly associated with the nutritional status of the female prior to and during early lactation. It is therefore appropriate to consider the data on milk composition in the context of data on fish stocks.

The annual catches for arrow squid in the Auckland Islands for the years 1988 to 2006 are shown in Figure 33. Year 1999 and 2001 was one of the lowest catches in the last decade (Figure 33) and coincides with i) the lowest milk quality in 2001 (see Figure 11, Chapter 3) and maternal body weight (see Figure 14a, Chapter 4) reported for NZSL, and ii) a maternal body condition below average (see Figure 14b, Chapter 4). The total squid catch for 2001 was at least three times higher than in 1999 and is a clear indication of a year of low squid availability in 1999 (Figure 33). This may suggest that there was less food for the sea lions in 1999. The highest milk quality reported in the present work was in 2005 (see Figure 11, Chapter 3) and this was a year with one of the highest annual arrow squid catches in the last decade (Figure 33). Evidently the relationship between arrow squid catches and maternal nutritional condition and milk quality is complex and should be investigated further. Defining if such relationship exists, directly or indirectly, and its nature, could contribute to the management of NZSL population and the arrow squid fisheries.

The data on milk composition have important implications for the conservation and current management of the NZSL population. The data indicated that the quality of milk is an index of the health and nutritional status of a population of marine mammal although its usefulness to monitor a population would be greatly improved if there was a better understanding of the factors that affect milk composition. For instance, the relationship between milk composition and

milk yield could be elucidated and eventually the correlation between milk yield and pup growth, offspring survival and maternal reproductive success. There is data available to estimate these relationships. Therefore it is important that these are considered and incorporated into management and biological models of the NZSL population.

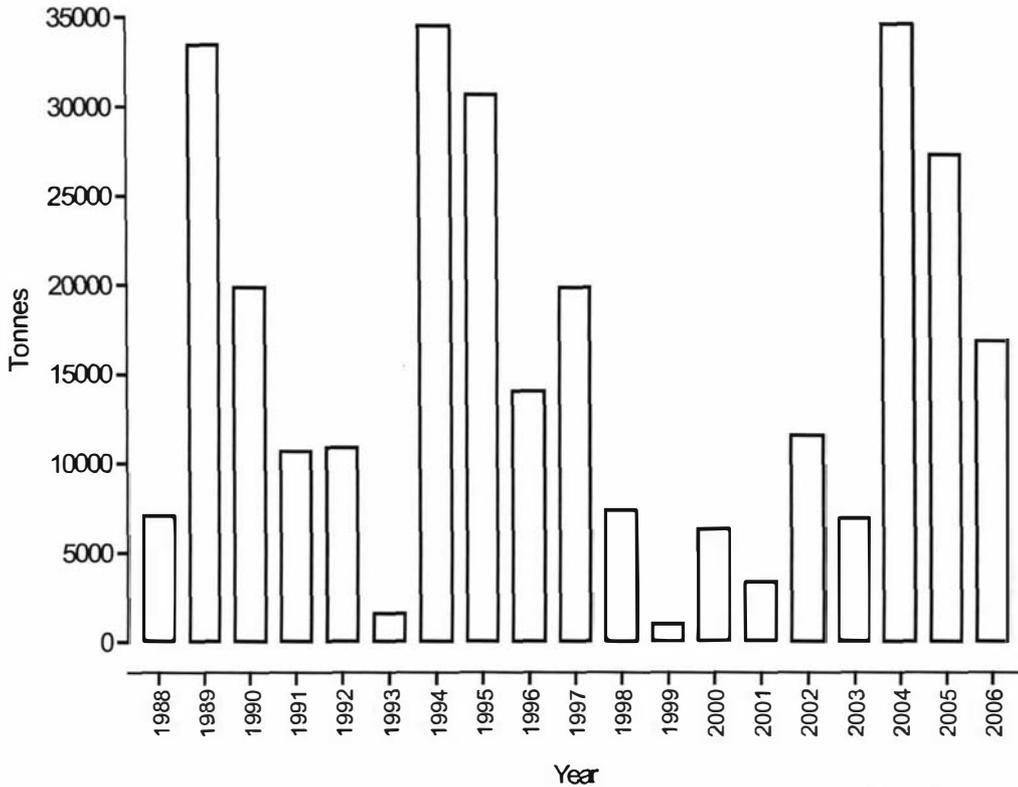


Figure 34. Fisheries annual catches (tonnes) of arrow squid (*Nototodarus sloanii*) at the Auckland Islands. (Data provided from Ministry of Fisheries, New Zealand).

Furthermore, there are limited data on the inter-annual variation in milk composition and thus milk quality in otariids species. Some authors have associated the changes in milk fat to changes in local food resources and while others have not (Arnould and Boyd, 1995; Lea *et al.*, 2002). For instance, Arnould and Boyd (1995) observed a significant decline in the body condition and body weight of Antarctic fur seals females in response to a local food shortage but the milk fat concentration increased and that of protein decreased throughout lactation. The higher fat concentration was explained by a significant increase in the foraging duration in the year of shortage of food (Arnould and Boyd, 1995). However, a more likely explanation is that

food intake was reduced and while milk yield was similarly reduced fat yield was buffered by drawing on body reserves and consequently fat concentration was increased. This conclusion is supported by the decrease in protein concentration, which is an indication of underfeeding or a shortage of food. This highlights the need for monitoring both the fat and protein concentrations as well as maternal body condition and growth rates of the pup for indirect assessments of the nutritional status of the lactating NZSL.

The fact that lactating NZSLs apparently have a low lactation performance and are operating near their physiological limits in a marginal habitat make them highly vulnerable to, direct and indirect, impacts from anthropogenic sources or changes in the local marine environmental conditions. At present, there are data to support the claim that there is ecological resource competition between NZSL and the squid fisheries but the data falls short of showing that the competition is serious. Thus there are temporal and spatial overlaps between the operations of the arrow squid fisheries and foraging grounds covered by lactating NZSLs so competition for ecological resources is considered very likely (Gales and Mattlin, 1997; Chilvers *et al.*, 2005, 2006). Secondly, presence of squid in the diet of NZSL bycatch and analysis of regurgitate (Childerhouse *et al.*, 2001; Bando *et al.*, 2005), is proof of competition during the summer; however, the contribution of squid to the diet of NZSL has not been quantified accurately because of the biases introduced by traditional methods to assess dietary intake. Resolution of the issue requires a more precise method estimating the contribution of arrow squid to the diet of NZSL.

In Chapter 5, data on the movements of FA of dietary lipid from the gut to the milk demonstrate that milk FA signatures can be used to infer diet. Therefore it is theoretically possible to firstly assess the contribution of arrow squid and other potential prey species to the diet of NZSL by using QFASA. Secondly, by collecting milk samples between years or throughout the lactation period information on shift in diet between years, lactation stages and seasons can

be assessed with FASA. Shift in the diet of pinnipeds can also be used as an indicator of the status of the various food resources in their feeding grounds.

The present study did not provide any data directly on the ecological resource competition between NZSL and the squid fisheries. Nor did it indicate the importance of arrow squid in the diet of NZSLs. However, it did demonstrate that resources around the Auckland Island varied between years. The inter-annual differences in the milk FA signatures in the present study could be associated with a shift in the composition of the diet. In Chapter 6, the temporal and spatial differences in milk FA were investigated by using FASA and these differences were assessed in relation to potential prey species. Based on the results from Chapter 6, milk FA signatures were different in 1997 and 2003 and from the five species incorporated in the study suggested that lactating NZSLs have a teleost fish based diet. Determining the diet of NZSL was not the scope of this study, but instead the study attempted to relate the differences to five potential prey species. The present study did not indicate that the differences in milk FAs signatures between years were associated with arrow squid (Chapter 5).

FUTURE RESEARCH

There are two broad areas of future research that it is considered important in the context of this thesis. Firstly, to design further studies to overcome some of the constraints on the present research. Secondly, to use the present research as base-line data to establish a long term monitoring program for NZSL at the Auckland Islands and on the New Zealand mainland (Otago) including FA signature analysis and ecological model approaches for management.

Solutions to constraints on the research

As outlined in the limitation section of this chapter, one of the main constraints of the present study is that milk composition represents early lactation and samples throughout the entire lactation period was not possible. This limits the ability to test the conclusions drawn in this thesis. Future research that replicates this study could test my conclusions, thereby being able to understand the trends in milk composition in NZSL and the factors that influence its composition during the entire lactation period. This could be done by developing a sampling regime in the Otago population where sea lions are accessible all year around.

Some directions for future research

The ultimate objective for research into NZSL is to provide an understanding of their biology so that effective conservation programmes can be put in place. The research that should follow from that described in this thesis concerns furthering our knowledge of lactation in NZSL so that studies on milk composition can be used to monitor the health and well being of the population and to aid in the characterisation of their diet. Projects of particular relevance include the following.

- The milk consumption in pups should be investigated to be able to better understand the lactation strategy of NZSL. Data are available to address this question.
- Collect samples from sea lions at Otago to investigate the milk composition during the entire lactation taken into account differences in prey sources and distance to feeding ground among other factors.
- Investigate the dietary preferences and dietary changes in sea lions from Dundas Island.

- Continue with extensive milk sampling to monitor the health of the population and by analysing the milk FAs signatures to assess changes in diet between years.
- The diet and the contribution of each prey species to the diet should be addressed to determine, a) the importance of the commercial arrow squid in the diet of NZSLs; b) the extent of the competition between NZSLs and the squid fisheries for the same prey source; and c) the inter-annual differences in milk FA in relation to diet. Research is underway to determine the diet of NZSLs via the analysis of the FAs composition of prey species.
- To investigate the fisheries catches around the Auckland Islands through the years and study the environmental factors such as sea temperature, primary productivity that influence prey availability.
- Investigations should focus on developing tools for the management of the NZSL population and fisheries. First, research on the distribution and abundance of prey around the Auckland Islands should be conducted to understand the ecosystem dynamics and the impact of fisheries catch on local food resources; second this information could be used to develop multi-species resource management and ecosystem management model; and third develop a management system or tool that includes the needs of predators that potentially compete with fisheries. This model could serve as an example to monitor other pinniped population that interact and compete with commercial fisheries.

GENERAL CONCLUSIONS

The main findings from the research presented in this thesis are:

- With appropriate calibration the Milkoscan FT 120 can be used to determine the milk composition of NZSL and other pinnipeds species in a fast and cost effective manner.
- NZSLs produce milk with the lowest energy content in early lactation reported for any otariid species and the quality of milk showed yearly

variation. This seems to be a result of their lactation strategy or to variable local food conditions that also affect body condition.

- Assessing the annual milk quality of a pinniped population (provide that the extensive sampling is conducted and factors that influence it are understood) may be a means to monitor the health of a population and useful as a management tool for pinnipeds species. Especially with a fast and cheap way of analysing the milk composition of pinnipeds as outlined in the present thesis.
- Stages of lactation, year and maternal characteristics have significant influences on the quality of milk produced (milk fat and protein concentrations and energy content). The variation in milk quality suggests that availability of food may indirectly reflect the nutritional condition of the mother and her reproductive success.
- Milk and blood FA composition were susceptible to changes in diet (due to the ingestion of the CoNVO) and the changes in diet can be detected within a few hours with the analysis of the FA composition. The timing of the incorporation and deposition of specific FAs were determined and suggested that this process is complex. This information could be used as a baseline for feeding experiment with pinnipeds addressing some of these questions.
- Biological and management models should integrate all the participants in the ecosystem, predators, prey (ecological resource) and fisheries and taken into account the dynamics of their interactions such as variability in prey abundance or increase in the fisheries catch effort, not to mention the effect of bycatch and epidemics in the NZSL population. The integration of these factors would develop into an ecosystem-based fisheries management programme.

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APPENDIX

Table 22. Milk composition of some mammalian species.

| Species | Milk composition | | | | | | Sources |
|--|------------------|------------------------|------------|----------|-------------|---------|--------------------------------------|
| | Fat (%) | Water ¹ (%) | Protein | | Lactose (%) | Ash (%) | |
| | | | Casein (%) | Whey (%) | | | |
| Black rhinoceros <i>Diceros bicornis</i> | 0.2 | 91.9 | 1.1 | 0.3 | 6.1 | 0.3 | (Aschaffenburg <i>et al.</i> , 1961) |
| Donkey <i>Equus asinus</i> | 1.4 | 88.3 | 1.0 | 1.0 | 7.4 | 0.5 | (Ofstedal, 1988) |
| Horse <i>E. caballus</i> | 1.5 | 88.8 | 1.3 | 1.2 | 6.7 | 0.4 | (Ofstedal, 1988) |
| Longnose bat <i>Leptonycteris sanborni</i> | 1.7 | 87.9 | | 4.4 | 5.4 | 0.6 | (Jenness, 1974) |
| Common zebra <i>Equus burchelli</i> | 2.2 | 88.1 | 1.2 | 1.1 | 7.0 | 0.3 | (Ofstedal, 1988) |
| Brown bear <i>Ursus arctos arctos</i> | 3.2 | 89.0 | | 3.6 | 4.0 | 0.2 | (Ben Shaul, 1962) |
| Red kangaroo <i>Macgaleia rufa</i> | 3.4 | 80.0 | 2.3 | 2.3 | 6.7 | 1.4 | (Ben Shaul, 1962) |
| Tapir <i>Tapirus terrestris</i> | 3.4 | 84.3 | | 5.7 | - | 1.0 | (Ormrod, 1967) |
| Mink <i>Mustela vison</i> | 3.4-13.4 | 78.8 | | 7.5 | 2.0-6.3 | 1.3 | (Jorgensen, 1961; Kinsella, 1971) |
| Hippopotamus <i>Hippopotamus amphibius</i> | 3.5 | 88.5 | | 5.3 | 4.3 | 0.8 | (Gray, 1959) |
| Orangutan <i>Pongo pygmaeus</i> | 3.5 | 88.5 | 1.1 | 0.4 | 6.0 | 0.2 | (Jenness, 1974) |

Table 22. (Continued).

| Species | Milk composition | | | | | | Sources |
|--|------------------|------------------------|------------|----------|-------------|---------|-------------------------------|
| | Fat (%) | Water ¹ (%) | Protein | | Lactose (%) | Ash (%) | |
| | | | Casein (%) | Whey (%) | | | |
| Chimpanzee <i>Pan satyrus</i> | 3.7 | 88.1 | 1.2 | | 7.0 | 0.2 | (Ben Shaul, 1962) |
| Cow <i>Bos taurus</i> | 3.7 | 87.3 | 2.8 | 0.6 | 4.8 | 0.7 | (Jenness, 1974) |
| Man <i>Homo sapiens</i> | 3.8 | 87.6 | 0.4 | 0.6 | 7.0 | 0.2 | (Jenness, 1979) |
| Guinea pig <i>Cavia porcellus</i> | 3.9 | 83.6 | 6.6 | 1.5 | 3.0 | 0.8 | (Baker <i>et al.</i> , 1963a) |
| Rhesus monkey <i>Macaca mulatta</i> | 4.0 | 84.6 | 1.1 | 0.5 | 7.0 | - | (Jenness, 1974) |
| Raccoon <i>Procyon lotor</i> | 4.2 | - | 3.0 | 3.1 | 4.8 | 1.1 | (Ben Shaul, 1962) |
| Dromedary <i>Camelus dromedarius</i> | 4.5 | 86.4 | 2.7 | 0.9 | 5.0 | 0.7 | (Kheraskov, 1961) |
| Goat <i>Capra hircus</i> | 4.5 | 86.8 | 2.5 | 0.4 | 4.1 | 0.8 | (Jenness, 1974) |
| Zebu <i>Bos indicus</i> | 4.7 | 86.5 | 2.6 | 0.6 | 4.9 | 0.7 | (Jenness, 1974) |
| Domestic cat <i>Felis catus</i> | 4.8 | - | 3.7 | 3.3 | 4.8 | 1.0 | (Folin <i>et al.</i> , 1919) |
| Golden hamster <i>Mesocricetus auratus</i> | 4.9 | 77.4 | 6.7 | 2.7 | 4.9 | 1.4 | (Jenness and Sloan, 1970) |
| Baboon <i>Papio papio</i> | 5.0 | 44.4 | | 1.6 | 7.3 | 0.3 | (Buss, 1968) |
| Bactrian camel <i>Camelus bactrianus</i> | 5.4 | 85.0 | 2.9 | 1.0 | 5.1 | 0.7 | (Kheraskov, 1961) |

Table 22. (Continued).

| Species | Milk composition | | | | | | Sources |
|---|------------------|------------------------|------------|----------|-------------|---------|--|
| | Fat (%) | Water ¹ (%) | Protein | | Lactose (%) | Ash (%) | |
| | | | Casein (%) | Whey (%) | | | |
| Mountain goat <i>Oreamnos americanus</i> | 5.7 | - | 11.4 | | 2.8 | 1.2 | (Lauer <i>et al.</i> , 1969) |
| Red fox <i>Vulpes vulpes</i> | 6.3 | 81.9 | 6.3 | | 4.6 | 1.0 | (Young and Grant, 1931) |
| Pig <i>Sus scrofa</i> | 6.8 | 81.2 | 2.8 | 2.0 | 5.5 | - | (Jenness, 1974) |
| Virginia opossum <i>Didelphis marsupialis</i> | 7.0 | 75.6 | 2.8 | 2.0 | 4.1 | - | (Bergman and Housley, 1968) |
| Water buffalo <i>Bubalus bubalis</i> | 7.4 | 82.8 | 3.2 | 0.6 | 4.8 | 0.8 | (Dastur, 1956) |
| Sheep <i>Ovis aries</i> | 7.4 | 80.7 | 4.6 | 0.9 | 4.8 | 1.0 | (Perrin, 1958) |
| African elephant <i>Loxodonta africana</i> | 9.3 | 79.1 | 5.1 | | 3.7 | 0.7 | (McCullagh and Widdowson, 1970) |
| Echidna <i>Tachyglossus aculeatus</i> | 9.6 | - | 7.3 | 5.2 | 0.9 | - | (Messer and Kerry, 1973; Griffiths <i>et al.</i> , 1984; Griffiths <i>et al.</i> , 1988) |
| Wolf <i>Canis lupus</i> | 9.6 | 76.9 | 5.8 | 2.1 | 3.1 | 1.2 | (Ben Shaul, 1962) |
| Norway rat <i>Rattus norvegicus</i> | 10.3 | 79.0 | 6.4 | 2.0 | 2.6 | 1.3 | (Jenness, 1974) |
| Giant panda <i>Ailuropoda melanoleuca</i> | 10.4 | 78.37 | 5.02 | 2.1 | 1.6* | 0.9 | (Nakamura <i>et al.</i> , 2003) |
| Indian elephant <i>Elephas maximus</i> | 11.6 | 78.1 | 1.9 | 3.0 | 4.7 | 0.7 | (Jenness, 1974) |

Table 22. (Continued).

| Species | Milk composition | | | | | | Sources |
|---|------------------|------------------------|------------|----------|-------------|---------|-------------------------------------|
| | Fat (%) | Water ¹ (%) | Protein | | Lactose (%) | Ash (%) | |
| | | | Casein (%) | Whey (%) | | | |
| Beaver <i>Castor fiber</i> | 11.7 | 75.9 | 6.2 | 1.9 | 2.6 | 1.1 | (Zurowski <i>et al.</i> , 1971) |
| Arctic fox <i>Alopex lagopus</i> | 11.8 | 70.9 | 12.0 | - | 5.4 | - | (Dubrovskaya, 1967) |
| Domestic dog <i>Canis familiaris</i> | 12.9 | 76.5 | 5.8 | 2.1 | 3.1 | 1.2 | (Ofstedal, 1984) |
| House mouse <i>Mus musculus</i> | 13.1 | 70.7 | 7.0 | 2.0 | 3.0 | 1.3 | (Ben Shaul, 1962) |
| Coati <i>Nasua nasua</i> | 14.9 | 65.1 | 3.5 | 3.9 | 6.4 | 1.0 | (Ben Shaul, 1962) |
| Pygmy sperm whale <i>Kogia breviceps</i> | 15.3 | 74.3 | 3.2 | 5.0 | 2.2 | 0.8 | (Jenness and Odell, 1978) |
| Lion <i>Panthera leo</i> | 17.5 | 69.8 | 5.7 | 3.6 | 3.4 | - | (Ben Shaul, 1962) |
| Spotted dolphin <i>Stenella plagiodon</i> | 18.0 | 69.0 | | 9.4 | 0.6 | - | (Eichelberger <i>et al.</i> , 1940) |
| Domestic rabbit <i>Oryctolagus cuniculus</i> | 18.3 | 67.2 | | 13.9 | 2.1 | 1.8 | (Coates <i>et al.</i> , 1964) |
| Canyon mouse <i>Peromyscus crinitus</i> | 18.8 | - | | 7.7 | 1.5 | - | (Zurowski <i>et al.</i> , 1971) |
| California mouse <i>P. californicus</i> | 18.8 | - | | 12.9 | 1.7 | - | (Zurowski <i>et al.</i> , 1971) |
| Florida manatee <i>Trichechus manatus latirostris</i> | 19.0 | - | | 9.6 | - | - | (Pervaiz and Brew, 1986) |
| Varying hare <i>Lepus timidus</i> | 19.3 | - | 19.5 | - | 0.9 | - | (Ben Shaul, 1962) |

Table 22. (Continued).

| Species | Milk composition | | | | | | Sources |
|--|------------------|------------------------|------------|----------|-------------|---------|--------------------------------|
| | Fat (%) | Water ¹ (%) | Protein | | Lactose (%) | Ash (%) | |
| | | | Casein (%) | Whey (%) | | | |
| Red deer <i>Cervus elaphus</i> | 19.7 | 65.9 | 10.6 | | 2.6 | 1.4 | (Ben Shaul, 1962) |
| Water shrew <i>Neomys fodiens</i> | 20.0 | 80.1 | 11.0 | | 3.2 | 0.8 | (Ben Shaul, 1962) |
| Sea otter <i>Enhydra lutris</i> | 20.9-26.4 | 56.6-61.8 | 4.1 | 7.6 | 0.4-1.4 | 0.9-0.8 | (Jenness <i>et al.</i> , 1981) |
| Grizzly bear <i>Ursus horribilis</i> | 22.3 | 59.4 | 6.7 | 4.4 | 0.6 | 1.5 | (Jenness <i>et al.</i> , 1972) |
| Otter <i>Lutra</i> sp. | 24.0 | 62.0 | 11.0 | | 0.1 | 0.8 | (Ben Shaul, 1962) |
| Black bear <i>Ursus americanus</i> | 24.5 | 55.5 | 8.8 | 5.7 | 0.4 | 1.8 | (Jenness <i>et al.</i> , 1972) |
| E. gray squirrel <i>Sciurus carolinensis</i> | 24.7 | 60.4 | 5.0 | 2.4 | 3.7 | 1.0 | (Ben Shaul, 1962) |
| Spotted porpoise <i>Stenella graffmani</i> | 25.3 | - | 4.7 | 3.6 | 1.1 | - | (Pilson and Waller, 1970) |
| Spinner porpoise <i>S. microps</i> | 26.2 | - | 7.1 | | 1.0 | - | (Pilson and Waller, 1970) |
| Beluga <i>Delphinapterus leucas</i> | 26.9 | 59.0 | 10.6 | | 0.7 | 0.8 | (Lauer and Baker, 1969) |
| Beluga whale <i>Delphinapterus physalus</i> | 26.9 | 59.0 | 10.6 | | 0.74 | 0.8 | (Lauer and Baker, 1969) |
| Nutria <i>Myocastor coypus</i> | 27.9 | 58.5 | 9.3 | 4.4 | 0.6 | 1.3 | (Jenness, 1974) |
| Sei whale <i>Balaenoptera borealis</i> | 28.2 | - | 8.1 | | - | 1.0 | (Jenness, 1974) |

Table 22. (Continued).

| Species | Milk composition | | | | | | Sources |
|---|------------------|--------------------|------------|----------|---------|-----|---|
| | Fat | Water ¹ | Protein | | Lactose | Ash | |
| | (%) | (%) | Casein (%) | Whey (%) | (%) | (%) | |
| Bottlenose dolphin <i>Tursiops truncatus</i> | 29.4 | - | 12.2 | | 2.2 | - | (Pervaiz and Brew, 1986) |
| Platypus <i>Ornithorhynchus anatinus</i> | 31.0 | 51.1 | 12.4 | | 1.7 | - | (Messer and Kerry, 1973; Griffiths <i>et al.</i> , 1984) |
| Polar bear <i>Thalarctos maritimus</i> | 33.1 | 52.4 | 7.1 | 3.8 | 0.3 | 1.4 | (Baker <i>et al.</i> , 1963b; Baker <i>et al.</i> , 1963c; Baker <i>et al.</i> , 1967; Cook <i>et al.</i> , 1970) |
| Fin whale <i>Balaenoptera physalus</i> | 32.4 | 54.2 | 4.8 | 8.0 | 0.3 | 1.0 | (White, 1953; Lauer and Baker, 1969) |
| Humpback whale <i>Megaptera novaeangliae</i> | 33.0 | 51.6 | 12.5 | | 1.1 | 1.6 | (Chittleborough, 1958) |
| White-toothed shrew <i>Crocidura russula monacha</i> | 35.5 | 48.8 | 9.4 | | 4.2 | - | (Mover <i>et al.</i> , 1989) |
| Sperm whale <i>Physeter catodon</i> | 36.4 | 56.5 | 3.8 | | - | - | (Jenness and Odell, 1978) |
| Blue whale <i>Balaenoptera musculus</i> | 42.3 | 42.9 | 7.2 | 3.7 | 1.3 | 1.4 | (White, 1953; Gregory <i>et al.</i> , 1955) |
| Harbor porpoise <i>Phocoena phocoena</i> | 45.8 | 58.9 | - | - | 1.3 | 0.6 | (Jenness, 1974) |
| Gray whale <i>Eschrichtius gibbosus</i> | 53.0 | 40.6 | - | - | - | - | (Jenness, 1974) |

¹ Water content (%) was calculated (100 - total solids) % ; * Total concentration of carbohydrates.

Table 23. Descriptive statistics for New Zealand sea lion, *Phocarcctos hookeri*, maternal traits and milk composition for each female age. Values indicate means (\pm SE) maternal weight (kg), maternal length (cm) and BCI (body conditions index, kg/cm) and percentage for each milk component.

| Ages (year) | Maternal characteristics | | | Milk composition | | | |
|----------------------|--------------------------|-------------------|------------------|------------------|------------------|---------------------|------------------|
| | Weight (kg) | Length (cm) | BCI (kg/cm) | Lipid (g/kg) | Protein (g/kg) | Protein Skim (g/kg) | Energy (kJ/g) |
| 3-4 _{n=7} | 93.98 \pm 5.06 | 159.82 \pm 9.46 | -8.65 \pm 3.67 | 14.21 \pm 5.46 | 9.20 \pm 1.60 | 10.69 \pm 2.52 | 7.60 \pm 2.17 |
| 5 _{n=6} | 110.45 \pm 3.43 | 164.90 \pm 9.46 | -0.71 \pm 2.88 | 20.58 \pm 3.04 | 10.40 \pm 0.88 | 13.42 \pm 1.40 | 10.29 \pm 1.22 |
| 6 _{n=16} | 101.63 \pm 3.39 | 159.90 \pm 9.46 | -6.21 \pm 2.39 | 18.30 \pm 2.78 | 8.64 \pm 0.81 | 10.51 \pm 1.30 | 9.01 \pm 1.12 |
| 7 _{n=26} | 105.31 \pm 2.65 | 163.21 \pm 9.35 | -2.92 \pm 1.98 | 18.57 \pm 2.12 | 8.87 \pm 0.62 | 10.88 \pm 1.00 | 9.20 \pm 0.86 |
| 8 _{n=39} | 108.86 \pm 2.32 | 164.00 \pm 9.31 | -0.24 \pm 1.67 | 19.20 \pm 2.01 | 9.36 \pm 0.58 | 11.59 \pm 0.96 | 9.56 \pm 0.82 |
| 9 _{n=59} | 110.08 \pm 2.17 | 166.95 \pm 9.30 | -3.00 \pm 1.46 | 21.93 \pm 1.90 | 8.80 \pm 0.55 | 11.37 \pm 0.90 | 10.45 \pm 0.77 |
| 10 _{n=40} | 113.81 \pm 2.24 | 166.17 \pm 9.31 | -0.78 \pm 1.64 | 29.00 \pm 2.03 | 9.51 \pm 0.58 | 13.76 \pm 0.96 | 13.32 \pm 0.82 |
| 11 _{n=35} | 114.84 \pm 2.29 | 165.46 \pm 9.32 | 1.23 \pm 1.72 | 20.13 \pm 2.16 | 8.94 \pm 0.62 | 11.28 \pm 1.01 | 9.83 \pm 0.87 |
| 12 _{n=30} | 117.15 \pm 2.25 | 167.78 \pm 9.31 | 0.85 \pm 1.76 | 21.50 \pm 2.21 | 8.44 \pm 0.64 | 10.90 \pm 1.04 | 10.20 \pm 0.89 |
| 13 _{n=32} | 119.43 \pm 2.75 | 167.89 \pm 9.32 | 2.70 \pm 1.72 | 22.90 \pm 2.12 | 9.42 \pm 0.61 | 12.73 \pm 1.00 | 10.94 \pm 0.86 |
| 14 _{n=13} | 121.18 \pm 2.75 | 170.36 \pm 9.38 | 5.79 \pm 2.47 | 21.28 \pm 2.95 | 9.37 \pm 0.85 | 12.34 \pm 1.36 | 10.38 \pm 1.18 |
| 15 _{n=12} | 122.39 \pm 3.27 | 174.89 \pm 9.44 | 4.20 \pm 2.80 | 23.87 \pm 3.06 | 8.74 \pm 0.88 | 11.59 \pm 1.41 | 11.18 \pm 1.23 |
| 16 _{n=11} | 116.77 \pm 3.19 | 167.53 \pm 9.43 | 2.24 \pm 2.70 | 21.53 \pm 2.98 | 9.07 \pm 0.87 | 11.79 \pm 1.40 | 10.38 \pm 1.19 |
| 17 _{n=4} | 117.37 \pm 6.86 | 170.46 \pm 10.1 | -1.72 \pm 4.75 | 22.25 \pm 4.55 | 8.40 \pm 1.34 | 10.82 \pm 2.10 | 10.48 \pm 1.81 |
| 18 _{n=4} | 114.32 \pm 4.88 | 170.62 \pm 9.75 | -0.70 \pm 4.39 | 23.00 \pm 3.97 | 9.21 \pm 1.17 | 11.98 \pm 1.85 | 10.95 \pm 1.59 |
| 19 _{n=3} | 118.61 \pm 7.86 | 174.16 \pm 10.3 | -7.46 \pm 5.47 | 18.44 \pm 4.54 | 8.30 \pm 1.34 | 10.24 \pm 2.11 | 9.02 \pm 1.80 |
| 20 _{n=7} | 127.86 \pm 5.65 | 173.68 \pm 9.81 | 2.80 \pm 3.82 | 21.81 \pm 4.03 | 10.33 \pm 1.18 | 13.30 \pm 1.87 | 10.77 \pm 1.81 |
| 21-23 _{n=8} | 121.70 \pm 5.54 | 170.74 \pm 9.45 | 2.88 \pm 3.39 | 25.71 \pm 3.25 | 9.74 \pm 1.04 | 13.14 \pm 1.74 | 12.09 \pm 1.32 |
| 25-26 _{n=2} | 137.42 \pm 10.6 | 169.21 \pm 10.7 | 21.27 \pm 6.66 | 36.59 \pm 7.85 | 7.03 \pm 2.58 | 11.05 \pm 4.38 | 15.58 \pm 3.20 |

Table 24. Measures of statistical fitness to assess agreement between standard method (Folch *et al.*) and alternative method (ASE) to extract milk lipid of New Zealand sea lions

| Fatty acids | Extraction Methods | | Measures of statistical fitness | | | | | |
|-------------------|--------------------|-------------|---------------------------------|--------|-------|------|-------|------|
| | Folch | ASE | r ² | r | MPE | ICC | BCF | CCC |
| C12:0 | 0.13 ±0.02 | 0.14 ±0.02 | 0.93 | 0.83 | 30.48 | 0.64 | 0.96 | 0.80 |
| C14:0 | 4.77 ±0.54 | 4.92 ±0.54 | 0.99 | 0.99 | 2.40 | 0.98 | 1.00 | 0.99 |
| C15:0 iso Br | 0.17 ±0.02 | 0.19 ±0.02 | 0.97 | 0.75 | 19.98 | 0.99 | 0.95 | 0.71 |
| C15:0 | 0.46 ±0.03 | 0.48 ±0.3 | 0.99 | 0.99 | 2.73 | 0.99 | 0.97 | 0.96 |
| C16:0 Br | 0.46 ±0.03* | 0.48 ±0.03 | 0.92 | 0.80 | 33.14 | 0.99 | 0.94 | 0.75 |
| C16:0 | 17.68 ±0.27 | 18.11 ±0.27 | 0.99 | 0.86 | 2.38 | 0.79 | 0.86 | 0.74 |
| C17:0 | 0.41 ±0.04 | 0.41 ±0.04 | 0.99 | 0.98 | 5.55 | 0.96 | 0.99 | 0.97 |
| C17:0 iso Br | 0.19 ±0.03 | 0.20 ±0.03 | 0.78 | 0.46 | 55.38 | 0.51 | 1.00 | 0.46 |
| C17:0 ante-iso Br | 0.13 ±0.02 | 0.15 ±0.02 | .001 | -0.04 | 38.79 | 0 | -1.00 | 0.04 |
| C18:0 | 2.53 ±0.18 | 2.58 ±0.18 | 0.99 | 0.98 | 3.77 | 0.98 | 1.00 | 0.98 |
| SAFA total | 25.68 ±0.75 | 25.82 ±0.75 | 0.99 | -0.005 | - | - | - | |
| C14:1 | 0.17 ±0.04 | 0.19 ±0.04 | 0.99 | 0.99 | 12.45 | 0.98 | 0.99 | 0.98 |
| C15:1 | 0.17 ±0.02 | 0.19 ±0.02 | 0.97 | 0.75 | - | - | - | |
| C16:1n-7 | 5.74 ±0.49 | 5.90 ±0.49 | 0.99 | 0.99 | 2.92 | 0.98 | 1.00 | 0.99 |
| C18:1n-9 | 25.38 ±1.58 | 26.50 ±1.66 | 0.99 | 0.99 | 0.63 | 0.78 | 0.99 | 0.98 |
| C18:1n-7 | 3.54 ±0.22 | 3.53 ±0.22 | 0.99 | 0.97 | 2.74 | 0.99 | 1.00 | 0.97 |

Table 24. Continued.

| Fatty acids | Extraction Methods | | Measures of statistical fitness | | | | | |
|----------------|--------------------|-------------|---------------------------------|------|-------|------|------|------|
| | Folch | ASE | r ² | r | MPE | ICC | BCF | CCC |
| Other C18:1? | 0.46 ±0.05 | 0.46 ±0.05 | 0.99 | 0.98 | 7.46 | 0.93 | 0.99 | 0.97 |
| C20:1n-11 | 4.31 ±1.12 | 3.88 ±1.12 | 0.92 | 0.90 | 37.45 | 0.89 | 0.99 | 0.89 |
| C20:1n-9 | 2.02 ±0.84 | 2.59 ±0.84 | 0.75 | 0.77 | 75.15 | 0.80 | 0.97 | 0.75 |
| ∑ 20:1 | 6.34 ±0.50 | 6.47 ±0.50 | 0.99 | 0.99 | 2.95 | 0.92 | 1.00 | 0.99 |
| C22:1n-13&n-11 | 0.73 ±0.10 | 0.74 ±0.10 | 0.99 | 0.99 | 5.85 | 0.86 | 1.00 | 0.99 |
| ∑ C22:1 | 0.96 ±0.14 | 0.96 ±0.14 | 0.99 | 0.98 | 9.00 | 0.92 | - | - |
| MUFA total | 36.88 ±3.15 | 37.02 ±3.20 | 0.99 | 0.99 | - | - | - | - |
| C16:2n-4 | 0.82 ±0.04 | 0.85 ±0.04 | 0.99 | 0.97 | 3.40 | 0.85 | 0.97 | 0.94 |
| C16:3n-4? | 0.55 ±0.04 | 0.59 ±0.04 | 0.99 | 0.93 | 8.39 | 0.93 | 0.95 | 0.88 |
| C18:4n-3 | 0.91 ±0.23 | 0.89 ±0.23 | 0.99 | 0.99 | 5.41 | 0.99 | 1.00 | 0.99 |
| C18:3n-3 | 0.53 ±0.05 | 0.52 ±0.05 | 0.99 | 0.95 | 8.69 | 0.91 | 1.00 | 0.95 |
| C18:2n-6 | 1.14 ±0.09 | 1.18 ±0.09 | 0.99 | 0.99 | 3.25 | 0.94 | 0.99 | 0.98 |
| C20:2n-6 | 0.28 ±0.02 | 0.28 ±0.02 | 0.99 | 0.90 | 8.94 | 0.91 | 1.00 | 0.90 |
| C20:4n-6 | 1.19 ±0.10 | 1.14 ±0.10 | 0.99 | 0.99 | 3.93 | 0.94 | 0.98 | 0.97 |
| C20:3n-3 | 0.27 ±0.03 | 0.25 ±0.03 | 0.96 | 0.91 | 22.73 | 0.88 | 0.92 | 0.84 |
| C20:4n-3 | 1.61 ±0.14 | 1.54 ±0.14 | 0.99 | 0.97 | 6.09 | 0.89 | 0.98 | 0.95 |
| C20:5n-3 (EPA) | 7.27 ±0.80 | 7.02 ±0.80 | 0.99 | 0.99 | 2.15 | 0.99 | 1.00 | 0.99 |

Table 24. Continued.

| Fatty acids | Extraction Methods | | Measures of statistical fitness | | | | | |
|-------------------|--------------------|-------------|---------------------------------|-------|------|------|------|------|
| | Folch | ASE | r^2 | r | MPE | ICC | BCF | CCC |
| C21:5n-3?? | 0.43 ±0.06 | 0.42 ±0.06 | 0.97 | 0.99 | 8.11 | 0.97 | 0.97 | 0.96 |
| C22:5n-3 (DPA) | 3.02 ±0.33 | 2.86 ±0.34 | 0.98 | 0.99 | 4.71 | 0.97 | 0.97 | 0.96 |
| C22:6n-3 (DHA) | 12.69 ±0.55 | 12.09 ±0.57 | 0.98 | 0.99 | 1.89 | 0.95 | 0.90 | 0.89 |
| PUFA total | 31.05 ±1.07* | 26.36 ±1.37 | 0.16 | -0.40 | - | - | - | - |
| Total Σ FA | 94.93 ±1.77 | 94.02 ±1.89 | 0.99 | 0.56 | 2.29 | 0.91 | - | - |

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