DETERMINING PREGNANCY STATUS OF EXTANT AND ANCIENT WHALES BY QUANTIFYING PROGESTERONE IN BLUBBER BIOPSIES AND BONE

MACKENZIE L. SHERIDAN

DETERMINING PREGNANCY STATUS OF EXTANT AND ANCIENT WHALES BY QUANTIFYING PROGESTERONE IN BLUBBER BIOPSIES AND BONE

by

Mackenzie L. Sheridan, B.Sc.

A thesis submitted to the

School of Graduate Studies

in partial fulfillment of the

requirements for the degree of

Master of Science

Cognitive and Behavioural Ecology Programme

Memorial University of Newfoundland

May 2004

St. John's Newfoundland

Your file Votre référence
ISBN: 0-612-99115-6
Our file Notre référence
ISBN: 0-612-99115-6

Abstract

This thesis had three major objectives: 1) to determine the most appropriate method for the short-term storage of blubber biopsy samples for hormonal analyses, 2) to determine whether or not progesterone could be quantified in blubber biopsy samples from free-ranging North Atlantic humpback (*Megaptera novaeangliae*) and North Atlantic right (*Eubalaena glacialis*) whales in order to determine pregnancy status, and 3) to determine whether or not immunoassayable progesterone could be quantified in ancient whale bone samples.

In order to determine the most appropriate method for storing blubber biopsies for hormone analyses, samples were collected from a known pregnant minke whale (*Balaenoptera acutostratus*) post-mortem and stored frozen for 2 months under different conditions – in ethanol, 100% dimethylsulfoxide (DMSO), 20% salt-saturated DMSO, and frozen. Biopsy samples stored in ethanol yielded less than 10% of the progesterone concentrations of those in the other three storage conditions ($p < 0.05$). However, once extracts of both the blubber samples that were in ethanol and the ethanol liquid fractions were combined, progesterone concentrations were comparable to those of the other three storage conditions ($p > 0.05$).

Progesterone concentrations were determined from blubber biopsy samples of free-ranging humpback ($n = 32$) and right ($n = 17$) whales in order to determine pregnancy status. We hypothesized that females pregnant at the time of sampling would have elevated blubber progesterone concentrations.

Thirteen of 21 female humpbacks (62%) were resighted the year following sampling. Three females with elevated blubber progesterone concentrations and one with

a moderate progesterone concentration were observed with calves. Seven mature females (two with elevated blubber progesterone concentrations and four with moderate blubber progesterone concentrations) were observed without calves, introducing the possibility of neonatal mortality, spontaneous abortion, pseudopregnancy, or possibly failure of the technique. Two females that had blubber progesterone concentrations comparable to males and immature whales were observed without calves. All adult males and immature individuals had low blubber progesterone concentrations.

All right whales, with the exception of one, were resighted following sampling. One mature female with an elevated blubber progesterone concentration was observed with a calf approximately six months following sampling. One mature female with a moderate blubber progesterone concentration was observed on the calving ground numerous times four to seven months after sampling but without a calf introducing the possibility of pseudopregnancy, spontaneous abortion, or neonatal mortality. All adult males and juveniles of both sexes had relatively lower blubber progesterone concentrations.

Progesterone concentrations were measured in ancient bone samples from bowhead (*Balaena mysticetus*) and right whales killed in the 16th century Basque fishery in Labrador. Immunoassayable bone progesterone concentrations from 0.19 - 13.07 ng/g. were detected. Bone progesterone concentrations of samples taken from bones that were known to have been underwater until relatively recently were significantly greater than those from bone samples collected in terrestrial deposits ($p < 0.05$). Reproductive status could not be determined on the basis of progesterone concentrations alone, and a preliminary analysis awaits the determination of sex for each of the samples.

Acknowledgements

I would like to thank Drs. Jon Lien, Atef Mansour, Donald McKay, and Charles Malsbury for their guidance and support. I am especially appreciative of Dr. Jon Lien for allowing me this opportunity in the first place.

Thank you to Dr. Moira Brown and Jooke Robbins (Center for Coastal Studies, Provincetown, MA, USA) for providing biopsy samples and helpful comments throughout the project. Carolyn Miller (Wood's Hole Oceanographic Institute, Wood's Hole, MA, USA) and Wayne Ledwell also provided blubber samples. Thank you to the Right Whale Research Consortium for providing access to their database. Thanks to Gehan Mabrouk for mentoring me when I first arrived in the lab.

Thanks to Elaine Anton, Martha Drake, and Penny Houlden (Provincial Museum of Newfoundland and Labrador, The Rooms Corporation of Newfoundland and Labrador, Government of Newfoundland and Labrador) for granting us permission to sample the bones and to Cindy Gibbons (Parks Canada, Red Bay) for facilitating access to the archived bones. Thank you to Drs. Selma and Michael Barkham and residents of the Labrador Straits for providing information on the Basque whaling industry. A special thanks to Brenna McLeod (Trent University) for being my field partner in Labrador. Thanks to Joanne Costello (Library Assistant, Map Library, Queen Elizabeth II Library, Memorial University of Newfoundland) for her assistance with the maps. As well, thanks to the Departments of Biology and Psychology and the Division of Basic Medical Sciences (Memorial University of Newfoundland) for administrative support.

Funding for this project was provided by the Endangered Species Recovery Fund (Environment Canada and World Wildlife Fund), the Center for Coastal Studies, the

Northern Scientific Training Programme and a Memorial University of Newfoundland Graduate Fellowship to the author.

Thanks to my colleagues in the Cognitive and Behavioural Ecology Programme and Dr. McKay's lab for providing support, inspiration and friendship. I am especially grateful to Robb Bowman, my parents (John and Sue), family (Tyler, Gavin, and Nan), the Bowman family, and my friends for their endless love, and support even from afar.

Table of Contents

List of Tables

List of Figures

List of Abbreviations and Symbols

Abbreviation / Symbol	Definition
RIA	radioimmunoassay
EIA	enzymeimmunoassay
DMSO	dimethylsulfoxide
v/v	volume per volume
HPLC	high performance liquid chromatography
rpm	revolutions per minute
w/v	weight per volume
ng/ml	nanogram per millilitre
p	probability
ng/g	nanogram per gram
COSEWIC	Committee on the Status of Endangered Wildlife in Canada
pers. comm.	personal communication
NMFS	National Marine Fisheries Service
CITES	Committee for International Trade in Endangered Species
$\bar{x}$	mean
SD	standard deviation
n	sample size
YoNAH	Years of the North Atlantic Humpback
DFO	Department of Fisheries and Oceans
WWF	World Wildlife Fund
IWC	International Whaling Commission

List of Appendices

Chapter 1: General Introduction

1.1. Reproductive endocrinology of cetaceans

Relatively little is known about the reproductive endocrinology of cetaceans, especially the larger baleen whales. Information has been limited to studies of captive animals, or from post-mortem examinations of whales from strandings or commercial whale hunts.

1.1.1. Captive whales

Captive animals are useful in the study of sex steroids (i.e. progesterone, testosterone, estradiol) because they can be trained to aid with the collection process, and testing can occur on a regular, longitudinal basis over a long period of time (Sawyer-Steffan *et al.,* 1983; Walker *et al.*, 1988; Ozharovskaya, 1990; Atkinson *et al.*, 1999). Collection of blood serum has been the most common method for the analysis of sex steroids (Sawyer-Steffan *et al.*, 1983; Schroeder and Keller, 1989; Owen, 1990). However, other biological samples have been used, including urine samples of killer whales, *Orcinus orca* (Walker *et al.*, 1988; Robeck *et al.*, 1993); saliva, ocular and vaginal secretions from false killer whales, *Pseudorca crassidens* (Atkinson *et al.*, 1999); and milk from bottlenose dolphins, *Tursiops truncatus* (West *et al.*, 2000). Using these methods, scientists have been able to determine ovarian cycles and/or the pregnancy status of individual captive cetaceans by quantifying progesterone and estrogens in the various tissues.

1.1.2. Free-ranging whales

Limited hormonal data on free-ranging cetaceans, including the quantification of progesterone from serum and muscle, has only been obtained post-mortem from minke whales, *Balaenoptera acutostratus* (Yoshioka *et al.*, 1994) and fin whales, *Balaenoptera physalus* (Kjeld *et al.*, 1992). Recently, Mansour *et al.* (2002) established a protocol for the extraction and quantification of progesterone from blubber samples of minke whales. The extraction technique purifies the sample as oil and then separates the steroids from other lipids, with approximately 80% efficiency. Pregnant females were distinguished from non-pregnant individuals by an almost 60-fold difference in progesterone concentration (Mansour *et al.*, 2002).

1.2. The role of progesterone

Progesterone is the most distinctive hormone between males and females (Kacsoh, 2000). Progesterone is a major ovarian sex steroid that is secreted by the corpus luteum of the ovaries as well as the placenta and is necessary for normal reproductive function during pregnancy (Kacsoh, 2000). High levels of serum progesterone sustained beyond 6 - 8 weeks in captive bottlenose dolphins are indicative of pregnancy (Sawyer-Steffan *et al.*, 1983). Initially elevated serum progesterone levels that fall after a brief period (< 6 – 8 weeks) suggest ovulation without pregnancy (Sawyer-Steffan *et al.*, 1983). In the past, quantification of progesterone has led to the accurate determination of pregnancy status in individual cetaceans such as bottlenose dolphins, fin and minke whales (Sawyer-Steffan *et al.*, 1983; Ozharovskaya, 1990; Kjeld *et al.*, 1992; Yoshioka *et al.*, 1994; Mansour *et al.*, 2002). However, determination of pregnancy status by these means has never been conducted on live, free-ranging whales.

1.3. Methods for measuring hormone levels

1.3.1. Radioimmunoassay (RIA)

Typically, RIA has been used to determine hormone concentrations. The basic principle of RIA is the competitive binding of a labelled hormone to a specific antibody by the unlabeled hormone that is contained in prepared standards or unknown samples. The basic protocol involves adding standards and unknowns to assay tubes, followed by a fixed amount of radiolabeled (for steroid hormones ^{3}H or ^{125}I usually) antigen, and antibody. Incubation, separation, and counting using a gamma (or beta) counter follow (Albertson and Swanson, 1996). Antibody coated tubes are also available, such that only the standards, unknowns and the radiolabeled antigen need to be added to the tubes. A standard curve of percent bound versus hormone concentration results, and the concentrations of unknowns are then obtained from such graphs.

1.3.2. Enzyme immunoassay (EIA)

EIA, which is cost-effective and can provide on-site determination of hormone concentrations, is an alternative to RIA. Equivalent results have been obtained using both methods in the study of dolphin sex steroids (Owen, 1990). EIA has also been used to study steroid hormones of other marine mammals (Boyd, 1991; Iga *et al.*, 1996; Suzuki *et al.*, 2001). The basic principle behind this method depends on the ability to measure the activity of an associated enzyme that is conjugated to either a primary or secondary antibody. Detection is acquired through systems ranging from visual to photometric, quantifying coloured, fluorescent or luminescent products (Albertson and Swanson, 1996).

1.4. Biopsy sampling technique

Small samples of skin and blubber can be collected from free-ranging cetaceans using a biopsy sampling technique (Brown *et al.*, 1991; Palsbøll *et al.*, 1991; Lambertsen *et al.*, 1994). Winn *et al.*, (1973) were the first to obtain skin and blubber samples successfully from free-ranging whales using a biopsy dart. The biopsy technique is a relatively non-invasive method, resulting in limited behavioural disturbances (Brown *et al.*, 1991; Weinrich *et al.*, 1992; Clapham and Mattila, 1993; Gauthier and Sears, 1999). Biopsy samples usually contain 0.035 - 0.582 g of blubber with a mean of 0.25 g (Gauthier and Sears, 1999). A significant amount of information has been obtained from free-ranging whales using the biopsy technique including studies of diet (Borobia *et al.*, 1995; Todd *et al.*, 1997), sex determination (Baker *et al.*, 1991; Palsbøll *et al.*, 1992; Brown *et al.*, 1994; Bérubé and Palsbøll, 1996*a*; Bérubé and Palsbøll, 1996*b*), levels of genetic variability (Schaeff *et al.*, 1997), phylogenetic relationships (Rosenbaum *et al.*, 2000), abundance (Palsbøll *et al.*, 1997) and pollutant loading (Woodley *et al.*, 1991; Gauthier *et al.*, 1997; Weisbrod *et al.*, 2000).

1.5. Ancient bone samples

Recently, significant amounts of lipids have been recovered from ancient bones of humans and animals (Evershed *et al.*, 1995). The lipids most likely correspond to the original blood-borne lipid or the fat component of the bone marrow (Stott *et al.*, 1997). Bone marrow is primarily located in the long bones, such as the humerus, ulna and radius (Brown and Cumbaa, 1999). Long bone shavings/dust samples from right (*Eubalaena glacialis*) and bowhead (*Balaena mysticetus*) whales killed during the 16th century Basque fishery in Red Bay, Labrador were initially collected for species identification and further

molecular analyses (Brown and Cumbaa, 1999). Hormonal analyses of ancient whale bones have not been conducted previously.

1.6. Objectives

1.6.1. Storage of biopsy samples

The first objective of this research was to determine the most appropriate method for the short-term storage of blubber biopsy samples for hormonal analyses. Storage methods of biopsies by different researchers vary, with some samples simply frozen and others stored either in ethanol or dimethylsulfoxide (DMSO) saturated with NaCl (Baker *et al.*, 1994; Smith *et al.*, 1999). A study by Amos and Hoelzel (1991) reported that skin samples from pilot whales (*Globicephala melaena*) could be preserved for up to one year in a 20% solution of DMSO saturated with NaCl. To evaluate this objective, a large sample of minke whale blubber post-mortem was divided into four sets of seven individual biopsies. Biopsies were stored in either ethanol, 100% DMSO, 20% DMSO saturated with NaCl, or kept frozen without preservative for 2 months.

1.6.2. Pregnancy status of free-ranging whales

The primary objective of this research was to determine if progesterone could be quantified in blubber biopsy samples from free-ranging North Atlantic right and North Atlantic humpback whales (*Megaptera novaeangliae*) from the Gulf of Maine in order to distinguish pregnant females from non-pregnant individuals. Mansour *et al.*, (2002) suggested that reliable progesterone concentrations might be obtained from biopsy samples as small as 0.02 g. It was hypothesized that females pregnant at the time of sampling would have elevated blubber progesterone concentrations and those that were not would have low concentrations. This objective is important because, if successful, it

may provide a minimally invasive and relatively inexpensive method for determining pregnancy status of free-ranging whales. An understanding of the reproductive endocrinology of free-ranging endangered whales is important for assessing reproductive success and developing recovery strategies.

1.6.3. Pregnancy status of ancient whales

The third objective was to determine whether or not immunoassayable progesterone could be extracted and quantified from ancient whale bone samples using the Mansour *et al.* (2002) technique. Considering the amount of lipid present in bone, it was hypothesized that hormones would be present and intact, and could be quantified using this method. If successful, this method could form the basis for estimating the reproductive status of the ancient whale populations and may contribute to a better understanding of the biology of endangered right and bowhead whales.

1.7. References

Albertson, B.D., and Swanson, J.R. 1996.Assay techniques available for the toxicologist. pp.32-34. *In* John A. Thomas (ed.) Endocrine methods, Academic Press Inc., San Diego, California.i-xxi + 458pp.

Amos, B., and Hoelzel, A.R. 1991. Long-term preservation of whale skin for DNA analysis. Report of the International Whaling Commission (Special Issue 13) 99-103.

Atkinson, S., Combelles, C., Vincent, D., Nachtigall, P., Pawloski, J., and Breese, M. 1999. Monitoring of progesterone in captive female false killer whales, *Pseudorca crassidens*. General and Comparative Endocrinology 115: 323-332.

Baker, C.S., Lambertsen, R.H., Weinrich, M.T., Calambokidis, J., Early, G., and O'Brien, S.J. 1991. Molecular genetic identification of the sex of humpback whales (*Megaptera novaeangliae*). Report of the International Whaling Commission (Special Issue 13): 105-111.

Baker, C.S., Slade, R.W., Bannister, J.L., Abernethy, R.B., Weinrich, M.T., Lien, J., Urban, J., Corkeron, P., Calmabokidis, J., Vasquez, O., and Palumbi, S.R. 1994. Hierarchical structure of mitochondrial DNA gene flow among humpback whales, *Megaptera novaeangliae*, world-wide. Molecular Ecology 3:313-327.

Bérubé, M., and Palsbøll, P.J. 1996a. Identification of sex in cetaceans by multiplexing with three ZFX and ZFY specific primers. Molecular Ecology 5: 283-287.

Bérubé, M., and Palsbøll, P.J. 1996b. Erratum of identification of sex in cetaceans by multiplexing with three ZFX and ZFY specific primers. Molecular Ecology 5: 602.

Borobia, M., Gearing, P.J., Simard, Y., Gearing J.N., and Béland, P. 1995. Blubber fatty acids of finback and humpback whales from the Gulf of St. Lawrence. Marine Biology 122: 341-353.

Boyd, I.L. 1991. Changes in plasma progesterone concentrations during the annual cycle and the role of prolactin in the maintenance of lactation and luteal development in the Antarctic fur seal (*Arctocephalus gazella*). Journal of Reproduction and Fertility 91:637-647.

Brown, M.W., and Cumbaa, S.L. 1999. Field report on sampling right and bowhead whale bones from the 16th century Basque fishery, stored in Red Bay, Labrador, for molecular analyses, July 9-13, 1999. Unpublished report, National Museum of Nature, Ottawa, Ontario.

Brown, M.W., Kraus, S.D., and Gaskin, D.E. 1991. Reaction of North Atlantic right whales (*Eubalaena glacialis*) to skin biopsy sampling for genetic and pollutant analysis. Report of the International Whaling Commission (Special Issue 13): 81-89.

Brown, M.W., Kraus, S.D., Gaskin, D.E., and White, B.N. 1994. Sexual composition and analysis of reproductive females in the North Atlantic right whale, *Eubalaena glacialis*, population. Marine Mammal Science 10: 253-265.

Clapham, P.J., and Mattila, D.K. 1993. Reactions of humpback whales to skin biopsy sampling on a West Indies breeding ground. Marine Mammal Science 9: 382-391.

Evershed, R.P., Turner-Walker, G., Hedges, R.E.M., Tuross, N., and Leyden, A.1995. Preliminary results for the analysis of lipids in ancient bone. Journal of Archaeological Science 22:277-290.

Gauthier, J. M., Metcalfe, C.D., and Sears, R. 1997. Chlorinated organic contaminants in blubber biopsies from northwestern Atlantic balaenopterid whales summering in the Gulf of St Lawrence. Marine Environmental Research 44: 201-223.

Gauthier, J., and Sears, R. 1999. Behavioral response of four species of balaenopterid whales to biopsy sampling. Marine Mammal Science 15: 85-101.

Iga, K., Fukui, Y., Miyamoto, A., Ishikawa, H., and Ohsumi, S. 1996. Endocrinological observations of female minke whales (*Balaenoptera acutorostrata*). Marine Mammal Science 12: 296-301.

Kacsoh, B. 2000. Reproductive endocrinology. pp. 448-564. *In:* B. Kacsoh (ed.), Endocrine Physiology. McGraw-Hill, New York City, New York. i-xxiv + 741pp.

Kjeld, J.M., Sigurjønsson, J., and Árnason, A. 1992. Sex hormone concentrations in blood serum from the North Atlantic fin whale (*Balaenoptera physalus*). Journal of Endocrinology 134:405-413.

Lambertsen, R.H., Baker, C.S., Weinrich, M., and Modi, W.S. 1994. An improved whale biopsy system designed for multidisciplinary research. *In:* M.C. Fossi and C. Leonzio (eds.), Nondestructive biomarkers in Vertebrates. Lewis Publishers, London, p. 219-243.

Mansour, A.A.H., McKay, D.W., Lien, J., Orr, J.C., Banoub, J.H., Øien, N., and Stenson, G. 2002. Determination of pregnancy status from blubber samples in minke whales (*Balaenoptera acutorostrata*). Marine Mammal Science 18: 112-120.

Owen, S.J. 1990. Use of enzyme immunoassay to determine progesterone status in the bottlenose dolphin (*Tursiops truncatus*). Journal of Zoo Wildlife Medicine 21:70-72.

Ozharovskaya, L.V. 1990. The female reproductive cycles of Black Sea bottlenose dolphins as revealed by analysis of plasma progesterone levels. Report of the International Whaling Commission 40: 481-485.

Palsbøll, P.J., Larsen, F., and Hansen, E.S. 1991. Sampling of skin biopsies from free-ranging large cetaceans at West Greenland: development of new biopsy tips and bolt designs. Report of the International Whaling Commission (Special Issue 13): 71-79.

Palsbøll, P.J., Vader, A., Bakke, I., and El-Gewely, M.R. 1992. Determination of gender in cetaceans by the polymerase chain reaction. Canadian Journal of Zoology 70: 2166-2170.

Palsbøll, P. J., Allen, J., Bérubé, M., Clapham, P.J., Feddersen, T.P., Hammond, P.S., Hudson, R.R., Jorgensen, H., Katona, S., Larsen-Holm, A., Larsen, F., Lien, J., Mattila, D.K., Sigurjønsson, J., Sears, R., Smith, T., Sponer, R., Stevick P., and Øien, N. 1997. Genetic tagging of humpback whales. Nature 388: 767-769.

Robeck, T.R., Schneyer, A.L., McBain, J.F., Dalton, L.M., Walsh, M.T., Czekala, N.M., and Kraemer, D.C. 1993. Analysis of urinary immunoreactive steroid metabolites and gonadotropins for characterization of the estrous cycle, breeding period, and seasonal estrous activity of captive killer whales (*Orcinus orchus*) Zoo Biology 12:173-187.

Rosenbaum, H.C., Brownell, R.L., Brown, M.W., Schaeff, C., Portway, V., White, B.N., Mali, S., Pastene, L.A., Patenaude, N.J., Baker, C.S., Goto, M., Best, P.B., Clapham, P.J., Hamilton, P., Moore, M., Payne, R., Rowntree, V., Tynan, C.T., Bannister, J.L., and DeSalle, R. 2000. World-wide genetic differentiation of *Eubalaena*: questioning the number of right whale species. Molecular Ecology 9: 1793-1802.

Sawyer-Steffan, J.E., Kirby, V.L., and Gilmartin, W.G. 1983. Progesterone and estrogens in the pregnant and non-pregnant dolphin, *Tursiops truncatus*, and the effects of induced ovulation. Biology of Reproduction 28: 897-901.

Schaeff, C.M., Kraus, S.D., Brown, M.W., Perkins, J.S., Payne, R., and White, B.N. 1997. Comparison of genetic variability of North and South Atlantic right whales (Eubalaena), using DNA fingerprinting. Canadian Journal of Zoology 75:1073-1080.

Schroeder, J.P., and Keller, K.V. 1989. Seasonality of serum testosterone levels and sperm density in *Tursiops truncatus*. Journal of Experimental Zoology 249:316-321.

Smith, T.D., Allen, J., Clapham, P.J., Hammond, P.S., Katona, S., Larsen, F., Lien, J., Mattila, D., Palsbøll, P.J., Sigurjønsson, J., Stevick, P.T., and Øien, N. 1999. An ocean-basin-wide mark-recapture study of the North Atlantic humpback whale (*Megaptera novaeangliae*). Marine Mammal Science 15: 1-32.

Stott, A.W., Evershed, R.P., and Tuross, N. 1997. Compound-specific approach to the $\delta^{13}C$ analysis of cholesterol in fossil bones. Organic Geochemistry 26: 99-103.

Suzuki, T., Morgue, T., Asada, M., Miyamoto, A., Netsuke, M., Ishikawa, H., Ohsumi, S., and Fukui, Y. 2001. Plasma and pituitary concentrations of gonadotropins (FSH and LH) in minke whales (*Balaenoptera acutostratus*) during the feeding season. Theriogeneology 55:1127-1141.

Todd, S., Ostrom, P., Lien, J., and Abrajano, J. 1997. Use of biopsy samples from humpback whale (Megaptera novaeangliae) skin for stable isotope ($\delta^{13}C$) determination. Journal of Northwest Atlantic Fishery Science 22: 71-76.

Walker, L.A., Cornell, L., Dahl, K.D., Czekala, N.M., Dargen, C.M., Joseph, B., Hsueh, A.J.W., and Lasley, B.L. 1988. Urinary concentrations of ovarian steroid hormone metabolites and bioactive follicle-stimulating hormone in killer whale (*Orcinus orchus*) during ovarian cycles and pregnancy. Biology of Reproduction 39: 1013-1020.

Weinrich, M.T., Lambertson, R.H., Belt, C.R., Schilling, M.R., Iken, H.J., and Syrjala, S.E. 1992. Behavioral reactions of humpback whales *Megaptera novaeangliae* to biopsy procedures. Fishery Bulletin, U.S. 90:588-598.

Weisbrod, A.V., Shea, D., Moore, M.J., and Stegeman, J.J. 2000. Organochlorine exposure and bioaccumulation in the endangered Northwest Atlantic right whale (Eubalaena glacialis) population. Environmental Toxicology and Chemistry 19: 654-666.

West, K.L., Atkinson, S., Carmichael, M.J., Sweeney, J.C., Krames, B., and Krames J. 2000. Concentrations of progesterone in milk from bottlenose dolphins during different reproductive states. General and Comparative Endocrinology 117:218-224.

Winn, H.E., Bischoff, W.L., and Taruski, A.G. 1973. Cytological sexing of Cetacea. Marine Biology 23:343-346.

Woodley, T.H., Brown, M.W., Kraus, S.D., Gaskin, D.E. 1991. Organochlorine levels in North Atlantic right whale (*Eubalaena glacialis*) blubber. Archives of Environmental Contamination and Toxicology 21:141-145.

Yoshioka, M., Okumura, T., and Aida, K. 1994. A proposed technique for quantifying muscle progesterone content in minke whales (*Balaenoptera acutorostrata*). Canadian Journal of Zoology 72: 368-370.

Co-authorship statement

Elements of my Master's project were in place prior to my arrival at Memorial University. The initial hormone work began in 1997 and I continued where the "team" (Drs. Mansour, McKay and Lien) left off. I conducted all lab work, statistical analyses and wrote the thesis with the guidance and financial support of the aforementioned committee members. Any publications that may result from my thesis work will have these members as co-authors. Material presented in Chapter 3 will be submitted to the Journal of Cetacean Research and Management (Mackenzie L. Sheridan, Atef A.H. Mansour, Jooke Robbins, Martine Bérubé, Donald W. McKay, and Jon Lien). Material in Chapter 4 will be submitted to Aquatic Mammals (Mackenzie L. Sheridan, Atef A.H. Mansour, Moira W. Brown, Donald W. McKay, and Jon Lien). As well, this research was presented at four conferences in 2003 (F.A. Aldrich Interdisciplinary Lecture and Conference, 7th Student Conference on Northern Studies, North Atlantic Right Whale Consortium Annual Meeting, and the 15th Biennial Conference on the Biology of Marine Mammals).

Chapter 2: Short-term preservation of whale blubber for hormonal analyses

2. Introduction

2.1. Current methods of preservation

At present, no work has been published on the preservation of whale blubber for hormonal analyses; however, a study on the preservation of whale skin for DNA analyses has been. Amos and Hoelzel (1991) examined pilot whale (*Globicephala melaena*) skin under different storage conditions to determine the best method of preservation for DNA-based genetic analysis. Results showed that samples could be preserved for up to one year in a 20% solution of dimethylsulfoxide (DMSO) in saturated NaCl. The function of DMSO is to increase cell permeability, which allows the preservative (NaCl) to rapidly enter the sample (Amos and Hoelzel, 1991). As well, salt denatures the majority of proteins at high concentrations (Amos and Hoelzel, 1991). Several studies of cetacean biology since have used samples preserved this way (i.e. Clapham and Mattila, 1993; Baker *et al.*, 1994; Todd *et al.*, 1997, Smith *et al.*, 1999; Waldic *et al.*, 1999).

Ethanol has also been used as a method for storing biopsy samples. Baker *et al.* (1994) used samples stored in 70% ethanol for the analysis of mitochondrial DNA. Other researchers simply keep samples frozen. Blubber samples used for assessing contaminant levels (Woodley *et al.*, 1991;Gauthier *et al.*, 1997) and those for DNA analyses (Brown *et al.*, 1991; Schaeff *et al.*, 1993) were kept frozen.

2.2. Statement of Purpose

With the collection of biopsy samples by several researchers it is important to establish a standard method for storing the blubber portion of biopsy samples for hormonal analyses. The purpose of this study was to determine the most appropriate

method (100% DMSO, 20% DMSO in saturated NaCl, ethanol or frozen without preservative) for short-term storage of blubber biopsy samples for hormonal analyses.

2.3. Methods

2.3.1. Blubber samples

A blubber sample from a known pregnant minke whale was used for this study because of its known high concentration of progesterone (Mansour *et al.*, 2002). A biopsy punch (8 mm in diameter) was used to collect 28 individual biopsies ranging in size from 0.25 - 0.46 g. Dichloroethane was used to clean the biopsy punch between samples. Each sample was placed in a separate 5 ml glass vial. Seven vials were filled with 100% DMSO, seven vials with 20% DMSO saturated with NaCl, seven vials with ethanol, and seven vials were kept frozen without preservative. All vials were stored at – 40 °C for two months.

2.3.2. Hormone extraction

Lab analyses were performed in the Faculty of Medicine, Memorial University of Newfoundland and experiments were approved by the University Animal Care Committee. All glassware was silanized with 1% v/v dichlorodimethylsilane $[(CH_3)_2Si(OAc)_2]$ (Kelly's Reagent) in toluene (HPLC grade), followed by rinsing with methanol twice. Tubes were then air-dried.

Hormone extractions were performed according to Mansour *et al.*, (2002) with modifications as follows. Frozen biopsy samples were immersed in liquid nitrogen and then ground to a powder with mortar and pestle. The powder was weighed and transferred to a 50 ml glass tube; 5 ml of ethanol (HPLC grade): acetone (HPLC grade) (4:1, v/v) was

added, vortexed for 5 min and then centrifuged (3,000 rpm, 22 °C, 20 min). The supernatant was collected in a 15 ml disposable glass centrifuge tube and the pellet re-suspended in 5 ml of ethanol: acetone, vortexed, and re-centrifuged. Both supernatants were combined and evaporated in a warm water bath (37 °C) under a pressurized flow of commercial grade nitrogen until only an oil extract remained.

To the oil extract, 5 ml of diethyl ether (PRA grade) was added, vortexed for 5 min and centrifuged (3,000 rpm, 22 °C, 15 min). To the original pellet, 3 ml of diethyl ether was added, vortexed for 5 min and centrifuged (3,000 rpm, 22 °C, 15 min). The supernatant was aspirated and the previous step repeated with 2 ml of diethyl ether. The three portions (10 ml total) of diethyl ether supernatant were combined in a 15 ml glass tube and evaporated under commercial grade nitrogen until only oil remained.

To separate the steroids from fats, 3 ml of acetonitrile (Optima grade) were added to the oily residue and mixed thoroughly 5 min using a vortex mixer. Optima grade hexane (3 ml) was then added and mixed 5 min. Centrifugation (3,000 rpm, 22 °C, 15 min) facilitated the solvent separation (hexane on top, acetonitrile on bottom). Steroids more readily dissolved in the acetonitrile, and fats in the hexane. Each solvent layer was collected and re-extracted with the other solvent twice. Finally, the hexane fraction was discarded and the acetonitrile portion was evaporated in a warm water bath (37 °C) under industrial grade nitrogen. The residue was placed under industrial grade argon and frozen at – 20 °C until the radioimmunoassay (RIA).

The ethanol liquid portion was transferred to a 15 ml centrifuge tube. The initial vials were rinsed with 5 ml of ethanol: acetone (4:1, v/v), which was combined with the

first step of the extraction. The extraction proceeded as usual.

2.3.3. RIA

All samples were dissolved (1:20, w/v) in a phosphate-buffered saline solution, pH 7.4. The samples were mixed thoroughly using a vortex mixer and were sonicated for 30 min in a Bransonic 3510 ultrasonic cleaner. Samples were measured in duplicate using a progesterone coated tube RIA kit (Active progesterone®, DSL-3900, Diagnostic Systems Laboratories, Inc., Webster, TX), with a standard curve of 0.3 - 60 ng/ml and a theoretical sensitivity of 0.12 ng/ml.

2.3.4. Data Analysis

Statistical analyses were performed using SPSS statistical package (version 11.5, SPSS Inc., Chicago, Illinois). A one-way analysis of variance (ANOVA) and multiple comparisons using Scheffé's test were used to compare the differences in progesterone concentrations among blubber biopsy samples stored in 100% DMSO, 20% DMSO saturated with NaCl, ethanol, and frozen without preservative.

2.4. Results

Blubber progesterone concentrations for samples in three of the four storage conditions (100% DMSO, 20% DMSO in saturated NaCl, and frozen) had progesterone concentrations that were not significantly different ($p > 0.05$). Blubber samples stored in ethanol yielded progesterone concentrations that were significantly lower than those of the other three conditions ($p < 0.05$) (Figure 2.1). However, there was no significant difference between progesterone concentrations in the three storage conditions and the combined concentration of the blubber samples in ethanol and the ethanol liquid fractions ($p > 0.05$) (Figure 2.2).

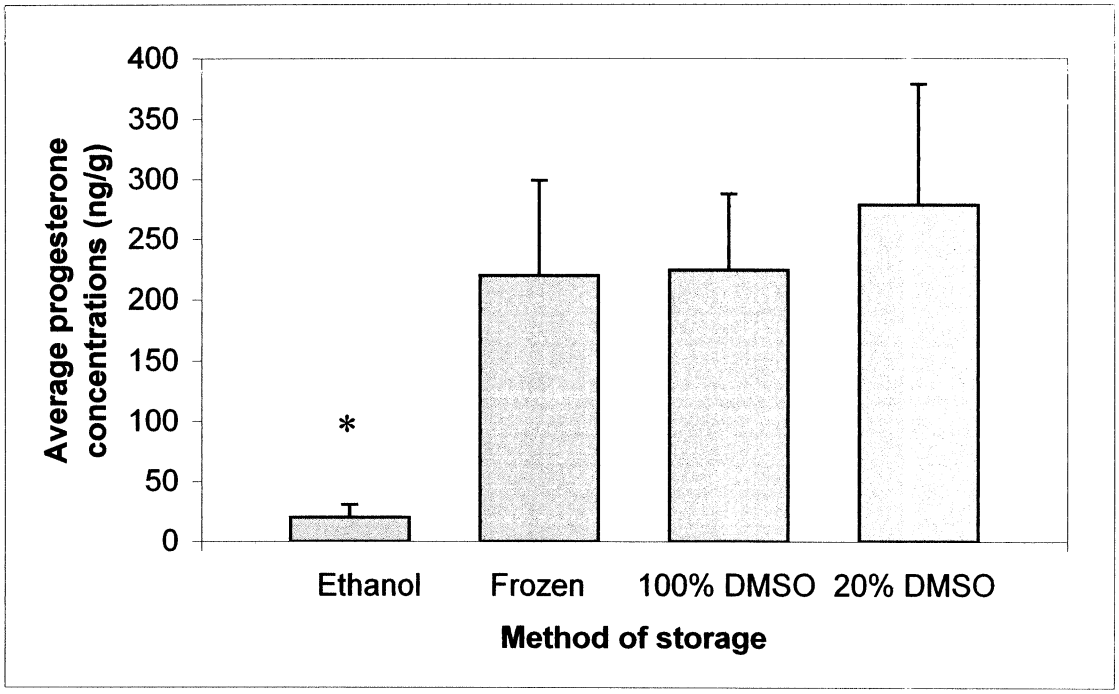

Figure 2.1. Average progesterone concentrations for biopsy samples stored under different conditions (* $p < 0.05$). Error bars represent standard deviation.

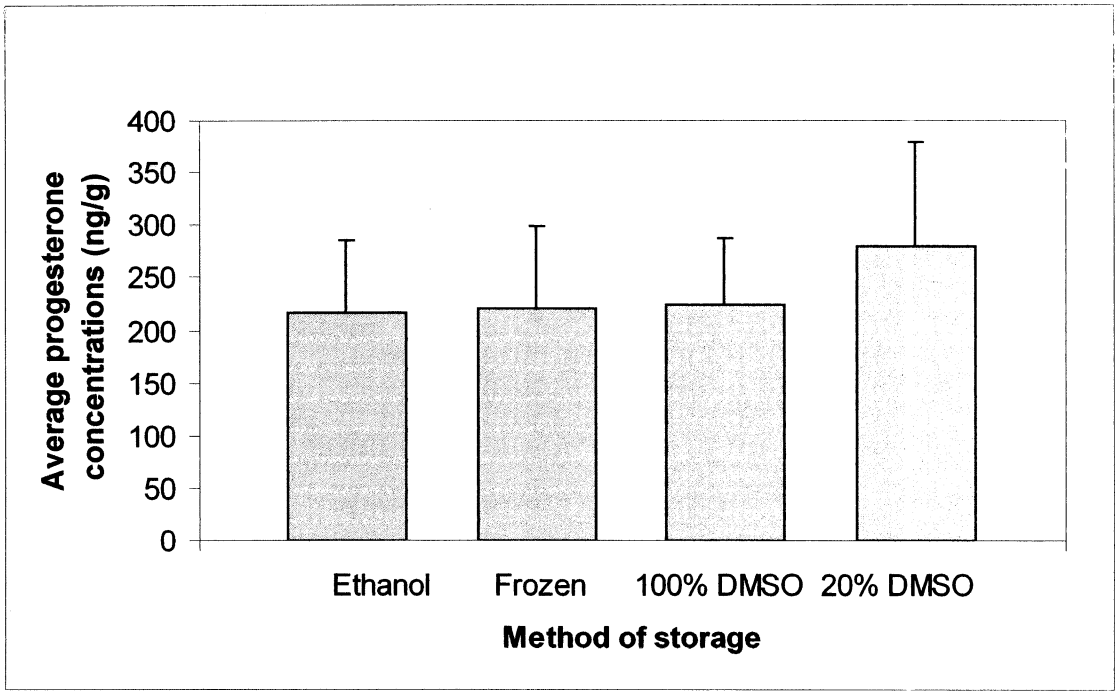

Figure 2.2. Average progesterone concentrations for biopsy samples stored under different conditions, after two ethanol portions combined ($p > 0.05$). Error bars represent standard deviation.

2.5. Discussion

DMSO increases the permeability of cells, allowing preservatives to rapidly enter the sample (Amos and Hoelzel, 1991). Biopsy samples stored in DMSO (100% and 20% saturated with NaCl) for two months have the same progesterone concentrations as the samples that were kept frozen without preservative. Blubber biopsy samples stored in ethanol for two months lost approximately 90% of the progesterone as compared to samples stored in the other three conditions. It was hypothesized that the progesterone seeped into the ethanol liquid fraction. To test this, the ethanol liquid fraction was extracted (as described on p. 15), assayed, and the progesterone concentrations combined with those of the blubber samples in ethanol. Once the progesterone concentrations from the ethanol liquid portion were added to those of the blubber samples in ethanol, progesterone concentrations were similar to those measured in the blubber samples stored under the other conditions.

Amos and Hoelzel (1991), studied pilot whale skin at 1 month, 3 months and 1 year to determine the long-term effectiveness of DMSO to preserve DNA. A similar long-term study should be undertaken with blubber, looking at the preservation of hormones over time under similar conditions. This is especially important if archived blubber biopsy samples are to be used for studies of hormone levels (Smith *et al.*, 1999). A long-term study was not feasible for us due to the time constraints of the Master's program.

Recent advances in the study of cetacean reproductive endocrinology (Mansour *et al.*, 2002), combined with the biopsy sampling technique may provide information regarding the reproductive health of individual whales and on a larger scale, may allow

for reproductive rates of populations to be estimated. In conclusion, blubber samples for hormonal analyses can be stored frozen for up to two months in solutions of DMSO (100% or 20% saturated with NaCl), and ethanol, with hormone concentrations remaining intact. This information will aid in the proper collection, transportation, and storage of blubber biopsy samples for hormone research.

2.6. References

Amos, B., and Hoelzel, A.R. 1991. Long-term preservation of whale skin for DNA analysis. Report of the International Whaling Commission (Special Issue 13) 99-103.

Baker, C.S., Slade, R.W., Bannister, J.L., Abernethy, R.B., Weinrich, M.T., Lien, J., Urban, J., Corkeron, P., Calmabokidis, J., Vasquez, O., and Palumbi, S.R. 1994. Hierarchical structure of mitochondrial DNA gene flow among humpback whales, *Megaptera novaeangliae*, world-wide. Molecular Ecology 3:313-327.

Brown, M.W., Kraus, S.D., and Gaskin, D.E. 1991. Reaction of North Atlantic right whales (*Eubalaena glacialis*) to skin biopsy sampling for genetic and pollutant analysis. Report of the International Whaling Commission (Special Issue 13): 81-89.

Clapham, P.J., and Mattila, D.K. 1993. Reactions of humpback whales to skin biopsy sampling on a West Indies breeding ground. Marine Mammal Science 9(4): 382-391.

Gauthier, J. M., Metcalfe, C.D., and Sears, R. 1997. Chlorinated organic contaminants in blubber biopsies from Northwestern Atlantic balaenopterid whales summering in the Gulf of St Lawrence. Marine Environmental Research 44: 201-223.

Mansour, A.A.H., McKay, D.W., Lien, J., Orr, J.C., Banoub, J.H., Øien, N., Stenson, G. 2002. Determination of pregnancy status from blubber samples in minke whales (*Balaenoptera acutorostrata*). Marine Mammal Science 18: 112-120.

Schaeff, C.M., Kraus, S.D., Brown, M.W., and White, B.N. 1993. Assessment of the population structure of western North Atlantic right whales (*Eubalaena glacialis*) based on sighting and mtDNA data. Canadian Journal of Zoology 71:339-345.

Smith, T.D., Allen, J., Clapham, P.J., Hammond, P.S., Katona, S., Larsen, F., Lien, J., Mattila, D., Palsbøll, P.J., Sigurjønsson, J., Stevick, P.T., and Øien, N. 1999. An ocean-basin-wide mark-recapture study of the North Atlantic humpback whale (*Megaptera novaeangliae*). Marine Mammal Science 15: 1-32.

Todd, S., Ostrom, P., Lien, J., and Abrajano, J. 1997. Use of biopsy samples from humpback whale (Megaptera novaeangliae) skin for stable isotope ($\delta^{13}C$) determination. Journal of Northwest Atlantic Fishery Service 22: 71-76.

Waldick, R.C., Brown, M.W., and White, B.N. 1999. Characterization and isolation of microsatellite loci from the endangered North Atlantic right whale. Molecular Ecology 8: 1763-1765.

Woodley, T.H., Brown, M.W., Kraus, S.D., Gaskin, D.E. 1991. Organochlorine levels in North Atlantic right whale (*Eubalaena glacialis*) blubber. Archives of Environmental Contamination and Toxicology 21:141-145.

Chapter 3: Determining pregnancy status of free-ranging North Atlantic humpback whales (*Megaptera novaeangliae*) from the Gulf of Maine by quantifying progesterone in blubber biopsies

3. Introduction

3.1. The humpback whale

3.1.1. Current population status

Protected from commercial whaling for almost half a century, the North Atlantic humpback whale (*Megaptera novaeangliae*) has shown significant signs of recovery (Smith *et al.*, 1999). Recently the western North Atlantic population was delisted from a special concern to "not at risk" by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC, 2003). The western North Atlantic population has been estimated at about 11,000 individuals (Smith *et al.*, 1999).

3.1.2 Migration

Humpback whales are highly migratory, traveling on average 10,000 km return each year (Baker *et al.*, 1994). The majority of North Atlantic humpback whales migrate from the West Indies, where they spend the winter breeding and calving, to high-latitude feeding areas during the summer and fall (Winn *et al.*, 1975; Martin *et al.*, 1984; Katona and Beard, 1990). Katona and Beard (1990) and Smith *et al.* (1999) suggest that there are at least five relatively distinct high-latitude feeding aggregations, specifically the Iceland-Denmark Strait; western Greenland; Newfoundland (including the Labrador Coast); Gulf of St. Lawrence; and the Gulf of Maine-Scotian Shelf. Individuals from these aggregations maintain strong philopatry, returning to the same area year after year (Martin *et al.*, 1984; Clapham and Mayo, 1987*a*; Katona and Beard, 1990; Clapham *et al.*,

1993). Members of these different feeding areas, however, come together on common wintering grounds to breed and calve (Mattila *et al.*, 1989; Katona and Beard, 1990).

3.1.3 Gulf of Maine feeding area

The Gulf of Maine feeding area, from Massachusetts Bay to southern Nova Scotia, has been studied intensively since 1979 using photoidentification of individual animals, which has facilitated long-term studies investigating such things as the reproductive biology of humpback whales (Clapham and Mayo, 1987*a*; 1987*b*; 1990; Clapham, 1992). Many individuals from this sub-population have been observed from their year of birth through successive seasons, which allows for detailed resighting histories of individuals (Clapham and Mayo, 1987*a*; 1987*b*; 1990). The Gulf of Maine stock of humpback whales has been estimated at approximately 350-450 individuals (Katona and Beard, 1990). More recent estimates of 500-900 have been reported (Clapham *et al.*, 2003). However, research is currently being conducted to strengthen these estimates (Jooke Robbins, pers. comm.).

3.1.4. Reproduction

Clapham and Mayo (1987*a*) determined several reproductive variables during an extensive study from 1979-1985 in the Gulf of Maine. Mean crude birth rate (the total number of calves observed in a given year divided by the total number of individuals observed in the same year) was observed to be 0.075, with calving intervals of 2.4 years and a mean of 0.43 calves per mature female per year born. Clapham and Mayo (1990) continued the previous study to include data through to 1987 and observed a relatively stable mean crude birth rate of 0.079 and mean calving interval of 2.35 years. Although a two- or three-year breeding cycle is common, annual reproduction has been observed

(Straley *et al.*, 1994). In Alaskan waters, five North Pacific female humpback whales were observed to have successfully produced calves during annual cycles, with no neonatal mortality (Straley *et al.*, 1994).

3.2. Statement of Purpose

In this study, progesterone concentrations in blubber biopsy samples were used to develop a method to predict the pregnancy status of free-ranging humpback whales from the Gulf of Maine. Ovulation in humpbacks occurs primarily during winter months (Chittleborough, 1954; 1965). Thus, elevated progesterone concentrations found in tissues sampled during the feeding season are most likely to be indicative of pregnancy. As the Gulf of Maine feeding population has been intensively studied using both photo-identification and biopsy techniques since the mid-1970s, maturational class, sex and calving data are known for many individuals. These data provided a unique basis for evaluating the potential use of progesterone-based pregnancy tests on free-ranging baleen whales.

3.3. Methods

3.3.1. Biopsy sampling

Biopsy samples were collected by researchers at the Centre for Coastal Studies, Provincetown, Massachusetts, in the Gulf of Maine from mature female humpback whales during July and August 2002 and from male and immature humpback whales from April to November 2002 (National Marine Fisheries Service (NMFS) permit number 633-1483-01). Samples were obtained in the following way (Jooke Robbins, pers. comm.). Once an individual was selected for the procedure, the vessel moved into position abeam and approximately 50 ft (15.24 m) from the whale. In most instances,

sample collection was performed when the animal arched to initiate its terminal dive. An experienced 'darter' collected the sample using a crossbow (150-lbs. draw) and specially designed CETA-DART bolts and tips (Palsbøll *et al.,* 1991). The bolt was a standard carbon fibre shaft, equipped with a pressed foam stop collar/float. CETA-DART cylindrical sampling tips are constructed from stainless steel and designed to collect a 2.5 cm x 9 cm sample. Samples were collected from the upper flank of the animal, generally below or posterior to the dorsal fin. Once the dart was fired, the animal was allowed to move out of the area before the vessel moved in to retrieve the floating bolt. After sampling, tips were thoroughly cleaned using a soap solution, followed by either flaming or an extended soak in a 10% hydrogen peroxide solution to remove any residual organic materials. Finally, tips were rinsed in 70% ethyl alcohol.

3.3.2. Sample shipment

A total of 34 blubber samples were shipped frozen in liquid nitrogen vapour to Memorial University of Newfoundland (Committee for International Trade in Endangered Species (CITES) permits #CA02CWIM0109 and #02US020950/9). Upon arrival at the lab, samples were stored at – 40 °C until extraction. All blubber samples were weighed prior to lipid extraction; they ranged from 0.08 - 0.60g ($\bar{x}$ = 0.22 g, SD = 0.13). Those samples weighing less than 0.1 g were too small to be used and were not processed (n = 2).

3.3.3. Hormone Extraction

Hormone extractions were performed according to those methods presented in Chapter 2 of this thesis. All lab tests were conducted 'blinded' with respect to field data that was provided just prior to statistical analyses. Solvent blanks were obtained by

completing the extraction protocol using only the extractions solvents with no sample added.

3.3.4. RIA

Samples were dissolved in the zero standard (standard 'A' of 0 ng/ml, DSL-3901, Diagnostic Systems Laboratories, Inc., Webster, Texas). Samples weighing $\geq$ 0.20 g after grinding were redissolved 2:1 (w/v), whereas samples weighing < 0.20 g were redissolved 1:1 (w/v). The samples were mixed thoroughly using a vortex mixer and sonicated for 30 min in a Bransonic 3510 ultrasonic cleaner. Prior to the assay, samples were sonicated for 10 min and mixed immediately before transferring to the assay tube. Duplicates of each sample were assayed using a progesterone double antibody RIA kit (DSL-3400, Diagnostic Systems Laboratories, Inc.), with a standard curve ranging from 0.3-70.0 ng/ml and a theoretical sensitivity of 0.10 ng/ml. All samples were run in a single assay, with an intra-assay coefficient of variation of 10.8% determined by ten replicate measurements of sample # CCS 2002-70. Results of the solvent blanks were below the sensitivity of the assay.

3.3.5. Assay validations

In order to validate the assay for humpback whale blubber, linearity and parallelism in the RIA was tested, remaining humpback blubber biopsy extracts were pooled and known amounts of progesterone were added. Using the least-squares method ($y = 0.15x + 1.42$, $R^2 = 0.989$) a regression line was obtained for the observed versus expected progesterone concentrations of the pooled samples (Figure 3.1). While linearity was obtained, parallelism was not. Thus, the assay was recovering less progesterone than

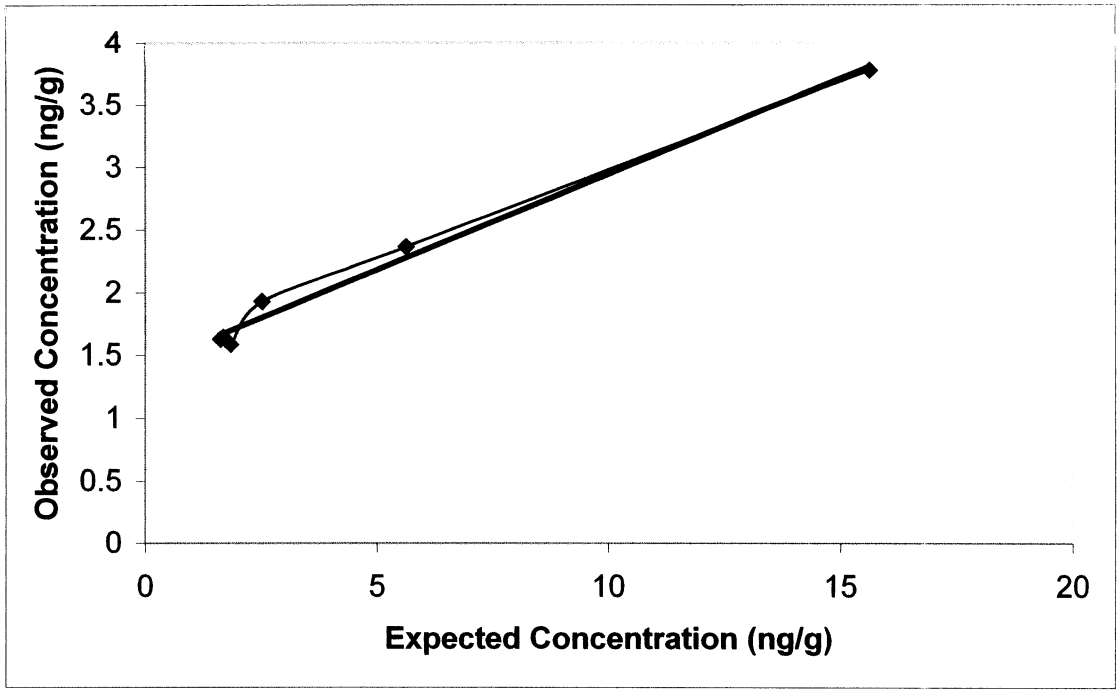

Figure 3.1. Linearity of addition of pooled humpback blubber extracts using RIA, obtained by the least-squares method.

was expected. A second test involved decreasing the amount of known progesterone added while increasing the amount of humpback blubber extract (i.e. first, 4/5 of known progesterone was added to 1/5 of blubber extract, then 3/5 progesterone and 2/5 blubber extract, and so on until 1/5 progesterone was added to 4/5 extract). Once again parallelism was not obtained. In order to rule out human error as the cause for the lack of assay parallelism a third test was performed. Instead of using humpback blubber extract, zero standard (standard 'A' of 0 ng/ml, DSL-3901) was used and known amounts of progesterone were added. In this case, observed progesterone concentrations were comparable to expected progesterone concentrations. Thus, it seems likely that there may be an unknown substance in the blubber that is interfering with the assay. As a result, the concentrations reported in this section do not represent the actual mass of progesterone per gram of blubber. However, as the assay was linear, the values derived from the assay can be used to determine greater or lesser amounts of progesterone per sample.

3.3.6. Individual data used to evaluate results

Once lab assay results were completed they were evaluated in light of known gender, maturational class and subsequent calf production. The sex of each individual was determined by molecular analysis of a tissue sample (Palsbøll *et al.*, 1992; Bérubé and Palsbøll, 1996 *a,b*). A maturational class was also assigned based on the length of an individual's sighting history or documented calving history (Jooke Robbins, pers. comm.). Females were considered to be mature if they were known to have produced at least one calf. The earliest documented age at sexual maturity in this population is 4 years (Clapham, 1992), a result that corresponds with findings for both male and female humpback whales in the Southern Hemisphere whaling literature (Chittleborough, 1965).

Females of at least four years, but which had not yet been observed with a calf, were considered to be of unknown maturational class. Immature whales were those first catalogued as calves and known to be less than fours years old in the year of sampling. Samples from mature males and juveniles of both sexes were considered 'negative controls' in this experiment, as those individuals could not have been pregnant when sampled.

The humpback whale gestation period is approximately 12 months (Chittleborough, 1958). Gulf of Maine calves remain in association with their mothers during their first feeding season (Clapham and Mayo, 1987*a*; Baraff and Weinrich, 1993). Thus, progesterone results of females could be validated based on the presence or absence of a calf the following year. A calf was identified in the field by its close, consistent association with a mature female at least twice its size (Jooke Robbins, pers. comm.). Calves exhibited stereotypical positioning and behaviours that are not observed in older animals; photo-identification confirmed that they were new to the catalogued population. When a mature female was resighted without a calf it was not possible to determine whether she had not been pregnant or had lost her calf prior to being observed.

3.3.7. Data Analysis

Statistical analysis was performed using SPSS statistical package (version 11.5, SPSS Inc., Chicago, Illinois). Kruskal-Wallis' tests followed by multiple comparisons using Mann-Whitney's test were used to analyze the differences among progesterone concentrations in blubber biopsy samples from mature females, lactating females, females of uncertain maturational class, mature males and immature females.

3.4. Results

3.4.1. Blubber progesterone concentrations

Biopsy samples from 32 individual humpback whales were identified as being from mature, non-lactating females ($n = 14$), lactating females ($n = 5$), females of uncertain maturational class ($n = 2$), and 11 negative control samples (4 mature males and 7 immature animals of either sex). One sample, from an immature male contained a progesterone concentration that fell below the sensitivity of the assay and was excluded from further analyses. Due to the lack of assay parallelism, blubber progesterone concentrations were normalized using the average concentration found in known males as a base value arbitrarily set at 1. Relative blubber progesterone concentrations for the remaining 31 whales are listed in Appendix A. Mature, non-lactating females had significantly higher relative blubber progesterone concentrations than all other groups, including lactating females, females of uncertain maturational class, and negative control groups ($p < 0.05$). There were no statistically significant differences in progesterone concentration between the other reproductive classes ($p > 0.05$).

3.4.2. Pregnancy test confirmations

Thirteen of 21 females (62%) in this study were resighted the following year (2003) (Figure 3.2). Three had elevated relative blubber progesterone concentrations ($\geq$ 12 ng/g), and the following year returned with calves. One female with a moderate blubber progesterone concentration (3 - 8 ng/g) also returned the following year with a calf. Seven mature females (two with elevated blubber progesterone concentrations and five with moderate blubber progesterone concentrations) returned without calves. Two females (one mature, non-lactating and one of unknown maturational class) with blubber

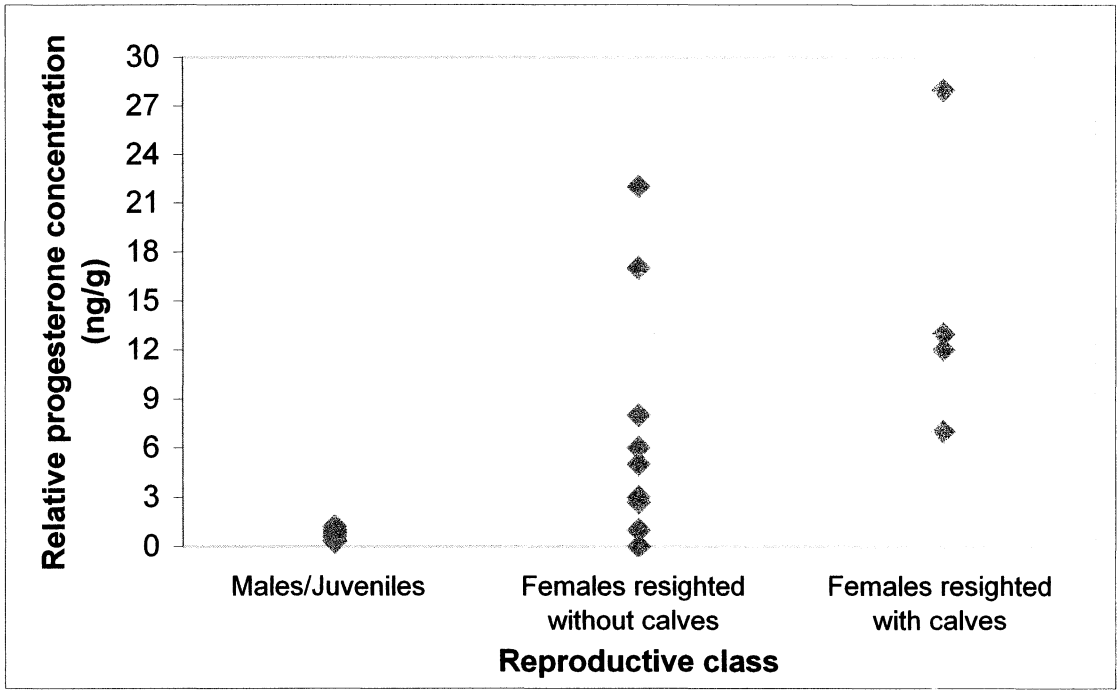

Figure 3.2. Progesterone concentrations for female humpback whales according to resighting class (n = 13) compared to progesterone concentrations for males and juveniles (n =10).

progesterone concentrations comparable to males and juveniles (0 - 1 ng/g) returned without calves.

3.5. Discussion

3.5.1. Determining pregnancy status

The results demonstrate that the combination of dart biopsy sampling and the progesterone extraction (Mansour *et al.*, 2002) provides a minimally invasive and relatively inexpensive method for determining pregnancy in free-ranging whales. Serum, muscle, and blubber progesterone are reliable indices of pregnancy in the bottlenose dolphin, *Tursiops truncatus* (Sawyer-Steffan *et al.*, 1983), fin whale, *B. physalus* (Kjeld *et al.*, 1992) and minke whale (Yoshioka *et al.*, 1994; Mansour *et al.*, 2002). Pregnant individuals produced higher levels of blubber progesterone than non-pregnant females and males (Mansour *et al.*, 2002). Higher levels of progesterone are also detectable in follicular fluid of pregnant minke whales versus immature females (Iga *et al.*, 1996).

Assay parallelism was not achieved in this study and therefore actual levels of blubber progesterone content are likely somewhat higher than reported. Despite this, elevated blubber progesterone concentrations were found in animals that were later resighted with calves. Immature whales, males and some mature females that returned without calves had relatively lower progesterone levels. Low progesterone concentrations among lactating females and females of unknown maturational class were not unexpected because in the Gulf of Maine, only 4% of lactating females return with a calf the following year (Clapham and Mayo, 1990) and some females of unknown maturational class were likely immature.

At least four possibilities may explain why some mature females with elevated or moderate blubber progesterone concentrations returned without calves. First, females may have had successful pregnancies but with subsequent neonatal mortality (Gabriele *et al.*, 2001). Second, females may have spontaneously aborted after the time of sampling. Third, females may have experienced pseudopregnancy, a phenomenon that has been reported in the bottlenose dolphin (West *et al.*, 2000), false killer whale, *Pseudorca crassidens* (Atkinson *et al.*, 1999) and several species of seals (Renouf *et al.*, 1994; Atkinson, 1997). A fourth, more parsimonious reason, is that this technique does not work for humpback whales.

3.5.2. Future research

While the resighting histories of individual Gulf of Maine humpback whales have allowed for long-term studies of reproduction (e.g. Clapham and Mayo, 1987*a*; 1990; Clapham, 1992; Barlow and Clapham, 1997), it is unknown how many females terminate pregnancies due to poor food/body resources, entanglements in fishing gear, or other factors. Monitoring individuals that are believed to be pregnant throughout the feeding season may provide information on rates of spontaneous abortion.

On a large scale, this method may be used to provide crucial information such as pregnancy rates and rates of spontaneous abortion within cetacean populations, which will help guide the development of management and recovery strategies. For example, the large archive of biopsy samples from the Years of the North Atlantic Humpback (YoNAH) study could provide the basis for such work (Smith *et al.*, 1999).

3.6. References

Atkinson, S 1997. Reproductive biology of seals. Reviews of Reproduction 2: 175-194.

Atkinson, S., Combelles, C., Vincent, D., Nachtigall, P., Pawloski, J., and Breese. M. 1999. Monitoring of progesterone in captive female false killer whales, *Pseudorca crassidens*. General and Comparative Endocrinology 115: 323-332.

Baker, C.S., Slade, R.W., Bannister, J.L., Abernethy, R.B., Weinrich, M.T., Lien, J., Urban, J., Corkeron, P., Calmabokidis, J., Vasquez, O., and Palumbi, S.R. 1994. Hierarchical structure of mitochondrial DNA gene flow among humpback whales, *Megaptera novaeangliae*, world-wide. Molecular Ecology 3:313-327.

Baraff, L., and Weinrich, M.T. 1993. Separation of humpback whale mothers and calves on a feeding ground in early autumn. Marine Mammal Science 9: 431-434.

Barlow, J., and Clapham, P.J. 1997. A new birth-interval approach to estimating demographic parameters of humpback whales. Ecology 78: 535-546.

Bérubé, M., and Palsbøll, P.J. 1996a. Identification of sex in cetaceans by multiplexing with three ZFX and ZFY specific primers. Molecular Ecology 5: 283-287.

Bérubé, M., and Palsbøll, P.J. 1996b. Erratum of identification of sex in cetaceans by multiplexing with three ZFX and ZFY specific primers. Molecular Ecology 5: 602.

Chittleborough, R.G. 1954. Studies on the ovaries of the humpback whale, *Megaptera nodosa* (Bonnaterre), on the Western Australian coast. Australian Journal of Marine and Freshwater Research 5: 35-63.

Chittleborough, R.G. 1958. The breeding cycle of the female humpback whale, *Megaptera nodosa* (Bonnaterre). Australian Journal of Marine and Freshwater Research 9:1-18.

Chittleborough, R. G. 1965. Dynamics of two populations of the humpback whale, *Megaptera novaeangliae* (Borowski). Australian Journal of Marine and Freshwater Research 16: 33-128.

Clapham, P.J. 1992. Age at attainment of sexual maturity in humpback whales, *Megaptera novaeangliae*. Canadian Journal of Zoology 70: 1470-1472.

Clapham, P.J., and Mayo, C.A. 1987*a*. Reproduction and recruitment of individually identified humpback whales, *Megaptera novaeangliae*, observed in Massachusetts Bay, 1979-1985. Canadian Journal of Zoology 65: 2853-2863.

Clapham, P.J., and Mayo, C.A. 1987*b*. The attainment of sexual maturity in two female humpback whales. Marine Mammal Science 3(3):279-283.

Clapham, P.J., and Mayo, C.A. 1990. Reproduction of humpback whales (*Megaptera novaeangliae*) observed in the Gulf of Maine. Report of the International Whaling Commission (Special Issue 8): 171-175.

Clapham, P.J., Baraff, L.S., Carlson, C.A., Christian, M.A., Mattila, D.K., Mayo, C.A., Murphy, M.A., and Pittman, S. 1993. Seasonal occurrence and annual return of humpback whales, *Megaptera novaeangliae*, in the southern Gulf of Maine. Canadian Journal of Zoology 71: 440-443.

Clapham, P., Barlow, J., Bessinger, M., Cole, T., Mattila, D., Pace, R., Palka, D., Robbins, J., and Seton, R. 2003. Abundance and demographic parameters of humpback whales from the Gulf of Maine, and stock definition relative to the Scotian Shelf. Journal of Cetacean Research and Management 5: 13-22.

Committee on the Status of Endangered Wildlife in Canada (COSEWIC). 2003. Species Assessment, May 2003.

Gabriele, C.M., Straley, J.M., Mizroch, S.A., Baker, C.S., Craig, A.S., Herman, L.M., Glockner-Ferrari, D., Ferrari, M.J., Cerchio, S., von Ziegesar, O., Darling, J., McSweeney, D., Quinn II, T.J., and Jacobsen, J.K. 2001. Estimating the mortality rate of humpback whale calves in the central North Pacific Ocean. Canadian Journal of Zoology 79: 589-600.

Iga, K., Fukui, Y., Miyamoto, A., Ishikawa, H., and Ohsumi, S. 1996. Endocrinological observations of female minke whales (*Balaenoptera acutorostrata*). Marine Mammal Science 12: 296-301.

Katona, S.D., and Beard, J.A. 1990. Population size, migrations and feeding aggregations of the humpback whale (*Megaptera novaeangliae*) in the western North Atlantic Ocean. Report of the International Whaling Commission (Special Issue 8): 295-305.

Kjeld, J.M., Sigurjønsson, J., and Árnason, A. 1992. Sex hormone concentrations in blood serum from the North Atlantic fin whale (*Balaenoptera physalus*). Journal of Endocrinology 134: 405-413.

Mansour, A.A.H., McKay, D.W., Lien, J., Orr, J.C., Banoub, J.H., Øien, N., and Stenson, G. 2002. Determination of pregnancy status from blubber samples in minke whales (*Balaenoptera acutorostrata*). Marine Mammal Science 18: 112-120.

Martin, A.R., Katona, S.K., Mattila, D., Hembree, D., and Waters, T.D. 1984. Migration of humpback whales between the Caribbean and Iceland. Journal of Mammalogy 65(2): 330-333.

Mattila, D.K., Clapham, P.J., Katona, S. K., and Stone, G.S. 1989. Population composition of humpback whales, Megaptera novaeangliae, on Silver Bank, 1984. Canadian Journal of Zoology 67: 281-285.

Palsbøll, P.J., Larsen, F., and Hansen, E.S. 1991. Sampling of skin biopsies from free-ranging large cetaceans at West Greenland: development of new biopsy tips and bolt designs. Report of the International Whaling Commission (Special Issue 13): 71-79.

Palsbøll, P.J., Vader, A., Bakke, I., and El-Gewely, M.R. 1992. Determination of gender in cetaceans by the polymerase chain reaction. Canadian Journal of Zoology 70: 2166-2170.

Renouf, D., Taylor, R., and Gales, R. 1994. Pseudopregnancy in harp seals (*Phoca groenlandica*). Journal of Reproduction and Fertility 101: 31-36.

Sawyer-Steffan, J.E., Kirby, V.L., and Gilmartin, W.G. 1983. Progesterone and estrogens in the pregnant and non-pregnant dolphin, *Tursiops truncatus*, and the effects of induced ovulation. Biology of Reproduction 28: 897-901.

Smith, T.D., Allen, J., Clapham, P.J., Hammond, P.S., Katona, S., Larsen, F., Lien, J., Mattila, D., Palsbøll, P.J., Sigurjønsson, J., Stevick, P.T., and Øien, N. 1999. An ocean-basin-wide mark-recapture study of the North Atlantic humpback whale (*Megaptera novaeangliae*). Marine Mammal Science 15(1): 1-32.

Straley, J.M., Gabriele, C.M., and Baker, C.S. 1994. Annual reproduction by individually identified humpback whales (Megaptera novaeangliae) in Alaskan waters. Marine Mammal Science 10:87-92.

West, K.L., Atkinson, S., Carmichael, M.J., Sweeney, J.C., Krames, B., and Krames J. 2000. Concentrations of progesterone in milk from bottlenose dolphins during different reproductive states. General and Comparative Endocrinology 117:218-224.

Winn, H.E., Edel, R.K., and Taruski, A.G. 1975. Population estimate of the humpback whale (Megaptera novaeangliae) in the West Indies by visual and acoustic techniques. Journal of the Fisheries Research Board of Canada 32: 499-506.

Yoshioka, M., Okumura, T., and Aida, K. 1994. A proposed technique for quantifying muscle progesterone content in minke whales (*Balaenoptera acutorostrata*). Canadian Journal of Zoology 72: 368-370.

Chapter 4: Quantification of progesterone from blubber biopsies of free-ranging North Atlantic right whales (*Eubalaena glacialis*) to determine pregnancy status

4. Introduction

4.1. The right whale

4.1.1 Current population status

Extensive exploitation of right whales (*Eubalaena glacialis*) for nearly ten centuries depleted populations and lead to protection by the International Convention for the Regulation of Whaling in 1935 (Aguilar, 1986). Despite over half a century of protection from commercial whaling, and recent efforts to reduce accidental encounters with ships, and entanglements in fixed fishing gear, the North Atlantic right whale remains one of the most endangered whales in the world with current population estimates, based on photo-identification methods, around 300-350 individuals (Knowlton *et al.*, 1994; DFO and WWF, 2000; IWC 2001). Predictions about the future of this population are not optimistic (DFO and WWF, 2000). Caswell *et al.*, (1999) conducted modeling studies that suggest the North Atlantic right whale population may become extinct in the next 191 years. The North Atlantic right whale has been listed as endangered by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) since April 1980 and is listed in Appendix I of the Committee for International Trade in Endangered Species (CITES).

4.1.2 North versus South right whale recovery

Low levels of recovery observed in the North Atlantic population contrast those observed in southern right whale (*E. australis*) populations (Best, 1990). Knowlton *et al.* (1994) estimated the population growth rate of the North Atlantic population to be 2.5%,

compared to Best's (1990) estimate of 6.8% for the Southern population. Brown *et al.*, (1994) indicated that only 38% of females in the North Atlantic population are known to have been reproductively successful in contrast to 54% of females in the South Atlantic population. Kraus *et al.*, (2001) presented data suggesting that the North Atlantic population reproductive rates were less than half the rates of populations in the Southern Hemisphere.

4.1.3. Causes of lack of recovery

Differences in recovery between these two species could partially be due to increased rates of abortion or neonatal mortalities in the northern species (Knowlton *et al.*, 1994) or alternatively that females become reproductively senescent. Reproductive senescence is not known to occur in right whales (DFO and WWF, 2000) and in general has never been documented in baleen whales (Marsh and Kasuya, 1986). North Atlantic right whales exhibit considerably less genetic variation than their southern relatives suggesting that inbreeding depression is a factor contributing somehow to the lower levels of recovery by the northern species (Schaeff *et al.*, 1997).

4.1.4. Migration

The majority of the right whale population migrates seasonally. Massachusetts Bay, Great South Channel, the Bay of Fundy (summer nursery), and the Nova Scotian Shelf, are high-use spring, summer, and fall feeding areas respectively (Kraus *et al.*, 1986*a*; Winn *et al.*, 1986; Hamilton and Mayo, 1990). Schaeff *et al.*, (1993) and Malik *et al.*, (1999) determined the existence of an alternative ("non-Fundy") summer nursery area(s), the location of which is still unknown. Kraus *et al.*, (1986*a*) presented data indicating that some females migrate nearly 1,800 miles from the northern summer

feeding grounds to the southeast U.S. coast (Georgia and Florida) where they give birth during the winter months. It is uncertain where the majority of non-calving adults are located during the winter months as they are rarely observed (Kraus *et al.*, 1986*a*; IWC, 2001).

4.1.5. The North Atlantic right whale catalogue

Collaborative efforts by researchers in the United States and in Canada (the Right Whale Consortium) have produced a database, which catalogues individual identification photographs of right whales from as far back as 1935 (Knowlton *et al.*, 1994). The catalogue includes information such as the date and location observed, age, sex, behaviour, and presence or absence of a calf. About 70% of the catalogued population is encountered on an annual basis (Kraus *et al.*, 1986*b*; 2001). Analyses of the accumulated data have been used to evaluate abundance (Hamilton and Mayo 1990), distribution (Winn *et al.*, 1986), migration (Kraus *et al.*, 1986*a*), reproduction (Knowlton *et al.*, 1994; Kraus *et al.*, 2001), and mortality (Kraus, 1990; Knowlton and Kraus, 2001).

Recent advances in molecular biology have also allowed for sex determination, mitochondrial DNA (mtDNA) analyses and levels of genetic diversity to be determined in right whales (Schaeff *et al.*, 1993; Brown *et al.*, 1994; Schaeff *et al.*, 1997; Malik *et al.*, 1999; Waldick *et al.*, 1999; Rosenbaum *et al.*, 2000).

4.1.6. Reproduction

Although the length of gestation in northern right whales is not precisely known, Best (1994) estimated a gestation of 12-13 months for southern right whales, which is similar to other mysticetes (Lockyer, 1984). The female right whale reproductive cycle is

assumed to include approximately 12 months of pregnancy, 10-12 months of nursing, and one to two years of reproductive rest (Knowlton *et al.*, 1994; Kraus and Hatch, 2001).

Right whales are highly sexually active. During summer and autumn feeding seasons right whales are often observed in surface active groups (SAGs), displaying behaviours that are consistent with courtship behaviours of other whales (Kraus and Hatch, 2001). If North Atlantic right whales have a comparable gestation period to those in the South Atlantic (Best, 1994) then these activities may not result in conception.

Knowlton *et al.*, (1994) and Kraus *et al.*, (2001) summarized the reproductive biology of the western North Atlantic right population using data obtained from 1980 through 1998. During 1987-1992 there were 51 reproductively active females; in 1997 and 1998 there were 75 and 70 respectively. Calving rate (the number of calves born per year) was highly variable during both study periods with between 5 and 17 calves ($\bar{x}$ = 11.15) for 1980-1992, and 6 and 22 calves ($\bar{x}$ = 11.32) for 1980-1998. The initial study found that mean age at first calving was 7.57 years and the calving interval of cows with two or more calves was 3.67 years (Knowlton *et al.*, 1994). The more recent study reported first calving at 9.53 years and a calving interval of 5.3 years (Kraus *et al.*, 2001). The source of this lower than expected reproductive rate is unknown. Several hypotheses have been suggested including inbreeding depression, body condition, and anthropogenic factors including chemical pollution and habitat degradation (IWC, 2001).

4.2. Statement of Purpose

Successful recovery of the North Atlantic right whale population is directly linked to their reproductive rates. Pregnancy rates and rates of spontaneous abortion have never

been determined in this population. This information is critical for determining if females fail to become pregnant or if there is an overall high rate of spontaneous abortion.

The objective of the current study was to determine if individual pregnant right whales could be identified using the hormonal analysis (Mansour *et al.*, 2002) of biopsy samples.

4.3. Methods

4.3.1. Biopsy sampling

Biopsy samples were collected by researchers from the Right Whale Research Consortium from March to September in 1999-2002 (permits issued by the Department of Fisheries and Oceans (DFO) and the National Marine Fisheries Service (NMFS) # 633-1483-01). Biopsies were collected according to the biopsy sampling procedure described by Brown *et al.*, (1991). Sampling occurred predominantly in the mouth of the Bay of Fundy, with one sample collected in Cape Cod Bay. All samples were obtained from the mid-dorsal body region of the whale. Each sample received a field identification number (e.g. 'MM' 21Jul99) until the whale could be identified in the right whale catalogue (i.e. #2135). The samples (n = 19) were initially stored in a solution of NaCl and 20% DMSO and then refrigerated until they were shipped (CITES #CA02CWIM0109 and #02US020950/9) to Memorial University of Newfoundland in liquid nitrogen vapour. Upon arrival at the lab, samples were stored at – 40 °C until extraction. Samples were weighed prior to extraction and ranged from 0.03 - 0.58 g ($\bar{x}$ = 0.29 g, SD = 0.15). Those samples weighing less than 0.1 g were too small to be used and were not processed (n = 2).

4.3.2. Hormone Extraction

Hormone extractions were performed according to those methods presented in Chapter 2 of this thesis. All lab tests were conducted 'blinded' with respect to field data until statistical analyses.

4.3.3. RIA

Samples were dissolved in the zero standard (standard 'A' of 0 ng/ml, DSL-3901, Diagnostic Systems Laboratories, Inc., Webster, Texas). Samples weighing $\geq$ 0.20g after grinding were dissolved 2:1(w/v), whereas samples weighing < 0.20g were dissolved 1:1(w/v) in order to have enough sample for the analysis. The samples were mixed thoroughly using a vortex mixer and sonicated for 30 min (Bransonic 3510 ultrasonic cleaner). Prior to transferring the samples to the assay tubes, samples were immediately sonicated for 10 min and mixed. Duplicates of each sample were assayed using a progesterone double antibody RIA kit (DSL-3400, Diagnostic Systems Laboratories, Inc, Webster, Texas), with a standard curve ranging from 0.3 - 70.0 ng/ml and a theoretical sensitivity of 0.10 ng/ml. All samples were run in a single assay with the intra-assay variation determined by eight replicate measurements of sample #1246. The intra-assay coefficient of variation was calculated to be 14.4%.

4.3.4. Assay Validations

Linearity and parallelism in the RIA were examined by adding known amounts of progesterone to a pooled sample of right whale blubber extract. Using the least-squares method ($y = 0.23x + 1.52$, $R^2 = 0.967$) a regression line was obtained for the observed versus expected progesterone concentrations of the pooled samples (Figure 4.1). While linearity was achieved, parallelism was not. As a result, the values reported for

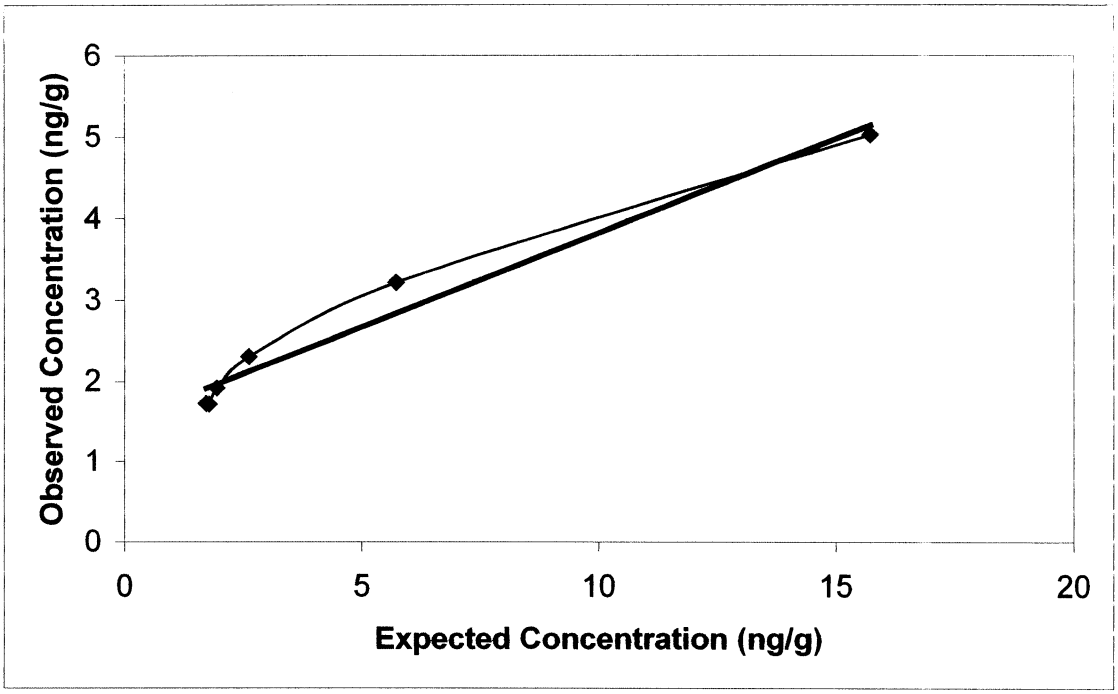

Figure 4.1. Linearity of addition of pooled right whale blubber extracts using RIA, obtained by the least-squares method.

progesterone are not absolute. As the assay showed linearity, the amount of progesterone measured can be reported in relative terms.

4.3.5. Individual data used to evaluate results

Once progesterone assay results were completed, field data (Right Whale Research Consortium) were examined. The sex of individuals was identified by photographic observation of the genital area, by molecular sex identification (Brown *et al.*, 1994), or for cows by consistent association with a newborn calf (Knowlton *et al.*, 1994).

Animals were classified into one of two age categories at the time of sampling. Individuals that were first identified as calves and were less than 9 years of age were classified as juveniles. Those animals that were at least 9 years of age, or after a 9 year sighting history were classified as adults (Hamilton *et al.*, 1988).

In general, right whale females follow a three-year calving interval so they are pregnant for a year, lactate for a year, then rest reproductively for a year before becoming pregnant again (Kraus and Hatch, 2001). Females that were classified as "overdue" were those females who rested for more than one year between lactation and pregnancy (Moira Brown, pers. comm.).

4.4. Results

4.4.1. Blubber progesterone concentrations

All right whales, with the exception of one, were resighted following sampling. Due to the lack of assay parallelism, progesterone levels were normalized using the average level found in known males as a basis value arbitrarily set at 1. Table 4.1 shows the relative blubber progesterone concentrations for the 17 right whales assayed. Relative

Table 4.1. Relative blubber progesterone concentrations (ng/g) from individually identified North Atlantic right whales.

Whale ID	Date Sampled	Relative progesterone concentration (ng/g)	Sex	Age Class (Age)	Reproductive Status
1946	14-Sep-02	90	f	M (13)	P
1158	23-Aug-00	16	f	M	R/N
2830	29-Aug-02	6	U	U	U
1911 or 1608	7-Mar-00	6	f	M	P or N
1701	8-Aug-01	6	f	M (14)	L/N
1503	21-Jul-00	5	f	M (15)	R/N
1812	31-Aug-00	3	f	M	R/N
2617	6-Aug-00	3	f	J (4)	N
1611	31-Jul-01	3	f	M (15)	L/N
1911	25-Aug-01	2	f	M (12)	L/N
1246	5-Aug-00	2	f	M	R/N
1281	28-Jul-01	2	f	M	L/N
2330	31-Aug-00	2	f	U	N
2780	26-Jul-00	1	m	U	
1503	30-Jul-99	1	f	M (14)	R/N
2135	21-Jul-99	1	m	J (8)	
2611	18-Jul-00	0	f	J (4)	N

Note:

U = Unknown
N = no viable calf produced

Sex: m = male, f = female

Age (at time of sampling): J = Juvenile (<9 years), M = Mature (at age 9 or after a 9 year sighting history),

Reproductive Status (at time of sampling): P = Pregnant (as confirmed by resighting with a calf), L = Lactating, R = Resting/Overdue

blubber progesterone concentrations ranged from 0 - 90 ng/g. Only one of the right whales (#1946) was confirmed to have been pregnant at the time of sampling, as indicated by the presence of a calf six months following sampling. This individual had a progesterone concentration of 90 ng/g. According to the right whale catalogue either right whale "G" (1911) or "F" (1608) was sampled on March 7, 2000. Right whale #1911 returned the following year with a calf while #1608 did not. Genetics are currently being conducted at Trent University to determine which individual was actually sampled that day. The sample from #1911 or #1608 yielded a progesterone concentration of 6 ng/g.

Resting/overdue females had relative progesterone concentrations ranging from 1 -16 ng/g, lactating females from 2 - 6 ng/g and males/juveniles from 0 - 3 ng/g.

4.5. Discussion

4.5.1. Pregnancy determinations

Pending the results of the genetic identification (if right whale #1911 was in fact the individual sampled) there may be a large variation in the progesterone concentrations between two individuals who returned with calves the following year, which should be addressed. Two possible explanations for the large variation include: the month of sampling, and the length of time the blubber biopsy samples were stored prior to extraction. The sample from female #1946 (with the higher blubber progesterone concentration) was collected in September, which suggests that the individual was approximately 8-10 months pregnant, assuming conception between November and January (DFO and WWF, 2000) and 12-13 months gestation (Best, 1994; Lockyer, 1984). The sample, from female #1911, with the lower blubber progesterone concentration, was collected in March and the individual was likely only 2-4 months pregnant. Blubber

progesterone levels are not likely constant throughout pregnancy. Progesterone is known to increase steadily throughout pregnancy with high levels of serum progesterone sustained beyond 6 – 8 weeks indicative of pregnancy in the bottlenose dolphin (Sawyer-Steffan *et al.*, 1983). With repeated sampling throughout gestation a limited progesterone profile could be created for pregnant individuals.

The differences in blubber progesterone concentrations between the pregnant individuals could also be attributed to the length of time the samples were stored prior to extraction. The biopsy sample from right whale #1911 was collected in March of 2000 and was stored frozen for almost 3 years prior to extraction, whereas the biopsy from right whale #1946 was collected in September of 2002 and only stored frozen for 3 months prior to extraction. Blubber biopsies stored frozen or in DMSO during a short-term period of 2 months however show no hormone loss over time (Chapter 2). A long-term study to determine how much, if any, progesterone is lost over longer periods may be necessary before interpreting hormone levels from archived biopsy samples.

With the exception of one sample, all samples from resting/overdue females, lactating females, males, and juveniles of both sexes had low levels of blubber progesterone and none were resighted with a calf the year following sampling. The sample from a resting female (#1158) exhibited a moderate progesterone level and the individual was resighted without a calf. Possibly, spontaneous abortion, neonatal mortality or pseudopregnancy, which has been reported in other marine mammals, (Renouf *et al.* 1994; Atkinson, 1997; Atkinson *et al.*, 1999; West *et al.*, 2000) occurred in this individual. Spontaneous abortion or neonatal mortality are more plausible explanations as this individual was sighted along the Georgia-Florida coast, the only

known right whale calving ground (Kraus *et al.*, 1986*a*), on December 8th, 2000 and on several other occasions from January through March 2001 (Moira Brown, pers. comm.). All other resting/overdue females were not observed in the southeast the year following sampling (Moira Brown, pers. comm.). It is also possible, however, that this method does not work for right whales and that is why a female with a moderate progesterone level returned without a calf the following year.

4.5.2. Future research

In order for the recovery efforts of North Atlantic right whales to be effective, researchers must answer the following question: is the low calving rate due to primary failure of females to become pregnant or a high rate of spontaneous abortion? With increased biopsy sampling of reproductively active females throughout the year it may be possible to determine pregnancy rates and rates of spontaneous abortion by using this method of pregnancy determination. This will directly address a recommendation of the Canadian North Atlantic Right Whale Recovery Plan to "monitor the physiological condition of right whales in relation to their reproductive performance" (DFO and WWF, 2000).

Comparing pregnancy rates of North Atlantic right whales with those in the South Atlantic, a population that has shown signs of recovery, may also provide important information about the nature of the problem in the reproductive cycle of the North Atlantic right whale.

4.6. References

Aguilar, A. 1986. A review of old Basque whaling and its effect on the right whales (*Eubalaena glacialis*) of the North Atlantic. Report of the International Whaling Commission (Special Issue 10): 191-199.

Atkinson, S. 1997. Reproductive biology of seals. Reviews of Reproduction 2: 175-194.

Atkinson, S., Combelles, C., Vincent, D., Nachtigall, P., Pawloski, J., and Breese, M. 1999. Monitoring of progesterone in captive female false killer whales, *Pseudorca crassidens*. General and Comparative Endocrinology 115: 323-332.

Best, P.B. 1990. Trends in the inshore right whale population off South Africa, 1969-1987. Marine Mammal Science 6: 93-108.

Best, P.B. 1994. Seasonality of reproduction and the length of gestation in southern right whales (*Eubalaena glacialis*). Journal of Zoology, London 232:175-189.

Brown, M.W., Kraus, S.D., and Gaskin, D.E. 1991. Reaction of North Atlantic right whales (*Eubalaena glacialis*) to skin biopsy sampling for genetic and pollutant analysis. Report of the International Whaling Commission (Special Issue 13): 81-89.

Brown, M.W., Kraus, S.D., Gaskin, D.E., and White, B.N. 1994. Sexual composition and analysis of reproductive females in the North Atlantic right whale, *Eubalaena glacialis*, population. Marine Mammal Science 10: 253-265.

Caswell, H., Fujiwara, M., and Brault, S. 1999. Declining survival probability threatens the North Atlantic right whale. Proceedings of the National Academy of Science USA 96: 3308-3313.

Department of Fisheries and Oceans (DFO) and World Wildlife Fund (WWF). 2000. Canadian North Atlantic right whale recovery plan, Department of Fisheries and Oceans, Halifax, Nova Scotia, 82 pp.

Hamilton, P.K., and Mayo, C.A. 1990. Population characteristics of right whales (*Eubalaena glacialis*) observed in Cape Cod and Massachusetts Bays, 1978-1986. Report of the International Whaling Commission (Special Issue 8): 203-208.

Hamilton, P. K., Knowlton, A. R., Marx, M. K., and Kraus, S. D. 1988. Age structure and longevity in North Atlantic right whales (*Eubalaena glacialis*) and their relation to reproduction. Marine Ecology Progress Series 171: 285-292.

International Whaling Commission (IWC). 2001. Report of the workshop on the status and trends of the western North Atlantic right whale. Journal of Cetacean Research and Management (Special issue 2): 61-87.

Knowlton A.R. and S.D. Kraus. 2001. Mortality and serious injury of Northern right whales (*Eubalaena glacialis*) in the western North Atlantic. Journal of Cetacean Research and Management (Special Issue 2): 193-208.

Knowlton, A.R., Kraus, S.D., and Kenney, R.D. 1994. Reproduction in North Atlantic right whales (*Eubalaena glacialis*). Canadian Journal of Zoology 72: 1297-1305.

Kraus, S.D. 1990. Rates and potential causes of mortality in North Atlantic right whales (*Eubalaena glacialis*). Marine Mammal Science 6: 278-291.

Kraus, S.D., and Hatch, J.J. 2001. Mating strategies in the North Atlantic right whale (Eubalaena glacialis). Journal of Cetacean Research and Management (Special Issue 2): 237-244.

Kraus, S.D., Prescott, J.H., Knowlton, A.R., and Stone, G.S. 1986*a*. Migration and calving of right whales (*Eubalaena glacialis*) in the western North Atlantic. Report of the International Whaling Commission (Special Issue 10): 139-144.

Kraus, S.D., Moore, K.E., Price, C.A., Crone, M.J., Watkins, W.A., Winn, H.E., and Prescott, J.H. 1986*b*. The use of photographs to identify individual North Atlantic right whales (*Eubalaena glacialis*). Report of the International Whaling Commission (Special Issue 10): 145-151.

Kraus, S.D., Hamilton, P.K., Kenney, R.D., Knowlton, A.R., and Slay, C.K. 2001. Reproductive parameters in the North Atlantic right whale. Journal of Cetacean Research and Management 2: 231-236.

Lockyer, C.H. 1984. Review of baleen whale (Mysticeti) reproduction and implications for management. Report of the International Whaling Commission, (Special Issue 6): 27-50.

Malik, S., Brown, M.W., Kraus, S.D., Knowlton, A.R., Hamilton, P.K., and White, B.N. 1999. Assessment of mitochondrial DNA structuring and nursery use in the North Atlantic right whale (*Eubalaena glacialis*). Canadian Journal of Zoology 77: 1217-1222.

Mansour, A.A.H., McKay, D.W., Lien, J., Orr, J.C., Banoub, J.H., Øien, N., and Stenson, G. 2002. Determination of pregnancy status from blubber samples in minke whales (*Balaenoptera acutorostrata*). Marine Mammal Science 18: 112-120.

Marsh, H., and Kasuya, T. 1986. Evidence for reproductive senescence in female cetaceans. Report of the International Whaling Commission (Special Issue 8): 57-74.

Renouf, D., Taylor, R., and Gales, R. 1994. Pseudopregnancy in harp seals (*Phoca groenlandica*). Journal of Reproduction and Fertility 101: 31-36.

Rosenbaum, H.C., Brownell, R.L., Brown, M.W., Schaeff, C., Portway, V., White, B.N., Malik, S., Pastene, L.A., Patenaude, N.J., Baker, C.S., Goto, M., Best, P.B., Clapham, P.J., Hamilton, P., Moore, M., Payne, R., Rowntree, V., Tynan, C.T., Bannister, J.L., and DeSalle, R. 2000. World-wide genetic differentiation of *Eubalaena*: questioning the number of right whale species. Molecular Ecology 9: 1793-1802.

Sawyer-Steffan, J.E., Kirby, V.L., and Gilmartin, W.G. 1983. Progesterone and estrogens in the pregnant and non-pregnant dolphin, *Tursiops truncatus*, and the effects of induced ovulation. Biology of Reproduction 28: 897-901.

Schaeff, C.M., Kraus, S.D., Brown, M.W., and White, B.N. 1993. Assessment of the population structure of western North Atlantic right whales (*Eubalaena glacialis*) based on sighting and mtDNA data. Canadian Journal of Zoology 71:339-345.

Schaeff, C.M., Kraus, S.D., Brown, M.W., Perkins, J.S., Payne, R., and White, B.N. 1997. Comparison of genetic variability of North and South Atlantic right whales (Eubalaena), using DNA fingerprinting. Canadian Journal of Zoology 75:1073-1080.

Waldick, R.C., Brown, M.W., and White, B.N. 1999. Characterization and isolation of microsatellite loci from the endangered North Atlantic right whale. Molecular Ecology 8: 1763-1765.

West, K.L., Atkinson, S., Carmichael, M.J., Sweeney, J.C., Krames, B., and Krames J. 2000. Concentrations of progesterone in milk from bottlenose dolphins during different reproductive states. General and Comparative Endocrinology 117:218-224.

Winn, H.E., Price, C.A., and Sorensen, P.W. 1986. The distributional biology of the right whale (*Eubalaena glacialis*) in the western North Atlantic. Report of the International Whaling Commission (Special Issue 10): 129-138.

Chapter 5: Progesterone concentrations in bone samples of bowhead (*Balaena mysticetus*) and right (*Eubalaena glacialis*) whales from the 16th century Basque fishery in Quebec and Labrador

5. Introduction

5.1.1. Selma Barkham's research

Extensive archival research in Spain by geographer and historian Selma Barkham investigated the Basque whale fishery in Labrador during the 16th century (Barkham, 1984). Barkham's research involved translating and analysing thousands of documents such as contracts, lawsuits, and wills, containing references to the Grand Bay (Tuck and Grenier, 1981). The Grand Bay, or the Strait of Belle Isle, was a popular route for whales traveling to and from the Gulf of St. Lawrence (Barkham, 1984). Barkham's research led to archaeological work by Memorial University of Newfoundland's Archaeology Unit and Parks Canada's Marine Excavation Unit (Tuck and Grenier, 1981), which through field studies documented whale bones, vessels, pieces of broken pottery, glassware, roofing tiles, and many other artifacts.

5.1.2. The Basque

Basque country is a coastal area on the Atlantic Ocean transcending the current borders of France and Spain. The Basque people prosecuted a whale fishery in their own waters as early as the 12th century (Barkham, 1984). Thus, when they arrived in Labrador during the 1530's, their whaling skills were well developed. This enterprise became Canada's first 'full-blown' industry (Thurston, 1983) in the modern sense of the term.

The Basques would arrive each spring and remain until December when ice conditions forced them to leave. Occasionally, they were forced to overwinter (Tuck and Grenier, 1981; Cumbaa, 1986).

5.1.3. The hunted whales

Bowhead whales (*Balaena mysticetus*), also known as Greenland right whales, are phylogenetically close relatives of right whales (*Eubalaena glacialis*) (McLeod *et al.*, 1993). The Basques preferentially hunted bowhead and right whales because they were slow swimmers, floated after death, and yielded large quantities of oil and baleen. They were, therefore, considered to be the "right" whales to hunt (Tuck and Grenier, 1981; Cumbaa, 1986; IWC, 2001). Cumbaa (1986) suggests that Basques hunted *E. glacialis* from late June or early July until about mid-October when they switched to *B. mysticetus*. *E. glacialis* traveled through the Straits beginning in the early summer as they migrated north, whereas *B. mysticetus* traveled into the Straits as they headed south from their Arctic feeding grounds in the fall and early winter (Cumbaa, 1986; Logan and Tuck, 1990; Fagan, 1993).

Written accounts indicate that the majority of whales killed were *E. glacialis*. Early osteological examinations concluded that approximately half the whales killed in Red Bay were *B. mysticetus*, and half were *E. glacialis* (Cumbaa, 1986). However, recent analysis of DNA from the bones suggests that the majority of the whales taken were actually *B. mysticetus* (Rastogi *et al.*, submitted; Brenna McLeod, pers. comm.).

While both the right and bowhead whales are currently listed by COSEWIC as endangered (COSEWIC, 2003), it is difficult to determine whether the 16th century Basque whale fishery was a significant factor leading to the current population states of

these two species. What is known from catch record data is that during the period of Basque whaling in Labrador, whalers came to depend increasingly on fall and early winter catches. This has been interpreted as leading to a commercial extinction of these species (Selma Barkham, pers. comm.).

5.1.4. Basque whaling harbours

Barkham's research involved matching 16th century place names from Basque documents to those on modern maps (Barkham, 1977). Figure 5.1 is a map of 16th century Basque whaling harbours along the north shore of Quebec and southern coast of Labrador. Table 5.1 lists selected Basque whaling harbours in Quebec and Labrador including current and 16th century names (Barkham, 1977, 1978, 1980). Figure 5.2 is a map of Red Bay, Labrador, the harbour where most historical Basque whaling references occur and where the most modern archaeological work has been conducted.

5.1.5. The process

Basque whaling was predominantly coastal (Barkham, 1984). Whales were harpooned and, once weakened, they were killed with a lance. The captured animal was then towed to shore for flensing of the blubber (Tuck and Grenier, 1981). Prior to flensing, flippers and tail flukes were cut off at a stage near the tryworks to streamline the carcass, which was rotated while flensing occurred (Thurston, 1983; Fagan, 1993; Brown and Cumbaa, 1999). Bones of the flippers (humerus, radius, ulna, metacarpals, and phalanges) were quickly covered by mud and silt, which along with the cold water allowed them to remain preserved until the present (Brown and Cumbaa, 1999). In Red Bay, Labrador the remainder of the carcass was towed across the harbour to what is locally called "Bony Shore/Beach" (Figure 5.2) (Thurston, 1983).

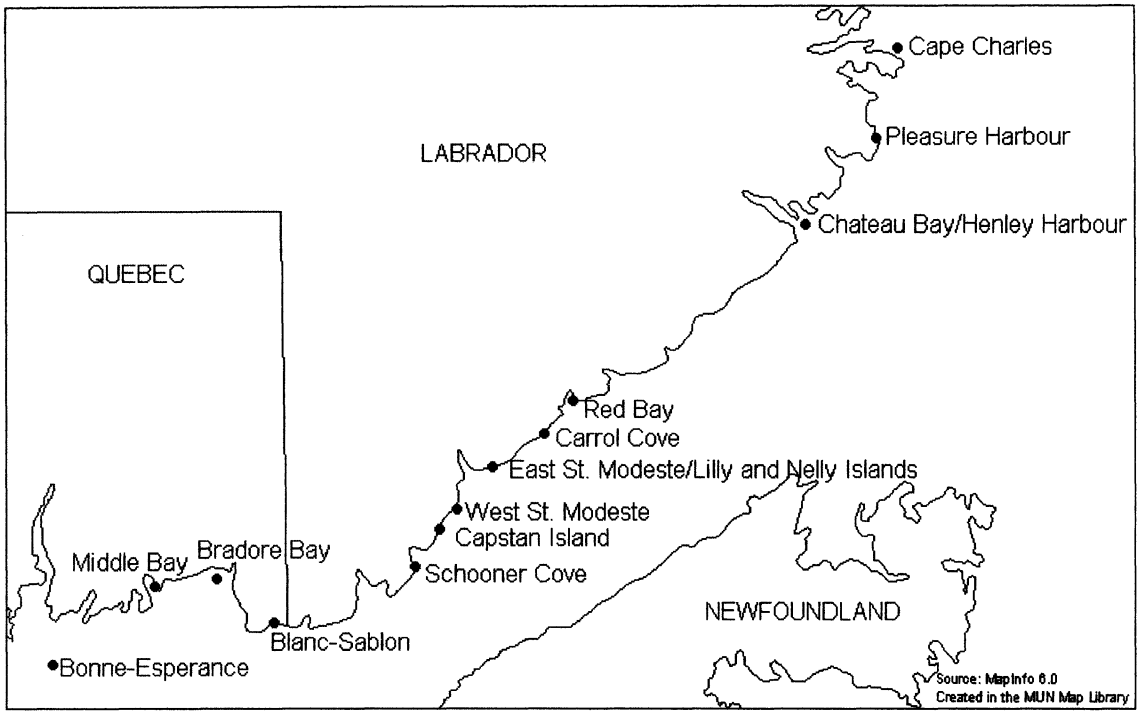

Figure 5.1. 16th century Basque whaling harbours along the Quebec north shore and the south coast of Labrador. Harbour locations are based on information from Barkham, 1977; 1978; 1980.

Table 5.1. Selected Basque whaling harbours along the Quebec north shore and the south coast of Labrador (Information abstracted from Barkham, 1977; 1978; 1980).

Current Name	**16th Century Name**
Middle Bay, Quebec	Gradun/Cradon
Blanc Sablon, Quebec	Blan Samon/Beau Sablom
Schooner Cove, Labrador	Baye de Ballennes/Baleetaco Baya
West St. Modeste, Labrador	Sembrero/Sant Maudet
Lilly & Nelly Islands/East St. Modeste, Labrador	Los Hornos/Labeeta/Les Fours
Carrol Cove, Labrador	Puerto Breton/Port de Ballene
Red Bay, Labrador	Havre des Buttes/Buetes
Chateau Bay/Henley Harbour, Labrador	Chateo/Chateaux
Pleasure Harbour	Puerto Nuevo/Port Neuf

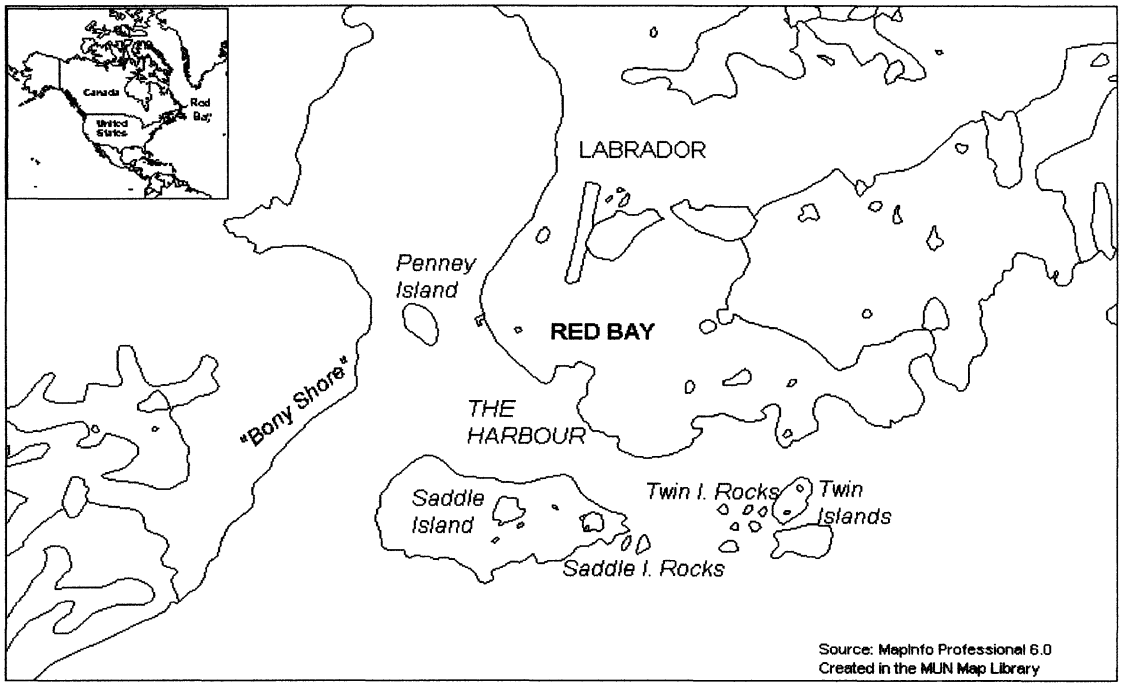

Figure 5.2. Red Bay, Labrador.

5.2. Statement of Purpose

The objective of the current research was to determine if immunoassayable progesterone could be extracted from the oil of ancient whale bone samples. If quantifiable, this method could form the basis for estimating the reproductive status of ancient whales and may contribute to a better understanding of the biology of extant endangered right and bowhead whales.

5.3. Methods

5.3.1. Bone sampling

5.3.1.1. Red Bay samples

As a result of archaeological work, approximately 1,500 bones from bowhead and right whales have been recovered from terrestrial and underwater deposits and are currently archived by Parks Canada and the Provincial Museum of Newfoundland and Labrador at the Red Bay Historic Site (Cumbaa, 1986). Humeri (n = 32) collected during the marine excavations (Tuck and Grenier, 1981) were used in this study (Figure 5.3). According to Cumbaa (1986) these bones were located in stratigraphic contexts indicating deposition during the Basque period. Access to the archived bones was granted by Elaine Anton, Penny Houlden and Martha Drake (Provincial Museum of Newfoundland and Labrador, The Rooms Corporation of Newfoundland and Labrador, Government of Newfoundland and Labrador) and was coordinated with Cindy Gibbons (Red Bay Historic Site, Parks Canada). The bones were catalogued based on a grid overlying the site (Cumbaa, 1986). Appendix B lists the catalogue numbers of humeri sampled from the Red Bay collection. Bones shavings and dust samples were collected according to Brown and Cumbaa (1999) as follows, with minor modifications. The sample area for each bone

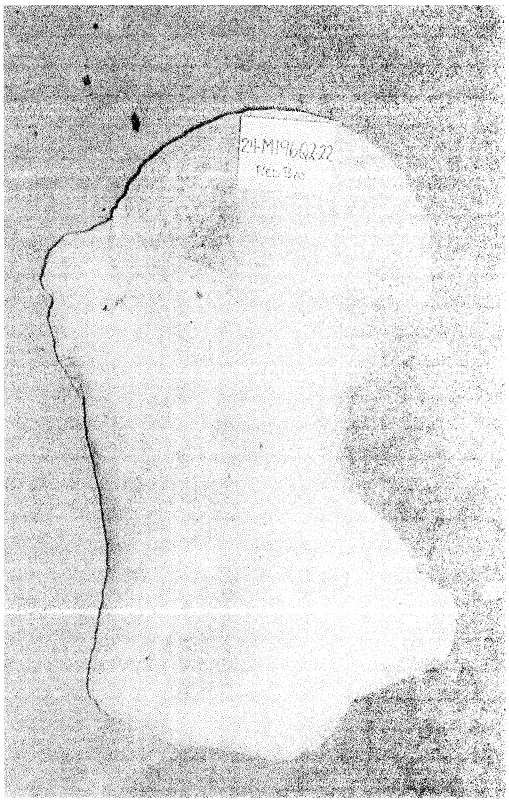

Figure 5.3. Humerus (#24M196Q2-2) recovered during marine excavations in Red Bay, Labrador during the 1970's and 1980's.

was cleaned prior to drilling with a scrub brush and hot, soapy water. The same solution was used to clean all drill bits prior to sampling and between samples, followed by rinsing with tap water then distilled water. A 14.4-volt cordless drill (Craftsman™) with a 10 mm drill bit was used to drill a shallow pilot hole through the outer surface. Any resultant material in the pilot hole or on the drill bit was removed with a toothpick and discarded. The same drill bit was used to continue drilling the length of the drill bit (11 cm), with the resultant bone shavings and dust collected on wax paper and transferred to a labelled glass scintillation vial. Upon arrival at the lab, samples were stored at – 20 °C until extraction.

5.3.1.2. Samples from other Basque sites

Other Basque sites have not been searched as intensively as Red Bay and were explored by the author and Brenna McLeod (Trent University, Peterborough, Ontario) during June 2003. The following sites were explored on the Quebec north shore: Middle Bay, Bradore Bay, and Blanc Sablon. Schooner Cove, Capstan Island, West St. Modeste, and Red Bay ("Bony Shore"), all located on Labrador's southern shore, were also investigated. Bones suitable for sampling were located at Middle Bay (n = 6), Capstan Island (n = 2), West St. Modeste (n = 1), and "Boney shore" (n = 30). Permission to sample bones found in these areas was granted by Elaine Anton, Martha Drake, and Penny Houlden, (Provincial Museum of Newfoundland and Labrador, The Rooms Corporation of Newfoundland and Labrador, Government of Newfoundland and Labrador). After sampling, bones remained in the area where they were found marked with an identification code (BMMS and an identification #), "BMMS" representing the

initials of Brenna McLeod and Mackenzie Sheridan. Appendix C lists the bones sampled at the various locations during the 2003 field trip.

Bone samples were obtained in a similar manner as the Red Bay archived humeri with a few modifications due to sampling logistics in the field (Figure 5.4). As hot water was not available at the remote sampling locations, the surface of the bones, and drill bits were cleaned with DECON 75 (an SDS (sodium dodecyl sulfate) based detergent, (BDH, Product no. B56019) and rinsed with distilled water. Upon arrival at the lab, samples were stored at –20 °C until extraction.

5.3.2. Hormone extraction

Hormone extractions were performed according to those presented in Chapter 2 with modifications as follows. A 1 g bone sample was ground to a powder with mortar and pestle, and then transferred to a 50 ml tube where it was weighed. After the extraction the residue was placed under argon and frozen at –20 °C until the enzyme immunoassay (EIA).

5.3.3. EIA

EIA was used as opposed to an RIA because of the greater sensitivity of the EIA and the uncertainty of what concentrations, if any could be measured in these ancient samples. Samples were diluted 1:5 with EIA buffer, mixed thoroughly using a vortex mixer, and sonicated for 30 min in a Bransonic 3510 ultrasonic cleaner. A few samples were diluted 1:10, 1:20 or 1:100 (w/v) in order to obtain progesterone concentrations in the middle range of the standard curve. Immediately prior to transferring the samples to the assay wells, samples were sonicated for 10 min and mixed. Duplicates of each sample

Figure 5.4. Drilling a whale bone on the shores of Capstan Island, Labrador.

were assayed using a progesterone ACE™ competitive EIA kit (Catalogue no. 582601, Cayman Chemical Company, Ann Arbor, Michigan), with a standard curve ranging from 7.8 - 1000.0 pg/ml.

5.3.4. Assay validations

Linearity and parallelism were tested in the EIA by using bone oil extract of #24M4P8-8 diluted ½, ¼, and ⅛ concentration using EIA buffer. Figure 5.5 shows the regression line obtained for the observed versus expected progesterone concentrations of the bone extract sample using the least-squares method ($y = 0.97x + 25.65$, $R^2 = 0.9957$). While linearity was obtained, parallelism was not. Thus, reported concentrations of progesterone in bone are not absolute values, but can be considered as larger values representing relatively more progesterone, whereas lower values representing relatively less progesterone.

5.3.5. Data analyses

Statistical analysis was performed using SPSS statistical package, version 11.5 (SPSS Inc., Chicago, Illinois). The independent samples t-test was used to analyze the difference between bone progesterone concentrations from bone samples collected from underwater deposits and those collected from terrestrial deposits.

5.4. Results

Due to the lack of assay parallelism, relative bone concentrations were calculated using the lowest concentration as a basis value normalized at 1. Relative bone progesterone concentrations for the 71 ancient bone samples ranged from 1 – 96 ng/g (Appendix D). Bones sampled from the Red Bay archives had progesterone

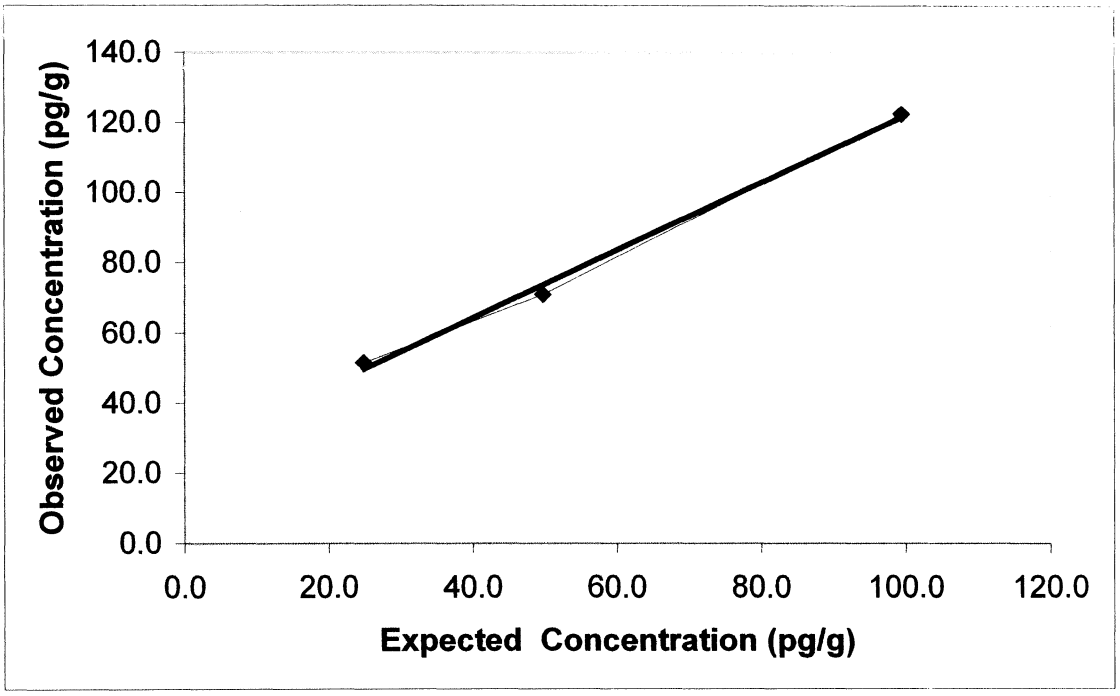

Figure 5.5. Linearity of dilution for bone sample #24M4P8-8 for the EIA obtained using the least-squares method.

concentrations ranging from 2 - 69 ng/g. Samples collected from terrestrial deposits had progesterone concentrations ranging from 1 – 10 ng/g. The archived bones, which were originally collected from underwater deposits, had greater progesterone concentrations than those that were collected from terrestrial deposits ($p < 0.05$) (Figure 5.6). Samples were run in a single assay with an intra-assay coefficient of variation determined by ten replicates of bone #24M4P8-8 (CV = 26.5 %).

5.5. Discussion

5.5.1. Progesterone concentrations

The results of this study show that immunoassayable progesterone can be found in oil extracts of ancient whale bones. Immunoassayable progesterone was found in all samples tested. Results also indicate that the location of the bones for the past 400-500 years may have had an impact on the preservation of the hormone. Progesterone concentrations from the 'Bony Shore' area and other terrestrial locations were consistently lower than those of the Red Bay archives. The physical condition of the bones is consistent with much more externally evident deterioration of bones that, at least in recent years, were not underwater. Another possibility is that humeri bones, or long bones in general, may contain greater progesterone concentrations than other bones.

Determining reproductive status of ancient whales by quantifying progesterone in bones will depend on knowledge of their species and their sex. The wide range of immunoassayable bone progesterone concentrations found in the Red Bay archive samples is consistent with a range of reproductive states where both male and non-pregnant whales possibly would have lower concentrations and pregnant animals would have greater concentrations. Similar observations have been made from determinations of

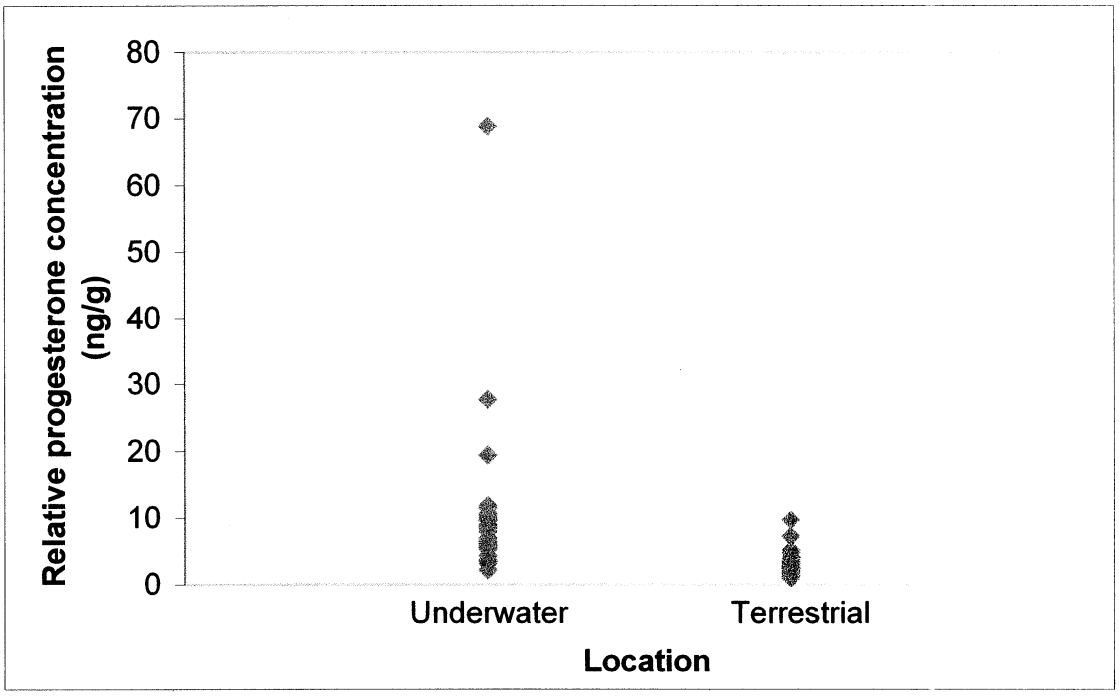

Figure 5.6. Relative progesterone concentrations (ng/g) for bones from underwater versus terrestrial deposits.

progesterone concentrations of blood, muscle, and blubber of contemporary whales. (Sawyer-Steffan *et al.*, 1983; Yoshioka *et al.*, 1994; Mansour *et al.*, 2002).

5.5.2. Future research

Further collaboration with researchers at Trent University (Peterborough, Ontario) where molecular analyses of the bones such as sex determination, and species identification are taking place will help clarify results of this study. Thus, this method, combined with genetic analyses, could form the basis for estimating the reproductive status of the ancient whales.

There are numerous sites along the Quebec north shore and Labrador south coast (i.e. Lilly and Nelly Islands, Carrol Cove, Chateau Bay/Henley Harbour, Pleasure Harbour) that remain virtually unexplored and unsampled. Increasing the sample size by sampling bones from these areas may allow for pregnancy rates of both ancient bowhead and right whale populations to be determined. The biological information gained from this research will not only supplement the knowledge of the Basque whaling industry, but it will also contribute to a better understanding of the historical and current reproductive dynamics of the endangered right and bowhead whales.

5.6. References

Barkham, S. 1977. The identification of Labrador ports in Spanish 16th century documents. The Canadian Cartographer 14: 1-9.

Barkham, S. 1978. The Basques: filling a gap in our history between Jacques Cartier and Champlain. Canadian Geographical Journal 96: 8-19.

Barkham, S. 1980. A note on the Strait of Belle Isle during the period of Basque contact with Indians and Inuit. Études Inuit 4: 51-58.

Barkham, S.H. 1984. The Basque whaling establishments in Labrador 1536-1632 – A summary. Arctic 37:515-519.

Brown, M.W., and Cumbaa, S.L. 1999. Field report on sampling right and bowhead whale bones from the 16th century Basque fishery, stored in Red Bay, Labrador, for molecular analyses, July 9-13, 1999. Unpublished report, National Museum of Nature, Ottawa, Ontario.

Committee on the Status of Endangered Wildlife in Canada (COSEWIC). 2003. Species Assessment, May 2003.

Cumbaa, S.L. 1986. Archaeological evidence of the 16th century Basque right whale fishery in Labrador. Report of the International Whaling Commission (Special Issue 10): 187-190.

International Whaling Commission (IWC). 2001. Report of the workshop on the status and trends of the western North Atlantic right whale. Journal of Cetacean Research and Management (Special issue 2): 61-87.

Fagan, B. 1993. Basques of Red Bay. Archaeology 46 (Sept./Oct.): 44-51.

Logan, J.A., and Tuck, J.A. 1990. A sixteenth century Basque whaling port in southern Labrador. Association for Preservation Technology (APT) Bulletin, Journal of Preservation Technology 22: 65-72.

Mansour, A.A.H., McKay, D.W., Lien, J., Orr, J.C., Banoub, J.H., Øien, N., and Stenson, G. 2002. Determination of pregnancy status from blubber samples in minke whales (*Balaenoptera acutorostrata*). Marine Mammal Science 18: 112-120.

McLeod, S.A., Whitmore Jr., F.C., and Barnes, L.G. 1993. Evolutionary relationships and classification. pp. 45. *In:* J.J. Burns, J.J. Montague, and C.J. Cowles (eds.), The Bowhead whale. Society for Marine Mammalogy, Lawrence, Kansas. i-xxxvi + 787pp.

Rastogi, T., McLeod, B.A., Frasier, T.R., Brown, M.W., Grenier, R., Cumbaa, S.L., Nadarajah, J., and White, B.N. Rewriting whaling history: analysis of four hundred year old whale DNA. Submitted.

Sawyer-Steffan, J.E., Kirby, V.L., and Gilmartin, W.G. 1983. Progesterone and estrogens in the pregnant and non-pregnant dolphin, *Tursiops truncatus*, and the effects of induced ovulation. Biology of Reproduction 28: 897-901.

Thurston, H. 1983. The Basque connection. Equinox 2: 46-59.

Tuck, J.A., and Grenier, R. 1981. A 16th-century Basque whaling station in Labrador. Scientific American 245: 180-190.

Yoshioka, M., Okumura, T., and Aida, K. 1994. A proposed technique for quantifying muscle progesterone content in minke whales (*Balaenoptera acutorostrata*). Canadian Journal of Zoology 72: 368-370.

Chapter 6: Summary

6.1. Summary

Limited information has been obtained regarding the reproductive endocrinology of cetaceans, especially the larger baleen whales. This information is critical for assessing and monitoring the reproductive success of free-ranging whales. One aim of this thesis was to start the process of expanding our knowledge on the reproductive endocrinology of free-ranging whales by quantifying progesterone in the blubber. Another aim was to analyze progesterone content of bone from ancient whales to determine whether current reproductive patterns were observed at a time when these whales were more abundant.

Blubber biopsies for hormonal analyses can be stored frozen, in 100% dimethylsulfoxide (DMSO) or in 20% salt-saturated DMSO for up to 2 months with progesterone concentrations remaining intact. When samples are stored in ethanol, both the blubber and ethanol liquid must be extracted and quantified in order for accurate progesterone concentrations to be measured. If archived biopsy samples, such as those from the Years of the North Atlantic Humpback (YoNAH) study (Smith *et al.*, 1999), are to be used for hormonal analyses then a long-term study is critical to see how storage condition affects hormone concentrations over a longer period of time.

The results of the research presented in Chapters 3 and 4 demonstrate that the combination of biopsy sampling and progesterone extraction (Mansour *et al.*, 2002) provides a minimally invasive and relatively inexpensive method for determining pregnancy in free-ranging right and humpback whales. Prior to this research determining pregnancy status of free-ranging whales was limited to observations following parturition,

when calves were in close association with their mothers. Cases where females were pregnant but later terminated would not have been detected by observational means of pregnancy determination.

The ability to determine pregnancy status during gestation may allow researchers to identify cases of spontaneous abortion. For endangered species, like the North Atlantic right whale, this is of critical importance in order to gain a better perspective into their poor reproductive success. Identifying the point at which a female's pregnancy is aborted may provide insight into the plausible cause or causes for the termination of the pregnancy. In addition, the research will address a recommendation of the Canadian Right Whale Recovery Plan, to "monitor the physiological condition of right whales in relation to their reproductive performance" (DFO and WWF, 2000).

Although the North Atlantic humpback whale is not currently endangered, future management plans need to be based on sound information about the reproductive biology of this species. For example, it is unknown how many females become pregnant and terminate their pregnancy because of poor food/body resources or other factors. Determining the pregnancy status of mature females early and late in the feeding season may provide researchers with this information. As well, assessing human-related impacts on reproduction may be achieved by using this method of pregnancy determination, e.g., determining the impact of a female's entanglement history on her fecundity.

The applicability of this research may also be expanded to include studies of other marine mammals and terrestrial animals in order to determine reproductive status and monitor an individual's pregnancy.

The results of the research in Chapter 5 indicate that immunoassayable progesterone can be quantified from oil extracts of ancient whale bone samples. As well, the location of the bones for the past 400-500 years may have had an impact on the preservation of the samples, as progesterone concentrations from the 'Bony Shore' samples were consistently lower than those of the Red Bay archives, which were collected from underwater deposits. Hopefully, with continued use of these methods of pregnancy determination, rates of pregnancy can be estimated for ancient and current right and bowhead whales. This information will contribute to a better understanding of the historical and current reproductive dynamics of the endangered right and bowhead whales.

6.2. Limitations

Assay parallelism was not achieved in any of the studies presented in the thesis, such that relative progesterone values were reported. Future work to improve this problem is necessary. Until this technique can be validated by both assay linearity and parallelism, we must admit the possibility that this technique does not work.

As well, currently, we do not have enough information to know whether or not we can distinguish progesterone levels of pregnancy from those found in the luteal phase. With additional studies, it may be possible to refine the method to enable the detection of 'ovulation' versus pregnancy. Possibly, elevated progesterone levels resulting from ovulation may be so brief in duration that concentrations are not reflected in the superficial layer of the blubber.

6.3. Future research

The research in this thesis presents a starting point. There are still many questions that need to be answered. For example, a study to compare progesterone levels in the blubber, muscle, blood, urine and bone of individuals across reproductive classes is necessary. Most importantly, comparing progesterone levels in blubber and bone may help us to better evaluate the results achieved in Chapter 5.

Another question that may be answered by extending this research is: how do levels of progesterone and other sex steroids vary both with depth of blubber and across the blubber profile? Results from harbour porpoises raise this question, as stratification and age-related differences in blubber fatty acids have been observed (Koopman *et al.*, 1996).

In conclusion, the data on the reproductive endocrinology of both ancient and current whales gathered in this project provides a basis for future research and may offer a tool for assessing reproductive success and developing realistic management and recovery strategies for current whale populations.

6.4. References

Department of Fisheries and Oceans (DFO) and World Wildlife Fund (WWF). 2000. Canadian North Atlantic right whale recovery plan, Department of Fisheries and Oceans, Halifax, Nova Scotia, 82 pp.

Koopman, H.N., Iverson, S.J., and Gaskin, D.E. 1996. Stratification and age-related differences in blubber fatty acids of the male harbour porpoise (*Phocoena phocoena*). Journal of Comparative Physiology B 165: 628-639.

Mansour, A.A.H., McKay, D.W., Lien, J., Orr, J.C., Banoub, J.H., Øien, N., Stenson, G. (2002) Determination of pregnancy status from blubber samples in minke whales (*Balaenoptera acutorostrata*). Marine Mammal Science 18: 112-120.

Smith, T.D., Allen, J., Clapham, P.J., Hammond, P.S., Katona, S., Larsen, F., Lien, J., Mattila, D., Palsbøll, P.J., Sigurjønsson, J., Stevick, P.T., and Øien, N. 1999. An ocean-basin-wide mark-recapture study of the North Atlantic humpback whale (*Megaptera novaeangliae*). Marine Mammal Science 15: 1-32.

Appendix A – Relative blubber progesterone concentrations (ng/g) of humpback whales from the Gulf of Maine, according to rank.

Sample ID	Relative progesterone concentration (ng/g)	Sex	Age Class	Reproductive Status
CCS 2002-23	28	f	M	P
CCS 2002-36	22	f	M	N
CCS 2002-50	17	f	M	N
CCS 2002-07	13	f	M	P
CCS 2002-09	13	f	M	U
CCS 2002-33	12	f	M	P
CCS 2002-51	8	f	M	N
CCS 2002-59	7	f	M	P
CCS 2002-42	6	f	M	N
CCS 2002-60	5	f	M	N
CCS 2002-69	4	f	M	U
CCS 2002-70	3	f	M	N
CCS 2002-39	3	f	PM	N
CCS 2002-79	1	m	M	N
CCS 2002-41	1	f	M	L/U
CCS 2002-49	1	f	M	L/U
CCS 2002-64	1	f	M	L/U
CCS 2002-43	1	f	M	U
CCS 2002-67	1	m	M	N
CCS 2002-32	1	f	M	L/U
CCS 2002-35	1	f	M	N
CCS 2002-37	1	f	J	U
CCS 2002-88	1	f	J/M	N
CCS 2002-87	1	f	J	U
CCS 2002-12	1	m	M	N
CCS 2002-74	1	f	M	L/U
CCS 2002-90	1	m	M	N
CCS 2002-83	0	f	J	U
CCS 2002-22	0	f	J	U
CCS 2002-29	0	f	J/M	U
CCS 2002-13	0	f	PM	N

Appendix A – continued.

Sex: m = male
f = female

Age class: J = Juvenile
J/M = Age 4 (earliest age of sexual maturity)
PM = Possibly mature (>4 years of age but no prior calving history)
M = Mature (at least 10 years of age and/or prior calving history)

Reproductive status: N = No viable calf produced or a male
L/N = Lactating at time of sampling and no viable calf produced
L/U = Lactating at time of sampling and pregnancy status unknown
U = Pregnancy status unknown
P = Pregnant, as confirmed by resighting with a calf

Appendix B – Catalogue numbers of humeri sampled from the Red Bay collection.

The bones were catalogued based on a grid overlying the site (Cumbaa, 1986).

Catalogue Number
24M10Q10-5
24M194Q1-1
24M196N2-2
24M196N2-6
24M196N2-7
24M196N7-23
24M196P7-10
24M196Q2-22
24M196R2-17
24M198P2-29
24M198P3-2
24M2M7-1
24M20R3-3
24M22R3-9
24M22R3-10
24M200P2-21
24M4J4-18
24M4M5-5
24M4M5-6
24M4M7-1
24M4P8-8
24M4Q3-19
24M6H5-2
24M6K7-13
24M903B2-2
24M903C2-7
24M903C2-8
24M903F2-1
24M903G3-4
24M903H1-2
24M903H3-6

Appendix C – Location of bones sampled during the 2003 field season.

BMMS represents the initials of the two researchers that sampled the bones (**B**renna **M**cLeod and **M**ackenzie **S**heridan).

Bone ID	Location	Latitude (N)	Longitude (W)
BMMS 01	Middle Bay, Quebec	51°27.686'	57°28.451'
BMMS 02	Middle Bay, Quebec	51°27.351'	57°28.483'
BMMS 04	Middle Bay, Quebec	51°27.296'	57°28.453'
BMMS 05	Middle Bay, Quebec	51°27.130'	57°28.627'
BMMS 08	Middle Bay, Quebec	51°27.119'	57°28.679'
BMMS 09	Middle Bay, Quebec	51°27.103'	57°28.716'
BMMS 11	West St. Modeste, NL	51°35.172'	56°42.689'
BMMS 12	Capstan Island, NL	51°34.156'	56°43.710'
BMMS 13	Capstan Island, NL	51°34.162'	56°43.697'
BMMS 14	Boney Shore, NL	51°43.663'	56°26.563'
BMMS 15	Boney Shore, NL	51°43.666'	56°26.549'
BMMS 16	Boney Shore, NL	51°43.688'	56°26.517'
BMMS 18	Boney Shore, NL	51°43.704'	56°26.498'
BMMS 19	Boney Shore, NL	51°43.702'	56°26.488'
BMMS 20	Boney Shore, NL	51°43.713'	56°26.482'
BMMS 21	Boney Shore, NL	51°43.708'	56°26.480'
BMMS 25	Boney Shore, NL	51°43.718'	56°26.459'
BMMS 26	Boney Shore, NL	51°43.718'	56°26.462'
BMMS 27	Boney Shore, NL	51°43.723'	56°26.462'
BMMS 28	Boney Shore, NL	51°43.716'	56°26.443'
BMMS 29	Boney Shore, NL	51°43.723'	56°26.445'
BMMS 30	Boney Shore, NL	51°43.723'	56°26.445'
BMMS 32	Boney Shore, NL	51°43.732'	56°26.433'
BMMS 33	Boney Shore, NL	51°43.732'	56°26.433'
BMMS 34	Boney Shore, NL	51°43.731'	56°26.429'
BMMS 35	Boney Shore, NL	51°43.737'	56°26.423'
BMMS 36	Boney Shore, NL	51°43.740'	56°26.419'
BMMS 37	Boney Shore, NL	51°43.740'	56°26.412'
BMMS 38	Boney Shore, NL	51°43.740'	56°26.412'
BMMS 40	Boney Shore, NL	51°43.740'	56°26.410'
BMMS 41	Boney Shore, NL	51°43.742'	56°26.407'
BMMS 42	Boney Shore, NL	51°43.742'	56°26.402'
BMMS 43	Boney Shore, NL	51°43.759'	56°26.369'
BMMS 44	Boney Shore, NL	51°43.757'	56°26.372'
BMMS 45	Boney Shore, NL	51°43.759'	56°26.370'
BMMS 46	Boney Shore, NL	51°43.760'	56°26.369'
BMMS 47	Boney Shore, NL	51°43.762'	56°26.371'
BMMS 48	Boney Shore, NL	51°43.764'	56°26.359'
BMMS 49	Boney Shore, NL	51°43.757'	56°26.372'

Appendix D – Relative progesterone concentrations (ng/g) for bones sampled, according to rank.

Rank	Bone ID	Relative Progesterone Concentration (ng/g)
1	24M4M5-6	69
2	24M196N2-2	28
3	24M4Q3-19	19
4	24M196N2-6	12
5	24M4J4-18	12
6	24M196R2-17	12
7	24M198P2-29	11
8	24M903H1-2	11
9	24M4P8-8	10
10	24M22R3-9	10
11	BMMS 27	10
12	24M20R3-3	10
13	24M194Q1-1	9
14	24M200P2-12	9
15	24M903B2-2	8
16	24M903C2-7	8
17	24M4M7-1	7
18	BMMS 47	7
19	24M4M5-5	7
20	24M2M7-1	7
21	24M903C2-8	6
22	24M186N7-23	6
23	24M903G3-4	6
24	24M198P3-2	6
25	24M196N2-7	6
26	24M200P2-21	6
27	24M6H5-2	6
28	24M196P7-10	5
29	24M22R3-10	5
30	BMMS 13	5
31	BMMS 16	5
32	24M903F2-1	4
33	BMMS 12	4
34	BMMS 46	4
35	24M6K7-13	4
36	BMMS 38	4
37	24M196Q2-2	4
38	BMMS 11	4
39	BMMS 41	4
40	BMMS 02	3
41	BMMS 14	3
42	BMMS 08	3
43	24M903H3-6	3
44	BMMS 43	3

Appendix D – continued.

Rank	Bone ID	Relative Progesterone Concentration (ng/g)
45	BMMS 40	3
46	BMMS 09	3
47	BMMS 45	3
48	BMMS 19	3
49	BMMS 18	3
50	BMMS 37	2
51	BMMS 15	2
52	BMMS 29	2
53	24M10Q10-5	2
54	BMMS 28	2
55	BMMS 48	2
56	BMMS 20	2
57	BMMS 25	2
58	BMMS 30	2
59	BMMS 35	2
60	BMMS 01	2
61	BMMS 42	2
62	BMMS 05	2
63	BMMS 21	2
64	BMMS 49	2
65	BMMS 04	2
66	BMMS 26	1
67	BMMS 33	1
68	BMMS 32	1
69	BMMS 44	1
70	BMMS 34	1
71	BMMS 36	1