Development of a non-invasive technique to determine reproductive hormones in cetaceans

Carolyn Hogg

PhD Thesis

University of Sydney

August 2005

This thesis is submitted to the University of Sydney in fulfilment of the requirement for the Degree of Doctor of Philosophy.

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

…………………………………

Carolyn Hogg

31 August 2005

Acknowledgements

Before I take the time to thank all of those that helped me succeed in this project I would like to thank all of those who said it would never work. This was probably the single most important factor that kept me going through the long hours and late nights of problem solving in the lab. My inherent belief that it would work and the knowledge that there was only a select few who believed me made the tedious hours pass by and it all worth it in the end.

I am completely indebted to my fabulous supervisors, Dr Tracey Rogers and Dr Russell Vickers, without whom this small and crazy idea would never have blossomed. Thank you to Tracey for believing in me when everyone else did not for your guidance, support and friendship. I will always be grateful. To Russell, for knowing that you are able to find the smallest and strangest things in saliva if only you have a secure grasp of chemistry and the tenacity to pursue it. Thank you for your support. You helped me discover that I could achieve anything if I was willing to chase it. Thank you to my Associate Supervisor Professor Chis Maxwell for valuable comments on the chapters. Without the great assistance of Sonia Gu, many of the finer points and inner workings of the LC-MS may never have been understood. Sonia was a great teacher, colleague and friend, who sat there and watched as this entire tale unfolded and became a reality. To Tanya Grassi, who kept me sane during those long months of getting the testosterone method to work. Tanya was there through the ups and downs of stability. Thank you for your reassurance that I was not mad and the results were correct and that the rest of them were in fact all wrong. Thank you for the hours of support and the copious amounts of red wine.

This program was made possible by a number of other amazing people. Thank you to Professor Laurie Mather for allowing whale snot to take over the corner of the lab for the past 3.5 years. Thank you also to the staff and management of Sea World Gold Coast for the use of their animals. For always being willing to provide saliva and blow at a moment's notice and ensuring that it all ran smoothly. Thanks in particular to Trevor Long and Guy Bedford and the dolphin trainers of Dolphin Cove and Dolphin Bay, Rosco, Clare, Tasha, Shari, Spike, Rachel, Macca, Todd, Kylie and everyone else.

The aim of this study was to design a method to collect blow from free-swimming whales. Thanks must go to all those who made humpback whale blow collection possible. Thank you to the members of HARC, namely Doug Cato (DTSO), Mike Noad (University of Queensland) and Dale Stokes (Scripps Oceanographic Institute) who made the field seasons possible. Thanks also to Tony White (DSTO) and Dave Clement for their comments on the original pole and bracket. Perhaps the greatest thank you goes to Alex Shorter and Kira Barton (Woods Hole Oceanographic Institute) who believed that with their pole and my snot collector anything was possible. Without their enthusiasm of the first season I believe that there would not have been a second. Thank you to Patrick Miller, Nicolletta Biasonetti and Jim Partan (WHOI) who were the extraordinary crew of the second season and the tagging/snot collecting experts. Thanks also to Michael Moore and his brother in-law who discovered the finer points of carbon fibre poles. However, perhaps the biggest thank you must go to Dave Paton, the most brilliant boat handler around whales I have ever seen. Thank you for your support, humour and long discussions at sea. The whale seasons would not have been the same if it weren't for a few others, Josh Smith (PhD partner-in-crime) who was always willing to share a beer and commiserate, Ceri Morris, Becca Dunlop, Alice Pope and Michaela Ciaglia.

Thanks must also go to my fellow PhD students in the lab, Angelo Jayammane, Hamish Ross, and David Lee, who travelled with me along the journey of character building, late nights and the insanity that constitutes a PhD. To the post docs who kept reminding me that failed experiments eventually become successful ones if you persevere for long enough. Dr Kristina Vikman and Dr Michelle Gerke, thank you for the words of wisdom and chats over coffee.

Lastly but not least to my family and friends who were there every step of the way. To Mum, Dad, Dunc and Ant for never really understanding what I was on about but still being so very proud of me. To Brad for believing that I was the most interesting dinner conversation in years; Susan, for making me meet my milestones; and Sue for always telling me this is what I was destined to achieve. Most importantly, thank you to Jeff, for his lack of interest in the great leviathans and who showed me that there was more to life than whales, who put up with the late night lab runs, waiting in the car when I needed to "just switch something off", for listening to the problems and trying to offer solutions, for giving me the biggest incentive to finish in a timely fashion and most of all for just being there. Thank you from the bottom of my heart.

Executive Summary

Reproductive physiology plays a vital role in population growth and vitality. Baseline data on reproductive physiology and a comprehensive knowledge of breeding biology are essential to conservation management. Great whales have been hunted from the 16^{th} century to the present day. Although many populations are increasing there are populations with low or declining reproductive rates. In 2001 it was recommended to the International Whaling Commission that new techniques be developed to assess the internal physiology of great whales. This study, based on this recommendation, aims to develop analytical methods to assess reproductive hormones in cetacean blow samples and determine the feasibility of its use with free-swimming great whales.

A method for the assessment of steroid hormone concentrations using liquid chromatography-mass spectrometry (LC-MS) was developed and validated. These methods were then used to determine testosterone and progesterone concentrations in saliva and blow of bottlenose dolphins. The stability of testosterone and progesterone was found to be a major issue. Without inhibitors, hormone concentrations increased by up to 65% over three hours at 21°C. Storing samples at low temperatures (-20°C or -80°C) slowed but did not cease the rate of change. The addition of inhibitors, manganese chloride and amoxycillin potassium/clavulanate, improved the stability of testosterone and progesterone. It is proposed that when using dolphin saliva and blow samples to measure reproductive hormones the samples are extracted as soon as possible after collection to prevent degradation. This study highlighted the need to address steroid hormone stability prior to any long-term biological program, to ensure that changes seen in hormone concentration are due to biological activity rather than storage.

A technique to collect blow samples from free-swimming great whales was developed. This technique, in conjunction with the specially developed LC-MS methods allowed for the determination of testosterone and progesterone concentrations in humpback whale blow. The techniques developed in this study to determine reproductive hormones in cetacean saliva and blow have applications for both captive and wild population studies. In captive institutions, saliva and/or blow can be used to monitor reproductive cycling in both females and males. As it is non-invasive it can be used on a daily basis with minimal stress to the animals. The use of blow sampling has the capacity to improve our understanding of reproductive cycling in great whales as it can be used to sample animals in both the breeding and feeding areas. This technique may allow us to now examine whether reproductive dysfunction is playing a role in the slow recovery of critically endangered species such as the North Atlantic right whale.

Table of Contents

Chapter 2 – General Methodology

Chapter 3 – Testosterone

Chapter 4 – Testosterone Stability

Chapter 5 – Female Reproductive Hormones

Chapter 6 – Progesterone Stability

Chapter 7 – Whale Blow Collection: is it feasible?

Chapter 8 – General Discussion

Appendices

Tables

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Abbreviations Used

ANOVA	Analysis of variance
ART	Assisted reproductive technology
CL	corpus luteum
Cr	Creatinine
Da	Daltons
DHT	5α-dihydrotestosterone
EDCs	Endocrine disrupting chemicals
EIA	Enzyme immunoassay
ESI	Electrospray ionisation
FSH	Follicle stimulating hormone
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GnRH	Gonadotropin releasing hormone
H	Proton
hCG	Human chorionic gonadotropin
HPLC	High performance liquid chromatography
hrs	Hours
IWC	International Whaling Commission
IS	Internal standard
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LC-MS-MS	Liquid chromatography-mass spectrometry-mass spectrometry
LH	Luteinising hormone
LOD	Limit of detection

LOQ	Limit of quantification
M	Molecular mass
MeOH	Methanol
min	Minute
mins	Minutes
$MnCl_2$	Manganese chloride
MS	Mass spectrometry
mw	Molecular weight
m/z	Mass-to-charge ratio
NI	No inhibitors
PdG	Pregnanediol-3α-glucuronide
PTP	Plasma transport protein
RE	Relative error
RIA	Radioimmunoassay
RSD	Relative standard deviation
RT	Retention time
scc	Side chain cleavage
secs	Seconds
SIM	Selected-ion monitoring mode
SPE	Solid phase extraction
SRM	Selected-reaction monitoring mode
TOF	Time-of-flight mass spectrometry

Scientific Names

Asian elephant	*Elephas maximus*
Bandicoot	*Isoodon macrourus*
Black Rhinoceros	*Diceros bicornis*
Bottlenose dolphin	*Tursiops truncatus*
Bowhead whale	*Balaena mysticetus*
California sea lion	*Zalophus californianus*
Capuchin monkeys	*Cebus apella*
Cattle	*Bos* sp.
Chimpanzee	*Pan troglodytes schweinfurthii*
Common dolphin	*Delphinus delphis*
Dairy cow	*Bos taurus*
False killer whale	*Pseudorca crassidens*
Feral horses	*Equus caballus*
Fin whale	*Balaenoptera physalus*
Goat	*Capra hircus*
Gorillas	*Gorilla gorilla*
Harbour seal	*Phoca vitulina*
Horse	*Equus caballus*
Human	*Homo sapiens*
Humpback whale	*Megaptera novaeangliae*
Killer whale	*Orcinus orca*
Minke whale	*Balaenoptera acutostrata*
Monk seal	*Monachus schauinslandi*
Muriquis	*Brachteles arachnoides*
Nile hippopotamus	*Hippopotamus amphibius*

North Atlatncic right whale	*Eubalaena glacilias*
One-humped camel	*Camelus dromedarius*
Pig	*Sus scrofa*
Polar bear	*Ursus maritimus*
Rat	*Rattus norvegicus*
River buffalo	*Bubalus bubalis*
Sei whale	*Balaenoptera borealis*
Sheep	*Ovis aries*
Short-finned pilot whale	*Globicephala macrorhynchus*
Siberian tigers	*Panthera tigris altaica*
Southern right whale	*Eubalaena australis*
Sperm whale	*Physeter macrocephalus*
Timber Wolf	*Canis lupis*
Western lowland gorilla	*Gorilla gorilla gorilla*
White-tailed deer	*Odocoileus virginianus*

"Instead of predicting new events with great precision, or explaining what has already been found, a great deal of scientific progress is linked to what is deemed possible and likely. Expectations, however vague and intuitive, lay the groundwork for discovery……"

Frans de Waal, 2001.