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Results of feasibility studies on novel non-lethal techniques to address the main objectives of NEWREP-A

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ABSTRACT

This paper presents the progress made in the research conducted to evaluate the feasibility of some novel non-lethal techniques. The techniques are DNA methylation for age determination and progesterone analysis for determining the reproductive status of whales. Age and reproductive status of whales are key information for addressing one of the main objectives of the NEWREP-A. Potentially both techniques can be used on biopsy samples collected from free ranging whales. Regarding the former technique, effort was spent to investigate differences in methylation rates between Antarctic minke and humpback whales. Regarding the latter technique, effort was made to examine progesterone from blubber of female Antarctic minke whales for which high qualitative reproductive data was already available. A final evaluation of both techniques will be made in the near future considering the protocol developed for evaluating novel non-lethal techniques (Mogoe, this issue).

INTRODUCTION

The Institute of Cetacean Research (ICR) conducts whale research using both lethal and non-lethal techniques. Lethal techniques are required for key research objectives of NEWREP-A such as the estimation of biological parameters based on age and reproductive data, and feeding ecology based on qualitative and quantitative analyses of stomach contents.

Routine non-lethal techniques used by ICR include systematic sighting surveys for abundance estimates, oceanographic surveys for ecological studies and photo-id and biopsy sampling for studies on movement, distribution and stock structure in blue, humpback and right whales. These routine non-lethal techniques have been used by ICR for many years in its whale research programs in the Antarctic and western North Pacific.

More recently some novel non-lethal techniques have been proposed, which potentially could be used to address some key objectives of NEWREP-A. For example, DNA methylation in skin to investigate the age of the animals, analysis of progesterone level in blubber to investigate the reproductive status of female whales, and stable isotopes in skin/blubber to study the feeding ecology of whales. These techniques can potentially be used with skin/blubber samples obtained by biopsy sampling.

ICR started the evaluation of the feasibility of using the non-lethal techniques mentioned above. The evaluation involves two aspects, the first is whether biopsy sampling (source of the samples) is feasible in the target species of the research programs. The other aspect is the analytical one, which involves laboratory and statistical analyses (see Mogoe, this issue).

The degree of difficulty of biopsy sampling is different among whale species, being more difficult for smaller and fast swimming species such as the minke whale. Biopsy sampling was considered feasible in the case of large whales such as sei and Bryde's whales in the North Pacific however it was considered not feasible for the case of North Pacific common and Antarctic minke whales (Yasunaga *et al.*, 2017; 2018).

This paper summarizes the laboratory and statistical procedures to investigate the feasibility of using non-lethal techniques to determine age and reproductive status in Antarctic minke whale, the target species for lethal sampling under the NEWREP-A. Tissue samples from the sampled Antarctic minke whale individuals were used as a proxy for biopsy sampling of skin and blubber. The outputs of these studies will be used for a final evaluation of non-lethal techniques based on some specified criteria (see Mogoe, this issue).

DNA METHYLATION FOR AGE DETERMINATION IN WHALES

DNA methylation as a tool for age determination

Chronological age is an important factor in animal ecology because many biological characteristics change with time (Jarman *et al.*, 2015). Biological ageing is a combination of programmed processes (Berdasco and Esteller, 2012; Horvath, 2013) and accumulated changes caused by unrepaired environmental damage (Kujoth *et al.*, 2005). Recent evidence suggests that epigenetic changes are both directing the process of ageing and being caused by it (Maegawa *et al.*, 2010; Koch *et al.*, 2011; Winnefeld and Lyko, 2012; Hannum *et al.*, 2013; Horvath, 2013).

The best studied class of epigenetic change in vertebrates is the methyl group presence or absence at the C5 position of Cytosine residues that are adjacent to Guanidine residues ('CpG sites') (Figure 1). CpG methylation levels play an important role in control of gene expression, where higher methylation levels ('hypermethylation') generally reduce gene transcription rate.

Methylation changes at specific CpGs have been linked to age in mice (Maegawa *et al.*, 2010) and humans (Christensen *et al.*, 2009; Grönniger *et al.*, 2010; Bocklandt *et al.*, 2011; Koch and Wagner, 2011; Hannum *et al.*, 2013). For the cetacean species, the DNA methylation approach was recently developed and applied to humpback whales with the aim of age determination in this species (Polanowski *et al.*, 2014).

Detection methods

DNA methylation can be detected by several methods, one of them being the pyrosequencing of bisulfite treated DNA, which was used in the case of the humpback whale (Polanowski *et al.*, 2014) and in the present study for Antarctic minke whales. This method implies the use of bisulfite treatment of DNA to determine its pattern of methylation. In animals it predominantly involves the addition of a methyl group to the carbon-5 position of cytosine residues of the dinucleotide CpG, and is implicated in repression of transcriptional activity. Treatment of DNA with bisulfite converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected. Therefore, DNA that has been treated with bisulfite retains only methylated cytosines. The percentage of DNA mismatch is recorded, this gives the user a percentage methylation per CpG site.

Research on DNA methylation at ICR

As a first step to evaluate the DNA methylation for age determination purpose, the same method used for humpback whale was applied to Antarctic minke whale. The purpose in this first step was to compare DNA methylation rates between two baleen whale species. The work conducted on Antarctic minke whale is explained in the sections below.

Samples

A total of 100 Antarctic minke whale samples taken during the JARPAII were used for the study. Each sample had good age information obtained by earplug reading. Details of the samples are shown in Table 1. DNA was extracted from each sample using standard protocols.

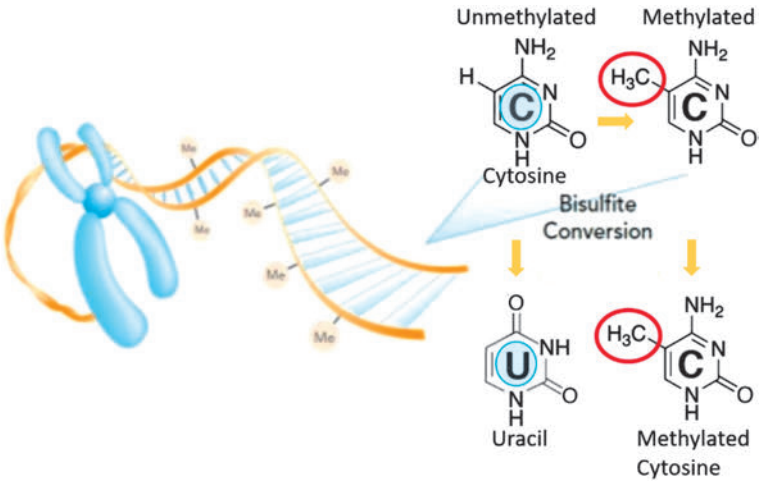

Figure 1. Methylation of cytosine to form 5-methylcytosine occurs at the same C5 position on the pyrimidine ring (upper right), the same position distinguishes thymine from the analogous RNA base uracil after bisulfite conversion (lower right), and then methylation rate is estimated by the ratio of thymine and cytosine.

Table 1

The sample sizes of Antarctic minke whales used for DNA methylation analysis by pyrosequencing method by sampling season, sex and age classes.

Season	Sex	Age					Total
		–10	11–20	21–30	31–40	41–	
2010/2011	F	10	16	9	3	3	41
	M	7	8	8	2		25
2011/2012	F	1	8	11	6	1	27
	M	1	3	1	1	1	7
Total		19	35	29	12	5	100

Figure 2. PYROMARK 24 Pyrosequencing System used in this study.

Identification of age-related epigenetic markers in Antarctic minke whale

The procedure for identification of age-related DNA methylation sites and measurement of methylation levels followed Polanowski *et al.* (2014). The following seven CpG sites in three genes were selected for this study because they showed significant correspondence between CpG methylation levels and age in humpback whales.

Gene TET2 (three sites: CpG+16; CpG+21 and CpG+31)
Gene CDKN2A (three sites: CpG+297; CpG+303 and CpG+309)
Gene GRIA2 (one site: CpG+202)

Measurement of cytosine methylation levels and correlation with age

Cytosine methylation levels were measured with Qiagen PyroMark assays. The pyrosequencing assays were designed using PYROMARK Assay Design Software (Version 2.0.1, Qiagen). Pyrosequencing was performed on a PYROMARK 24 Pyrosequencing System (Qiagen) (Figure 2). The PYROMARK Q24 software gave percentage methylation values for each CpG site (Figure 3).

DNA methylation rates in the seven sites were scored successfully, and regressions of each CpG methylation rate against age determined by earplug reading were made (Figure 4).

Correlations of all seven CpG sites in three loci with age based on earplug reading were lower than in the case of the humpback whale study (Table 2). The standard deviation for Antarctic minke whale by the Leave One

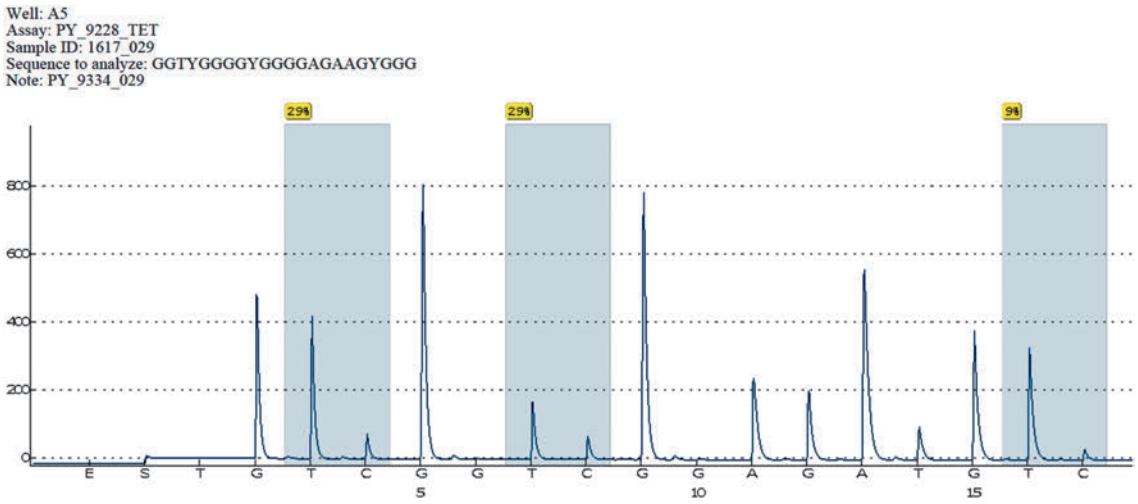

Figure 3. An Example of output of CpG Pyrogram report by PYROMARK 24 Pyrosequencing System of three CpG sites in TET2 gene. Methylation rate of each CpG site is estimated by the ratio of the integral of Thymine to Cytosine in gray shadow.

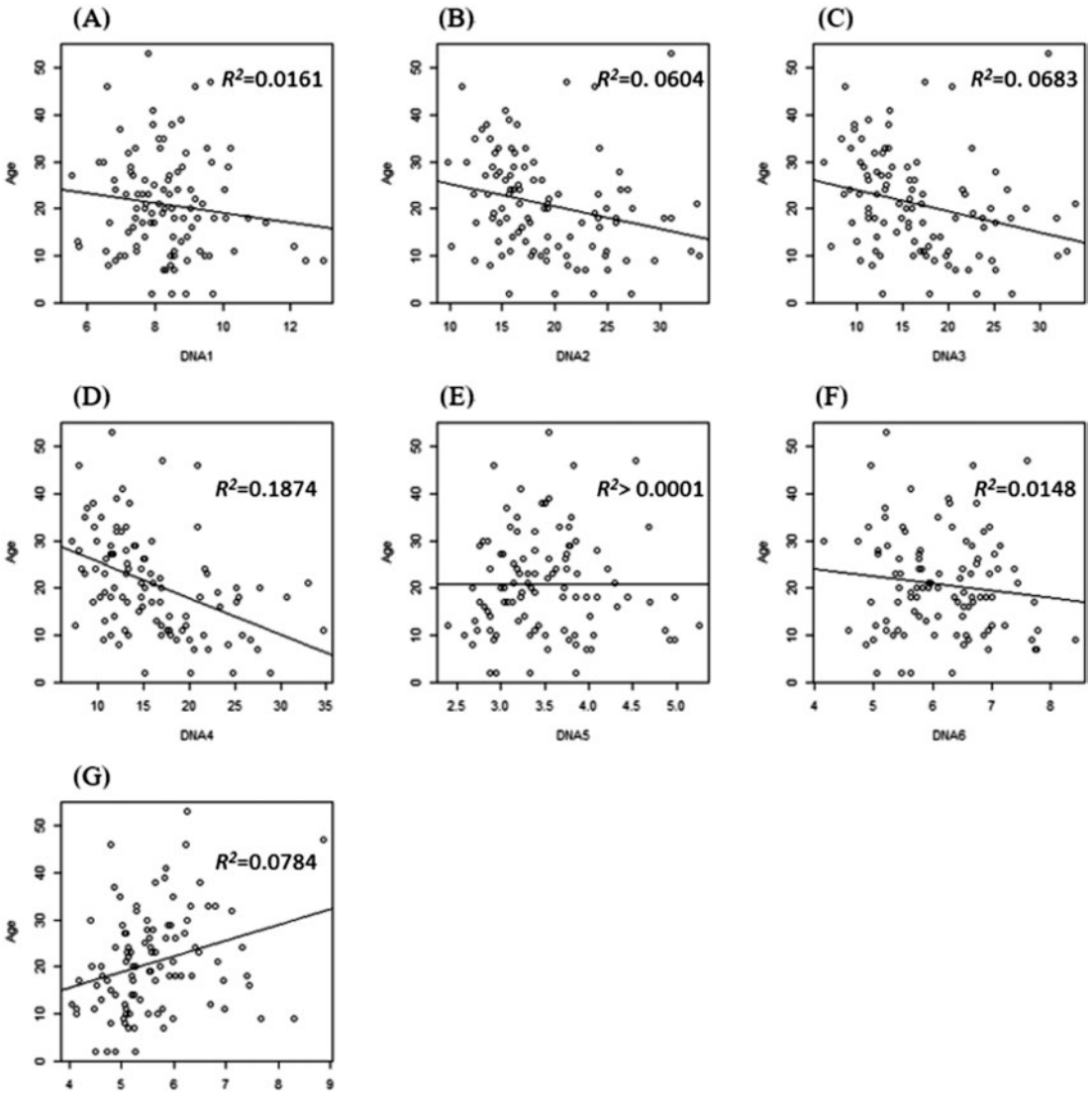

Figure 4. Regressions between CpG methylation and age determined by earplug at seven sites (n=100). CpG sites are: (A) GRIA2_CpG+202, (B) TET2_CpG+16, (C) TET2_CpG+21, (D) TET2_CpG+31, (E) CDKN2A_CpG+297, (F) CDKN2A_CpG+303 and (G) CDKN2A_CpG+309.

Table 2

Comparison of the coefficient of determination (R^2) between methylation rates of seven CpG sites and age determined from earplug reading in humpback (Polanowski *et al.*, 2014) and Antarctic minke (this study) whales.

Gene and CpG position	Humpback whale	This study
GRIA2_CpG+202	0.469	0.0161
TET2_CpG+16	0.174	0.0604
TET2_CpG+21	0.189	0.0683
TET2_CpG+31	0.409	0.1874
CDKN2A_CpG+297	0.409	>0.0001
CDKN2A_CpG+303	0.211	0.0148
CDKN2A_CpG+309	0.344	0.0784

Out Cross Validation (LOOCV) method was estimated at 8.865 years (Goto *et al.*, 2018), while that for the humpback whale was estimated at 2.991 years using the same method (Polanowski *et al.*, 2014). Therefore the main conclusion of this study is that the methylation rates for a same gene and sites are different between the two baleen whale species.

Future works

The ICR is planning investigation of additional informative methylation sites. Such investigation should include analyses on how the methylation rates changes among body tissues as well among body parts of the whale, among other factors.

With all information at hand the feasibility of using the DNA methylation technique for age determination in Ant-

arctic minke whale can be further evaluated.

HORMONE ANALYSIS FOR DETERMINATION OF REPRODUCTIVE STATUS IN WHALES

Progesterone analysis for determination of reproductive status in female whales

In cetacean species female reproductive status has been determined by direct observation of reproductive organs such as ovaries and uteruses. Progesterone is one of the sex hormones produced from ovaries and placenta (when an individual gets pregnant) and it has role in ovulation and maintaining pregnancy.

Previous studies suggested that an elevation in the concentration of progesterone in serum can be indicative of ovulation and pregnancy diagnosis in captive cetaceans (Sawyer-Steffan *et al.*, 1983; Schroeder and Keller, 1990; Kirby, 1990). Recently, the relationship between progesterone and reproductive status has been studied based on samples which potentially can be obtained by non-lethal approach from free ranging cetaceans (Table 3). Several of those studies reported that progesterone can be detected from the samples used, and that its concentration was substantially higher in pregnant than immature females.

Methods

There are two main groups of methods to measure progesterone level, one is liquid chromatography (e.g. HPLC and LC-MS in Table 3) and the other is immunoassay. The latter has been used in the studies conducted by the ICR.

Immunoassay

Immunoassay is a method that measures the presence or concentration of target analytes (like hormones) in solution using antigen-antibody reactions. The antigen-antibody reaction is a specific binding between an antigen and an antibody. The reaction can be used for the detection of antigens or antibodies specific for analytes.

Immunoassay measures the formation of antigen-antibody complexes by labeling or labeling-free format. There are several types of immunoassays depending on labeling material, for example, labeling by radioisotope is called radioimmunoassay (RIA), labeling by enzyme is called enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA). The immunoassay used at the ICR is the latter one (ELISA) (Figure 5).

Research on progesterone at ICR

In the ICR, the relationship between concentration of progesterone in blubber and reproductive status in the Antarctic minke whale was investigated by examining female Antarctic minke whales. The main question was whether or not progesterone concentration in blubber can be used as an indicator of the reproductive status of female whales.

Samples

Antarctic minke whales used in this study were collected between December 2015 and February 2016 by NEWREP-A in the Pacific sector of the Antarctic Ocean. Whales were sampled randomly from a predetermined zigzag track line designed to cover the whole research area. A total of 333 Antarctic minke whales (103 males and 230 females) were sampled during the survey. All sampled females (230) were used in this study. The blubber tissues were obtained from the lateral side of each whale by researchers on board the research base ship. The tissues were stored at −20°C until analyses at the laboratory.

Determination of reproductive status

Reproductive status was determined by standard procedures (Lockyer 1984; 1987). The sexual maturity of

Table 3

Summary of the non-lethal studies to measure sex hormones in whales.

Species	Samples	Methods	References
Mysticeti			
Blue whale *Balaenoptera musculus*	Feces	RIA, HPLC	Valenzuela-Molina *et al.* (2018)
North Atlantic right whale *Eubalaena glacialis*	Feces	RIA	Corkeron *et al.* (2017)
	Blow	LC-MS	Hogg *et al.* (2009)
Humpback whale *Megaptera novaeangliae*	Blubber	EIA	Clark *et al.* (2016)
	Blubber	EIA	Pallin *et al.* (2018)
	Blow	LC-MS	Hogg *et al.* (2009)
Odontoceti			
Beluga *Delphinapterus leucas*	Blow	ELISA	Richard *et al.* (2017)

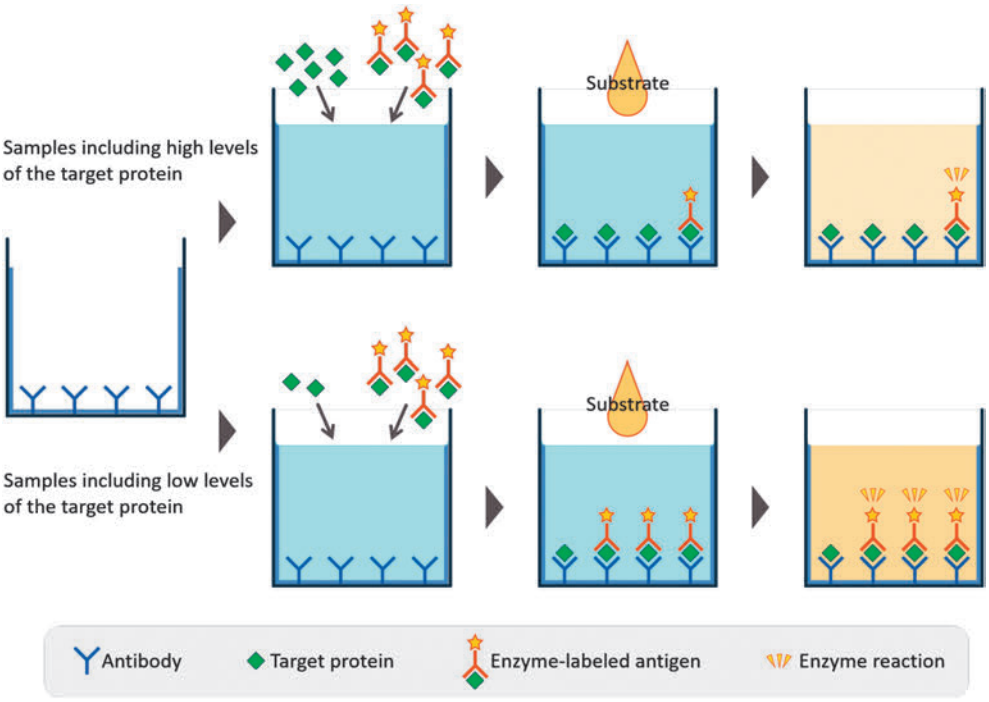

Figure 5. Schematic representation of ELISA. An antibody specific for a target protein is immobilized on the surface of microplate wells. Then it is incubated with samples containing the target protein and a known amount of enzyme-labeled target protein. After the reaction, the substrate is added and the activity of the microplate well-bound enzyme is measured. When the antigen level in the sample is high, the level of antibody-bound enzyme-labeled antigen is lower and the color is lighter. Conversely, when it is low, the level of antibody-bound enzyme-labeled antigen is higher and the color is darker.

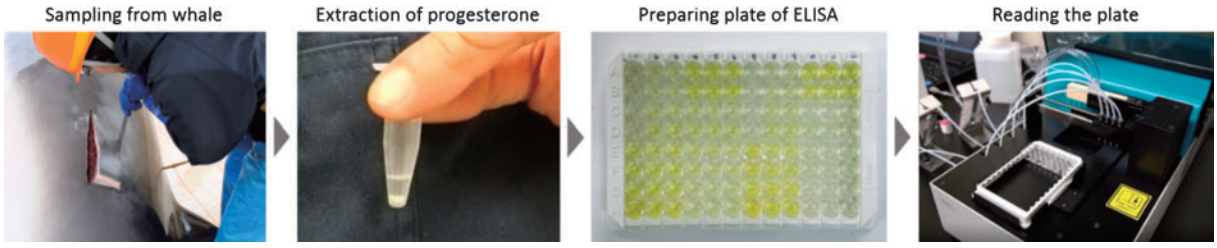

Figure 6. Flow from collection of blubber sample to measurement of progesterone concentration.

females was determined by the presence of at least one corpus luteum or corpus albicans in either ovary. In the case where no corpus luteum and corpus albicans was observed, the female was categorized as immature. Reproductive status of mature female whales was classified into three categories (resting, ovulating and pregnant), based upon observation of the ovary and uterus.

Extraction and measurement of progesterone

The procedures for progesterone extraction and defatting treatment were performed according to Kellar *et al.* (2013) and Nagata *et al.* (1996), respectively. Progesterone concentrations were determined by a commercially available ELISA kit, using the Crocodile Mini Workstation (Titertek Berthold, Germany). All samples were processed and quantified in triplicate. Figure 6 shows the steps followed in the progesterone concentration study.

Results

Table 4 shows the results of the reproductive status determined by direct observation of reproductive organs, progesterone concentrations by reproductive status and other biological data for the total sample of 230 female whales.

Seventy-six percent of the samples were mature females, and 90% of the mature females were pregnant. About 73% of the immature whales were below the detection limit of the assay for progesterone concentration (0.2 ng/g). The pregnant females had the highest median of 72 ng/g (range: 13–740 ng/g), with wider concentrations of progesterone. All pairwise comparisons apart from the ovulating-pregnant comparison, resulted in

Table 4
Biological data, determined reproductive status and progesterone concentration in the female Antarctic minke whales.

	Immature	Resting	Ovulating	Pregnant
Body length (m)				
Median	7.37	8.81	9.01	8.78
Range	5.17–8.51	7.65–9.32	8.45–9.40	7.71–10.06
Ovary				
total CL				
Mean	0	0	1	1
Range	0	0	1–1	1–2
total CA				
Mean	0	18	16	13
Range	0	1–44	0–45	0–40
Progesterone Concentration (ng/g)				
Median	<0.2	15	26	72
Range	<0.2–2.6	<0.2–34	24–150	13–740
N	56	11	6	157

significant statistical differences (Steel-Dwass post-hoc test, $p<0.05$). However, overlap of progesterone concentrations was observed between each reproductive status with the exception of the cases immature/ovulating and immature/pregnant.

Summary

In this study, significant differences were found in median progesterone concentration between all reproductive categories except in the case between ovulating and pregnant females. However, the ranges of progesterone concentration overlapped between each reproductive category with the exception of the cases immature/ovulating and immature/pregnant. The results of this study indicated that the progesterone concentration in blubber, which potentially can be obtained by biopsy sampling, cannot be used as an accurate diagnostic index to determine the reproductive status in the Antarctic minke whale.

CONCLUDING REMARKS

Progress has been made in the work to evaluate novel non-genetic techniques that potentially could be used to address some important objectives of NEWREP-A. The degree of progress is different among the specific technique examined and obviously further refinement work is required. Stable isotopes are also being examined to elucidate the feeding habits of some large baleen whale species in the Antarctic. As stated earlier a final evaluation of the feasibility of those techniques will be made in the near future following specific criteria (see Mogoe, this issue).

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