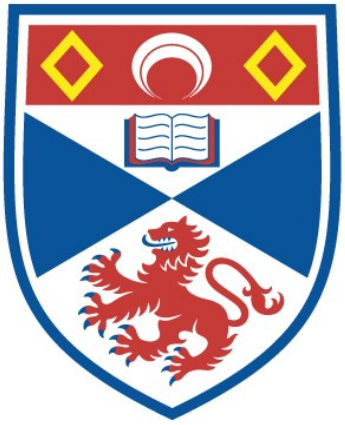
NOT JUST FAT: INVESTIGATING NOVEL PHYSIOLOGICAL STATE BIOMARKERS IN CETACEAN BLUBBER

Joanna Kershaw

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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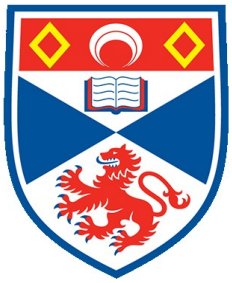

University of
St Andrews

This thesis is submitted in partial fulfilment for the degree of

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ABSTRACT

Despite its importance as a predictor of fitness, no consensus has been reached about how to assess the nutritive body condition of free-ranging cetaceans. Standard indices of condition used in terrestrial ecology were assessed in cetaceans using data and samples from stranded animals. The most appropriate morphometric indices were identified as mass/length^2 and girth/length (where mass data were unavailable). Blubber thickness, and blubber lipid content were poor indicators of condition. Variation in lipid content suggested that there may be trade-offs occurring between different blubber functions involved in energy storage, controlling buoyancy, and preserving thermoregulatory properties in species with different life-history strategies.

Novel blubber biomarkers of physiological state were investigated as objective and widely applicable tools for estimating body condition. Blubber cortisol concentrations were negatively correlated with morphometric indices of condition in stranded animals from two cetacean families. Blubber cortisol concentrations were thus identified as a candidate biomarker. When applied to a 13-year dataset of biopsy samples from female humpback whales (*Megaptera novaeangliae*), cortisol concentrations showed significant inter-annual variation. Concentrations were weakly correlated with annual pregnancy rates. High individual variability in blubber cortisol concentrations was likely a result of the multi-functional nature of cortisol. Cortisol concentrations thus probably provide a 'snap shot' of the metabolic state of the tissue when sampled.

A proteomic approach was developed to extract and identify proteins in blubber. A range of proteins involved in numerous metabolic processes and pathways were identified. These proteins likely capture the range of physiological processes experienced by individuals at the time of sampling. This new proteomic approach will help to assign novel functions to marine mammal blubber in keeping with current understanding of the multi-functional role of adipose tissue as an endocrine organ in mammals. It will also allow the future development of protein biomarkers of health and physiological state.

CHAPTER 1 : GENERAL INTRODUCTION

Body condition can be defined in various ways. Here, the focus is on nutritive condition quantified as the energy stores of an individual (Aguilar and Borrell, 1990; Pitt et al., 2006). The size of these energy stores reflects an individual's foraging effort and success, as well as their reproductive needs throughout their life cycle (Aguilar and Borrell, 1990). Animals in poorer condition have smaller energy stores than those in better condition. In females, overall body condition is a good predictor of fitness by affecting both survival and reproductive success (Hall et al., 2001; Lockyer, 1986; Pitcher et al., 1998; Pomeroy et al., 1999; Williams et al., 2013). For males, individual body condition also affects reproductive success because larger energy reserves are advantageous when Fighting for access to, or defending receptive females (Forsyth et al., 2005; Lane et al., 2010). Body condition will also affect an individual's ability to resist infectious diseases, and its ability to survive periods of food shortage (Møller et al., 1998; Verrier et al., 2011). More broadly, body condition has also been shown to affect an animal's behavioural patterns in relation to foraging strategies, predator avoidance, and migratory movements (Bêty et al., 2003; Heithaus et al., 2007).

Variation in body condition has been linked to environmental quality and anthropogenic activities and disturbance in a number of species (Bourbonnais et al., 2014; Delgiudice et al., 2001; Harwood et al., 2000; Parker et al., 2009). Therefore, determining how body condition influences vital rates and population dynamics is important for understanding species ecology, and measuring resilience to environmental or anthropogenic stresses. Moreover, top marine predators like marine mammals, can be valuable sentinel species of environmental quality as their health may ultimately reflect the health of the ecosystems of which they are part.

Accurate measurement of body condition across age and sex classes is therefore a valuable tool for conservation physiologists to assess individual health. The intrinsic complications of studying cetaceans, however, make direct measurements of energetic reserves impossible in live animals as the 'standard' set of morphometric measurements and biological samples (blood, faeces and urine) cannot be routinely obtained. There are currently increasing efforts to develop standardized methodologies for robust, minimally invasive measures that can be applied across large numbers of individuals for population monitoring programmes.

Cetaceans have a thick layer of subcutaneous adipose tissue called blubber which is their main site of lipid energy storage (Koopman et al., 2002; Lockyer, 1987). It typically makes up anywhere between 15% and 55% of the body mass, depending on the species (McLellan et al., 2002; Ryg et al., 1993). For this reason, body condition and energy stores of cetaceans were traditionally examined using measurements of blubber thickness, total blubber volume, and blubber lipid content of carcasses in conjunction with whaling operations (Konishi, 2006; Lockyer, 1986; Lockyer, 1987; Víkingsson, 1995). Now, blubber samples from *live* animals are routinely collected. Dart biopsying has rapidly become a common collection method for obtaining biological tissue samples (Hunt et al., 2013).

Dart biopsy samples are collected using either a crossbow or a pneumatic rifle with modified dart tips. These tips are hollow, thin-walled, surgical grade stainless-steel or titanium cylinders,

between 4 - 6 mm in diameter and 20 - 60 mm in length. Each dart has a cutting lead edge and small internal barbs to retain the sample after impact (Barrett-Lennard et al., 1996; Larsen, 1998). The core sample collected from the dart is typically between 4 - 6 mm in diameter with varying lengths, usually between 5 and 30 mm, depending on the species and the impact site. This core sample consists of both skin and underlying blubber tissue. The species-specific thickness of the skin and the blubber will determine the depth to which the core sample penetrates through the tissue, and therefore how representative it is of the full blubber layer.

Biopsy samples have previously provided information about diet through fatty acid analysis of the blubber (Waugh et al., 2012) and stable isotope analysis of the skin (Gavrilchuck et al., 2014). Pollutant exposure has been assessed through contaminant concentrations measured in the blubber (Krahn et al., 2007; Metcalfe et al., 2004). Genotype profiling of DNA from skin samples has provided information on population genetics (Morin et al., 2017; Palsbøll et al., 1997). The analysis of biopsy samples can therefore provide a wide range of ecological information. Biopsy samples are also increasingly being used to study different physiological processes in sampled individuals. For example, altered stress response protein expression has been measured in skin samples (Dizon et al., 2002; Southern et al., 2002). It was suggested that this method may be suitable for assessing chronic stress rather than the acute stress associated with sampling. The genes involved in the vitamin D_3 pathway have been detected in the skin and may provide information on immune system function (Ellis et al., 2009). Finally, cytochrome-P related enzymes in the mammalian liver are effective biomarkers of pollutant burden. These enzymes have now been measured in skin samples from several cetacean species and appear to be useful indices of contaminant exposure (Fossi et al., 2010; Waugh et al., 2011).

To date, physiological state markers in the blubber have focused on lipophilic steroid hormones to investigate reproductive status (Clark et al., 2016; Pallin et al., 2018a; Pallin et al., 2018b; Perez et al., 2011), sexual maturity (Kellar et al., 2009), and adrenal function and the stress response (Champagne et al., 2017; Champagne et al., 2018; Kellar et al., 2015; Schwacke et al., 2014; Trana et al., 2016). Recent advances in molecular sequencing and biochemical detection techniques have led to the application of transcriptomic analyses of blubber tissues to investigate adipogenesis, lipolysis and fasting metabolism in phocid seals (Khudyakov et al., 2017; Martinez et al., 2018). This approach has not yet been applied to cetacean blubber samples. The blubber is therefore now being increasingly recognised as a valuable tissue for conservation physiologists for three main reasons. Firstly, the blubber plays a key role in energy storage and metabolic homeostasis. Secondly, there is a growing field of research to maximise the ecological and physiological information available in biopsy samples. Thirdly, blubber is easily accessible for sampling.

The next stage is to identify measurable parameters, or biomarkers, in the tissue that can serve as reliable and cost-effective indicators of nutritive body condition and physiological state in order to make inferences about individual health. A biomarker is defined as a naturally occurring molecule, gene, or characteristic which allows a particular pathological or physiological process to be identified (Atkinson et al., 2001). The application of biomarker techniques is currently widely used in human medicine and ecotoxicology (Steffen et al., 2016; Zhang et al., 2004). New biomarker approaches applied to cetacean research will benefit from

interdisciplinary collaborations between cetacean biologists and experts in other areas of health and medical sciences for the integration of different methods.

The development of new biomarkers is an emergent and timely approach as the recent 'omics' revolution has provided new tools and methods for both the qualitative and the quantitative assessment of a wide range of biomolecules from the genome to the metabolome level (Gomez-Cabrero et al., 2014; Horgan and Kenny, 2011). The term 'omics' refers to a group of technologies that characterise and quantify biological molecules in order to explore their roles in cells and tissues. The 'omics' suffix has been added to describe the use of these technologies to examine proteins (proteomics), metabolites (metabolomics) and RNA (transcriptomics) (Horgan and Kenny, 2011). As a result, explorative analysis (instead of hypothesis driven research) has become applicable to a range of different cell types, tissues and organs (Gomez-Cabrero et al., 2014). Here, these approaches will improve our ability to determine body condition in its broadest sense because they encompass a range of processes that contribute to an individual's heath and physiological state at the time of sampling. This kind of information may not be captured in basic measures of morphometrics, lipid stores or fatty acid profiles, for example.

For effective method development and biomarker identification, full depth blubber samples are required from individuals of a known body condition and physiological state for 'ground truthing' purposes. Samples with accompanying individual covariates from stranded animals are therefore particularly important for this kind of work. Specifically, samples from individuals of different species with varied life-history strategies are important for comparison. Samples associated with post-mortem data including basic case descriptors, morphometrics, identification of pathological processes and, where possible, a cause of death are especially valuable. The use of different biomarkers of nutritive condition can thus be established using samples from fresh, dead, stranded animals first in order to accurately interpret results from live biopsy samples and to put them into a population-level context.

There are broadly two main themes of this work. The first is the validation of previously used, standard indices of body condition in cetaceans. The second is the investigation of novel blubber biomarkers of body condition, health and overall physiological state. These two main themes are split into the following aims:

1) Identify the most appropriate morphometric indices of overall body condition that reflect total body energy stores.
2) Investigate the use of blubber lipid content, a previously used index of body energy stores, as a biomarker of overall condition applicable to remotely obtained biopsy samples.
3) Investigate the use of blubber cortisol and blubber progesterone as endocrine biomarkers of body condition and pregnancy status, respectively.
4) Use a proteomic approach to explore the proteome of cetacean blubber tissue and investigate other potential biomarkers of health and physiological state.

The identification of the most appropriate morphometric indices of body condition for cetaceans can be used as a reference for 'ground-truthing' blubber biomarker development and

validation investigations. The assessment of both previously used and novel blubber biomarkers of body condition, physiological state and pregnancy status has important applications for the analysis of remote biopsy samples that can be rountinely collected from free-ranging cetaceans. The identification of useful biomarkers will thus help to develop and establish potential population health monitoring tools that can be applied across a range of different species. Finally, this work will improve our understanding of the potential functions of cetacean blubber as an endocrine tissue involved in the metabolic control of many of the extreme life-history strategies characteristic of these animals.

CHAPTER 2 : EVALUATING MORPHOMETRIC INDICES OF BODY CONDITION OVER A 425:1 MASS RANGE IN CETACEANS

The harbour porpoise data analysis and discussion part of this chapter has been published in *Ecology and Evolution* (2017).

Kershaw JL, Sherrill M, Davison NJ, Brownlow A, Hall AJ. Evaluating morphometric and metabolic markers of body condition in a small cetacean, the harbor porpoise (*Phocoena phocoena*). *Ecology and Evolution*. 2017;7(10):3494-3506.

1. ABSTRACT

No consensus currently exists on the best measure to quantitatively estimate body condition from body measurements in many species, including cetaceans. Here, the morphometric body condition index most diagnostic of nutritional status based on a set of biologically plausible hypotheses was assessed across three families of cetaceans. Morphometric data collected from stranded cetaceans by the Scottish Marine Animal Strandings Scheme from Phocoenids, Ziphiids and Balaenopterids were used to identify an informative condition index based on its ability to distinguish between individuals of varying condition according to their cause of death and their age class. Across all three species groups, blubber thickness was a consistently poor indicator of condition. The mass/length^2 ratio was the most appropriate morphometric index of 10 indices tested for the Phocoenids, explaining 50% of the variation in condition in stranded, male harbour porpoises (*Phocoena phocoena*) with different causes of death and across age classes (n = 291). The scaling relationship between mass and length was important to include for this species in order to obtain a condition index that was largely independent of body size and thus applicable across different age classes. Mass data were not available for all the Ziphiiid and Balaenopterid individuals, but estimations of mass using previously published relationships between girth and length were poor fits to the data for the small number of individuals where mass had been recorded. Thus, the girth/length ratio was identified as the most appropriate index for both the Ziphiids and the Balaenopterids in the absence of mass data.

The girth/length index was a good fit to the data from male minke whales (*Balaenoptera acutorostrata*) (n = 14), explaining 64% of the variation in the data with the expected relationships between cause of death and age class. The same index, while the best fitting of the four indices tested, was a poor fit to the Ziphiid data (n = 18), explaining just 16% of the variation. Here, the expected relationships between cause of death and age class were not observed, and there was little variation in overall condition between individuals. This unexplained variation could be a result of the bias in the data towards juveniles, and because the cause of death was not established for all animals. The lack of variation could also be specific to the Ziphiid family that shows less variability in their energy reserves. The morphometric condition indices identified here as the most appropriate to assess the nutritive body condition of these species can be used in comparative studies to assess other potential markers and indices of condition where a ‘ground truthing’ approach is required to evaluate novel methods.

2. INTRODUCTION

Several methods have been used to assess the body condition of mammals, and these generally fall into two categories : direct and indirect measures (Pitt et al., 2006). Direct measures quantify the amount of stored lipids within an individual which typically involves sacrificing the animal and measuring the amount of subcutaneous, visceral and muscle fat. Measuring the condition of *live* animals is more difficult and indirect measures are used that generally relate body mass to linear measures of body size. These morphometric measures therefore provide a non-destructive means of estimating an individual's body condition (Pitt et al., 2006). The ultimate goal of using a relationship between body mass and length measurements is to interpret variations of body mass for a given body size as an attribute of the individual's condition in terms of the size of its energy reserves, and therefore it's overall wellbeing (Peig and Green, 2010).

A wide range of different formulae and statistical methods have been proposed to standardise body size measurements, but there is still much debate about which methods are the most suitable to provide informative condition indices (Peig and Green, 2010). The most conventional methods include simple ratios between mass and length, and also girth and length, but other, more complex relationships have also been established as indicators of body condition in a number of different taxa. These include the Fulton Index, Quetelet's Index, Relative Condition, Relative Mass and the Residual Index (Peig and Green, 2010). Currently, no overall consensus has been reached about the best condition index, or criteria which allow the objective determination of the most appropriate index for any given species or a particular study. Instead, different disciplines tend to follow the traditions within their field, for example, the Residual Index is typically used in terrestrial ecology while mass to length ratios are consistently used in fisheries and health sciences. However, there have been a growing number of studies that criticise these approaches for being overly simplistic (Albrecht et al., 1993; Freckleton, 2002; Garcia-Berthou, 2001; Green, 2001; Packard and Boardman, 1999). This is because they assume that the proportion of mass associated with energy reserves is independent of body size, and do not take into account normal growth processes. This makes them inappropriate measures of condition (Green, 2001; Peig and Green, 2009, 2010; Schulte-Hostedde et al., 2005).

As most of the fat stores in cetaceans are found in the blubber, it has been previously assumed that indirect indices which reflect the amount of blubber an individual has, such as blubber thickness and body girth, are good indicators of nutritive condition (Koopman, 1998; Koopman et al., 2002; Kuiken et al., 1994; Lockyer, 1986; Lockyer et al., 1985; Moore et al., 2001). For this reason, blubber thickness continues to be used as a standard condition index despite evidence that this measure may not provide an accurate indication of overall condition as it assumes that blubber thickness is directly proportional to changes in lipid mass, and by extension, body energy stores that reflect nutritive condition (Aguilar et al., 2007; Caon et al., 2007; Evans et al., 2003; Gómez-Campos et al., 2011; Koopman, 2007; Read, 1990; Ruchonnet et al., 2006). This assumption may be incorrect because cetacean blubber serves other functions as well as energy storage including thermoregulation, streamlining and buoyancy (Iverson, 2009). These other functions limit the extent to which the blubber can change in mass and in

thickness if they are to be preserved. In addition, Christiansen et al. (2013) modelled total blubber volume of minke whales (*Balaenoptera acutorostrata*) using blubber thickness and morphometric measurements, and concluded that across body variations in blubber deposition in this species are too large to be captured by a single thickness measurement (Christiansen et al., 2013). This may be the same for other species too. For these reasons, more robust morphometric measures of body condition that directly reflect the energy stores of the individual should be investigated rather than relying on a single measurement of blubber thickness.

The body condition of cetaceans has also been estimated using various other measurements obtained from whales killed during whaling operations. For example, total lipid content of blubber, muscle and visceral fat have been estimated for a number of species (Ackman et al., 1975a; Lockyer, 1986; Lockyer, 1987; Lockyer et al., 1985). These data are generally only available from whaling datasets, and typically only one measurement was taken in each case, so there are few data available to compare different indices. Other common indices used in cetaceans are maximum girth, length and mass (Read, 1990; Víkingsson, 1995). More recent efforts have considered different scaling relationships between these measures (Hart et al., 2013), and estimations of total blubber volume (Christiansen et al., 2013). In addition, live-fisheries of killer whales (*Orcinus orca*) for aquaria, have generated valuable data on mass-length relationships (Bigg and Wolman, 1975), but because of the size-selective nature of captive animals, there is little information on the largest individuals. Captive cetaceans have also provided information on the health, physiology, metabolism and energy requirements (Rechsteiner et al., 2013; Rosen and Trites, 2013; Worthy et al., 2013), but these data are unlikely to be representative of wild populations. Thus, there are few data sets available to fully assess which metrics are best used to estimate the condition of cetacean species. Data collected from stranded animals can help to fill this gap as often a suite of measures are available for each individual, and can be used to assess different indices of condition simultaneously (Caon et al., 2007; Gómez-Campos et al., 2011; Read, 1990). These data are ideal for comparative studies as a range of individuals from a population across different age and sex classes can be sampled.

The aim of this work was therefore to identify the most appropriate morphometric condition index across three families of cetaceans; Balaenopteridae, Ziphiidae and Phocoenidae, chosen here for comparison due to their different life-history strategies, and thus their varying reliance on endogenous fat stores. Here, morphometric data collected from stranded, male cetaceans by the Scottish Marine Animal Strandings Scheme were analysed to identify a morphometric index most diagnostic of nutritional status based on a set of biologically plausible hypotheses. Females were not included in the analysis so as to remove the confounding effects of reproductive state. The most appropriate morphometric condition index identified for each family will later be used to investigate variations in alternative physiological biomarkers of condition in Chapters 2 to 6.

3. METHODS

3.1 Morphometrics Dataset

Data collected by the Scottish Marine Animal Strandings Scheme (SMASS) from stranded male cetaceans around Scotland were collated between January 1991 and January 2018 to assess different morphometric measures of body condition. These data were from 327 individuals from five different species across three cetacean families, *Balaenopteridae, Ziphiidae* and *Phocoenidae*. These were minke whales, Sowerby's beaked whales (*Mesoplodon bidens*), Cuvier's beaked whales (*Ziphius cavirostris*), Northern bottlenose whales (*Hyperodon ampullatus*) and harbour porpoises *(Phocoena phocoena).* These data are summarised in Table 2.1.

For these animals, the cause of death (COD) was determined either by necropsy, or based on visual observations of the carcass showing signs of trauma, disease or emaciation. Two COD categories were generated: 'acute' cases were individuals that died of an acute trauma (bycatch, entanglement, storm damage, and predatory attacks e.g. bottlenose dolphin or grey seal attacks on harbour porpoises), and 'chronic' cases were individuals that died of general debilitation either though infectious disease (parasitic, bacterial, viral, or mycotic infections), starvation (severely emaciated animals that died of starvation/hypothermia) or chronic injury (e.g. long-term entanglement). Measurements of mass, length (tip of rostrum to fluke notch), girth (immediately anterior of the dorsal fin) and blubber thickness (immediately anterior of the dorsal fin) to the dorsal fin along the dorsal, lateral and ventral axes were taken (Kuiken and Hartmann, 1991), but due to the logistical constraints presented by their larger body size, mass was not recorded for all the ziphiids or the minke whales. Females were not included in the analysis as the reproductive status (pregnant, lactating or resting) was not known for all individuals sampled and measured over the same time period. Reproductive status would likely have affected the estimated condition of the individuals as pregnant females increase in both mass and girth irrespective of their condition. Thus, without knowing the pregnancy status of females, all metrics that contain mass and girth are inherently confounded as they represent changes in both body condition and pregnancy status.

Table 2.1 – Summary of the mysticete and odonotcete data across three cetacean families used for analysis.

Family	Species	Sample Size
Phocoenidae	Harbour porpoise (*Phocoena phocoena)*	291
Ziphiidae	Sowerby's beaked whale (*Mesoplodon bidens*)	12
	Cuvier's beaked whale (*Ziphius cavirostris*)	2
	Northern bottlenose whale (*Hyperodon ampullatus*)	8
Balaenopteridae	Minke whale (*Balenoptera acutorostrata*)	14

Harbour porpoises: Over a ten-year period, between 2006 and 2016, data from 291 male harbour porpoises were used for analysis. For these animals, the cause of death had been determined, and a full set of morphometric measurements had been taken. The individuals were grouped into age classes based on the size of males at sexual maturity. Adults were ≥ 135cm (Ólafsdóttir et al., 2002), calves were ≤ 90cm (Lockyer, 1995), and others were classed as juveniles. These data were collected across all months of the year and were further grouped into two different life history stages to investigate potential annual changes in condition as a result of the different energetic costs of the harbour porpoise life cycle. The data were separated into 'Breeding' and 'Non-Breeding' stages of the life cycle. As the peak calving months are June and July in the North Sea (Lockyer, 1995), and a spike in male testes mass in August suggests that mating occurs largely in this month (Lockyer, 1995), data from June, July and August were grouped as 'Breeding' (n= 66) and the rest of the year was considered 'Non-Breeding' (n = 225).

Ziphiids: As a result of the small sample size of ziphiid individuals collected by the SMASS, data were collated between 1994 and 2017 from 22 male individuals of three species (Table 2.1). These individuals had accompanying morphometric measurements of girth, length and three blubber thickness measurements. Mass was recorded for only four of the individuals. Here, individuals were assigned cause of death categories of chronic and acute as before. As a result of the smaller sample size, individuals where the ultimate cause of death was live stranding, but where the underlying causes of the stranding had not been determined were retained for analysis and classed as 'Live Stranding' cases as well. These were identified based on evidence of asymmetry in the lungs as well as bruising and damage to the body associated with the stranding event. The beaked whales include some of the world's most cryptic and difficult to study mammals, and little is known about their behaviour and ecology (MacLeod, 2018). For this reason, size estimates at maturity are less well documented and their breeding and reproductive cycles are largely unknown in the North Sea and the eastern North Atlantic. Species-specific body size estimates were used to classify them as adults, juveniles and calves. Northern bottlenose whales are thought to measure between 7-9m in length, with calves

measured between 3.0-3.3m (Whitehead et al., 1997). Here, individuals of greater than 7m were considered as adults, and smaller than 3.3m were calves. All eight individuals measured here were juveniles. Cuvier's beaked whales grow to between 5-7m in length, with no significant size difference between the sexes (Heyning, 2002). Here, individuals larger than 5m were classed as adults. The two individuals measured were an adult and a juvenile. Finally, adult Sowerby's beaked whales were the smallest of the beaked whales measured here and are between 4.5-5.5m in length with calves measuring between 2.4-2.7m (Hooker and Baird, 1999; Mead, 1989). Individuals smaller than 3m were classified as calves, and larger than 4.5m were classified as adults. Here, two calves, six juveniles and four adults were measured. Few data are available regarding the reproductive or migratory patterns of these species, the data were not classified into breeding and non-breeding animals as was the case for the harbour porpoises.

Minke whales: Data from 14 male minke whales collected between 1995 and 2016 were used here for analysis. Morphometric measurements of girth, length and three blubber thickness measurements were collected, but mass was not recorded. The cause of death was established through post-mortem examination, and individuals were classed as acute or chronic cases as before. Adult males were > 6m, calves were <4.5m and the rest were considered as juveniles (Hauksson et al., 2011). A total of three adults, four juveniles and seven calves were measured. Like most mysticete species, minke whales undertake long-distance seasonal migrations between highly productive feeding grounds in the summer and less productive breeding grounds in the winter. Breeding and non-breeding parts of their life-cycle are therefore both temporally and spatially segregated. Summer feeding grounds are located in various areas across the North Atlantic, but breeding grounds are unknown, and are believed to be located close the equator (Vikingsson and Heide-Jørgensen, 2005). As little is known about the reproductive cycle or biology of this species away from their summer feeding grounds, and as these data all came from northerly latitudes that are thought to be more important for feeding rather than breeding, these data were not split into seasons for analysis.

3.2 Body Condition Indices Calculations

Seven body condition indices commonly used to assess condition in mammals were calculated based on previous work across a number of vertebrate species (Peig and Green, 2009, 2010) (Table 2.2). As well as these seven indices, the thickness of the ventral blubber and dorsal blubber were also investigated as potential body condition indices based on previous work that suggests that blubber thickness at these two sites can be used to estimate condition in small odontocetes (Koopman, 1998; Koopman et al., 2002) and baleen whales (Lockyer, 1986; Lockyer et al., 1985). Across the species groups sampled, there is currently no evidence to suggest that deposition and mobilisation of lipids occurs laterally as a result of energy store variation. So, the blubber thickness of this site was not incorporated into any of the condition indices investigated here. Two additional condition metrics, blubber thickness/girth and blubber thickness/length were also assessed as potentially more useful indices than blubber thickness alone that may vary as a function of overall size of the individual irrespective of its relative condition. Mass was not measured for the majority of the ziphiids, or any of the minke whales as a result of the logistical constraints associated with measuring such large animals. Attempts were therefore made to estimate mass based on published mass-length-girth

relationships for these species. Previous work by Bloch and colleagues (1996) estimated the relationship between mass and length in northern bottlenose whales for both sexes as:

$W = 0.0000131 \times L^{3.07}$

Where W is mass in kg and L is length in cm.

This equation was used to estimate the mass of the ziphiids based on their length measurements here, but it was found that this overestimated the mass by between ~40% and ~80% where mass had been recorded for four individuals (Fig. 2.1a). This is likely because this relationship is not appropriate for some of the smaller beaked whale species that were sampled here because it was proposed for Northern bottlenose whales (Bloch et al., 1996). In addition, using length alone is likely an inappropriate way to estimate mass, because it cannot account for any changes in the body composition of the individuals in varying condition which will affect their mass, and an extra metric, such as body girth, should be included to obtain better estimates.

Based on whaling records for baleen whales, the estimated relationship between mass, length and girth in fin and sei whales (*Balaenoptera borealis*) (Lockyer and Waters, 2006; Víkingsson et al., 1988) was calculated as:

$W = aG^kL^{k'}$

Where W is mass in tonnes, G is the mean of three girth measurements taken anterior (n = 2) and caudal (n = 1) to the dorsal fin in meters, and L is the length in meters.

For sei whales, as the smaller of the two species that are therefore closer in size to minke whales, $a = 0.11$, $k = 1.32$ and $k' = 1.097$. This equation was used to estimate the mass of four minke whale *females* that were not included in the larger dataset because of their sex, but their mass had been recorded. These were two juveniles and two calves. Their mass was calculated using this equation based on their length and a single girth measurement, and used for comparison with the measured mass of the individuals (Fig. 2.1b). Similarly to the ziphiids, the calculated masses were overestimated by between ~50% and ~100% (Fig. 2.1b). There may be very different species-specific scaling relationships between these morphometric measurements for balaenopterids which means that these model parameters are a poor fit to data from other species. This likely led to the disparity between the estimated and the measured masses here. It is also likely that these relationships between girth, length and mass are biased towards larger, adult animals which were typical of whaling catches. For this reason, they were not as appropriate for the much smaller calves and juveniles for which data were available here.

Had these estimated masses been used for the calculation of any of the mass-dependent body condition indices, the conclusion about the condition of the animals would be different. For this reason, the morphometric indices using a mass measurement were not calculated and used for evaluation for the ziphiids or the minke whales.

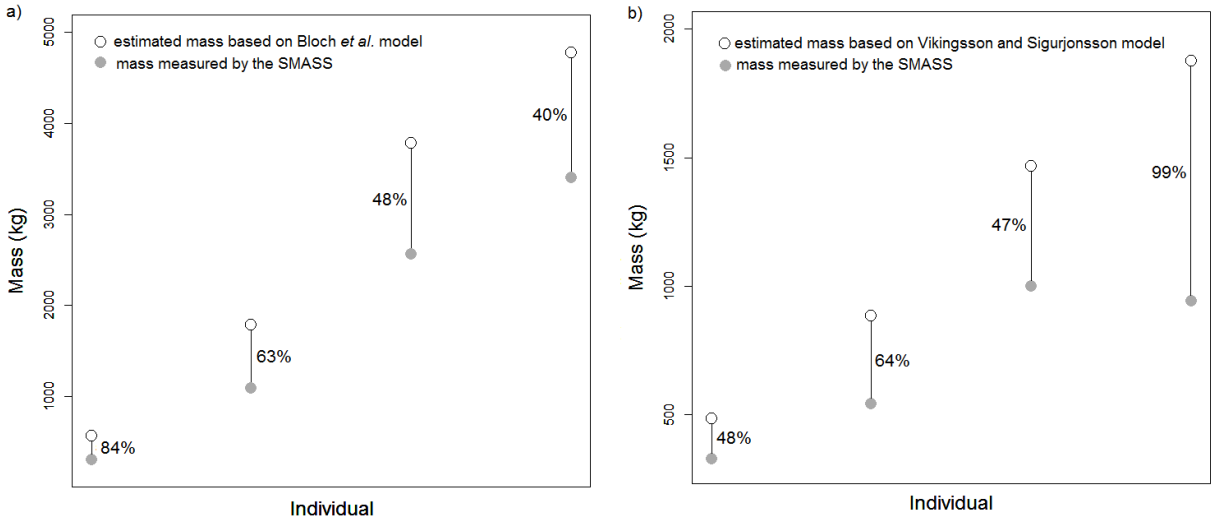

Fig. 2.1. Measured and estimated masses of ziphiids and minke whales. a) Estimated masses of four male ziphiids were calculated based on the relationship between mass and length in northern bottlenose whales published by Bloch and colleagues (1996). All mass estimates were higher than the measured masses of the individuals by between 48% and 84%. b) Estimated masses for four female minke whales were calculated based on the relationship between mass, length and girth in Sei whales published by Vikingsson and colleagues (1988). All masses were also overestimated for these animals by between 47% and 99%.

Table 2.2 – Body condition indices and their calculation. With the exception of ventral blubber thickness, ventral blubber : girth and ventral blubber : length, formulae are taken from Peig and Green, 2010.

Index	**Formula and Explanation**
Blubber Thickness	Full ventral blubber depth (Phocoenids, and Ziphiids) Full dorsal blubber depth (Balaenopterids)
Ventral Blubber : Girth	Full ventral or dorsal blubber depth / girth
Ventral Blubber : Length	Full ventral or dorsal blubber depth / length
Girth : Length	Girth / Length
Mass : Length	Mass / Length
Fulton's Index	$K = Mass/Length^3$
Quetelet's Index	$BMI = Mass/Length^2$
Relative Condition	$Kn = M_i/M_i^*$ (individual Mass/Predicted Mass) $M_i^* = a\ Li^b$ where a and b are determined by ordinary least squares (OLS) regression of M against L for the whole study population
Residual Index	R_i, the residuals from an OLS regression of M against L, after log transformation
Scaled Mass Index	$\widehat{M}i = Mi\ \left[\frac{Lo}{Li}\right]^{b_{SMA}}$ Where M_i and L_i are the mass and length of an individual. ^bSMA is the scaling component estimated by the standardized major axis (SMA) regression of lnM on lnL. L_0 is the arithmetic mean of the length of the study population. $\widehat{M}i$ is the predicted body mass of the individual when the linear body measure is standardised to L_0.

3.3 Statistical Analysis

All statistical analyses were performed using the statistical package, R, version 3.1.2 (R Core Development Team, 2014).

The most representative morphometric index of body condition was investigated based on three ground truthing assumptions:

1. 'Chronic' cases will have a lower body condition index than 'acute' cases because the acute cases died as a result of critical physical injury and did not become debilitated with a gradual decline in their health.
2. Overall, juveniles will have a lower condition index than adults. These individuals are foraging independently for the first time making them vulnerable to nutritional stress. This age group is also most likely to be physiologically stressed due to immunological challenge by parasites encountered for the first time, and any extra energy acquired is likely used for growth rather than retained as fat stores.
3. Body condition in the breeding season will be lower than the rest of the year because males expend energy on reproduction.

A reliable condition index should therefore be able to differentiate between individuals with different CODs, age classes and life-history stages. Each species group was modelled individually. The condition of an individual was assumed to be independent of the condition of others, there was no evidence of different standard deviations across the COD classes, age classes, or season groups for each index (leveneTest in the *Rcmdr* R package to test for homogeneity of variance between groups, p values all > 0.05), and all indices were normally distributed for each species group. Linear regression models were used to investigate the relationship between the index and the suite of covariates discussed above as well as an interaction between season and age class in the phocoenids. Backwards model selection was performed using the *dredge* function (*MuMIn* package in R) which identifies the variables and/or interactions that best explain the variation in the data. *Dredge* performs automated model selection with subsets of a supplied 'global' model including all covariates and interactions of interest, and optional choices of other model properties (such as different link functions). All possible combinations of covariates and their interactions were generated, and the model with the lowest AIC value (Akaike Information Criterion) with at least a value of two smaller than the next smallest AIC value, was identified as the best model for each index, and was used for further interpretation. For each index, the model with the smallest AIC met these criteria, so there were no models that would have been considered of equivalent fit to the data.

Visual inspections of the model residual plots and fitted values were used to check the fit and assumptions of the final models. When residuals are plotted against the fitted values, if the model is suitable and has not violated any major assumptions, there should be no obvious patterns or trends across the data points. If the model fits well, the fitted values should be similar to the observed values and when plotted together would lie approximately along the 45° line. If this is not the case, there may be some autocorrelation in the data and some over- or under-prediction occurring in the model.

The relationships between the covariates retained after model selection for each index were evaluated, and the model fit was assessed using the Adjusted R^2. As well as evaluating the model fit, the variance explained by each variable retained in the final models was also assessed. When there is more than one predictor, the partial eta-squared (partial η^2) is the proportion of the variance explained by a given predictor after excluding variance explained by the others (Levine and Hullett, 2002). For each body condition index model, partial η^2 values were used to assess the importance of each variable retained after model selection. Partial η^2 values were calculated using the '*etasq*' function in the *heplots* library in R; the sums of squares for the effect of interest was divided by the combined sums of squares of the effect of interest and the associated error term.

The condition index that showed the expected relationships based on the ground truthing assumptions and was a good fit to the data (a high adjusted R^2) and thus had good predictive power, was identified as the most appropriate morphometric index for that species group.

4. RESULTS

4.1 Harbour porpoises

The interaction between age class and season was not retained in any of the linear models. The predominant COD varied by age class. Fewer calves died as a result of trauma (25%) compared to both juveniles and adults (47.8% and 49.1% respectively). There was a significant effect of COD on all body condition indices (Table 2.3). All indices showed that the chronic cases were in poorer condition than the acute cases, as hypothesised based on the ground truthing assumptions, but the results varied by age class. Five of the indices suggested that adults were in better condition than the other age classes, and the other five suggested that there was either no effect or they were in poorer condition (Table 2.3).

As it is unlikely that, overall, adults would have smaller energy reserves than both juveniles and calves, these condition indices (blubber thickness/girth, blubber thickness/length and Fulton's Index) were not used for further interpretation. As girth/length and the Scaled Mass Index showed no differences between age classes, these were also dropped for further interpretation. Of the remaining indices (blubber thickness, mass/length, Quetelet's Index, Relative Condition and the Residual Index), there was little variation in the effect size for COD between the final models for each index with partial η^2 values ranging from 0.11 to 0.19 (Table 2.3). However, there was more variation in the effect size for age class between these models, with partial η^2 values ranging from 0.06 to 0.69 (Table 2.3). The model for mass/length was the best fit to the data with an adjusted R^2 value of 0.72 (Table 2.3), but, as seen with the partial η^2 values for COD and age class, the variation is largely explained by the age class, and thus the different size of individuals.

Generally, mass shows a strong non-linear relationship with length, which was also the case for these male porpoises (Fig. 2.2a), such that much of the variation in mass is accounted for by changes in length (Hayes and Shonkwiler, 2001). Thus, indices of condition that are not explicitly based on allometric models are affected implicitly by the scaling of mass and length (Hayes and Shonkwiler, 2001). For this reason, the simplest mass/length condition index, identified here as the best fit to the data, is correlated with body size (Fig. 2.2b). Mass/length as a condition index is therefore flawed because it inherently incorporates an association between length and condition and can therefore be misleading because the effects of condition are likely confounded with the effects of size for this species.

The second best fitting model for these data was the Quetelet's Index (mass/length^2) with an adjusted R^2 value of 0.50. In addition, for mass/length^2, the effect size for COD was highest with a partial η^2 value for COD of 0.19. Thus, using differences in COD as the most relevant ground truthing assumption for detecting changes in condition, this index explains the most variation in the COD data. In general, the relationship between mass and length can be modelled as:

$\text{mass} = \delta + \alpha\ \text{length}^{\beta}$ (Hayes and Shonkwiler, 2001).

This equation shows that mass is a power function of length plus an intercept term, and the parameters of this equation can be estimated empirically by non-linear regression. When the

goal is to generate a condition index that is independent of size, use of the mass/length ratio may be reasonable if mass scales with length such that $\beta = 1$ and δ is close to 0 (Hayes and Shonkwiler, 2001). If the scaling exponent does not equal 1, then obtaining a size-independent condition ratio requires that some scaling exponent other than 1 should be used.

The above non-linear model was fitted to the harbour porpoise data (*nls* function in the R package *nlstools*) (Fig. 2.2a). The δ parameter estimate was -3.17 with large 95% confidence interval of -9.10 to 1.66. As a negative intercept is not biologically plausible, this is likely driven by a lack of data for very small calves and foetuses. As this non-linear model would not be used to extrapolate beyond the smallest individuals measured here, and the intercept parameter estimate confidence interval incorporates 0, the intercept is assumed to be around 0. The β exponent was estimated as 2.33 (with 95% confidence intervals of 1.89 - 2.77) which suggests that while mass/length^2 showed a poorer overall fit to the data compared to mass/length, it is a better index to use here in order to obtain an index largely independent of body size (Fig. 2.2c). Using this index, the condition of adults and juveniles can be better compared (Fig. 2.2c), but the condition index of calves compared to the other two age classes should be interpreted with caution. Using mass/length^2, acute cases were in significantly better condition than the chronic cases (p value < 0.005) (Fig. 2.3a), and adults were in the best condition and calves in the poorest (p values all < 0.005) (Fig. 2.3b), but season was not retained in the best-fitting model (Table 2.3).

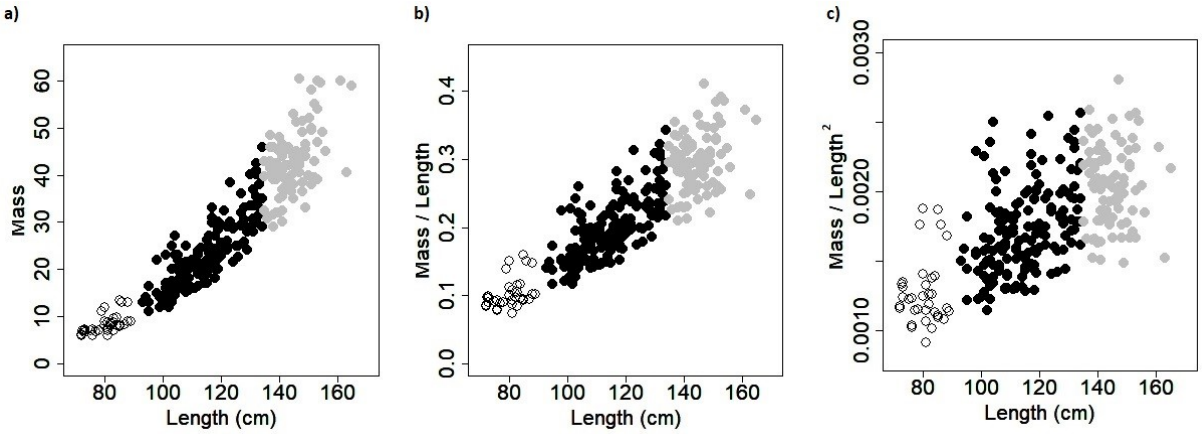

Fig. 2.2. Relationships between mass and length and their ratios for male harbour porpoises. a) Non-linear scaling relationship between mass and length for male harbour porpoises modelled using non-linear least squares regression where mass = -3.71 x 0.0004length$^{2.33}$. b) Positive correlation between mass/length and length with calves shown in white, juveniles in black and adults in grey. Using mass/length as a condition index is therefore not independent of body size. c) Relationship between mass/length^2 and length demonstrating that mass/length^2 is a more appropriate index for this species to compare between adults and juveniles as it is largely independent of body size.

Table 2.3 - Summary of the covariates used in the linear regression models for each body condition index for the harbour porpoise data. * indicates that the variable was retained after model selection. ** indicates that the variable was retained and statistically significant ($p < 0.05$). If ground truthing assumptions were met, the relationships are marked with a '+', while different relationships are marked with '-'. Partial η^2 is the proportion of variance explained by that variable in the final model after excluding variance explained by the other variables.

Body Condition Index Model		Model Covariates used in Model Selection			Model Adjusted R^2
		COD	Age Class	Season	
Ventral Blubber Thickness	Variable Retained	**	**	**	0.22
	Assumptions Met	+	+	+	
	Partial η^2	0.15	0.06	0.01	
Ventral Blubber / Girth	Variable Retained	**	**	**	0.09
	Assumptions Met	+	-	+	
	Partial η^2	0.14	0.05	0.01	
Ventral Blubber / Length	Variable Retained	**	**	*	0.15
	Assumptions Met	+	-	+	
	Partial η^2	0.14	0.05	0.01	
Girth / Length	Variable Retained	**		*	0.19
	Assumptions Met	+		-	
	Partial η^2	0.19		0.01	
Mass/Length	Variable Retained	**	**		0.72
	Assumptions Met	+	+		
	Partial η^2	0.17	0.69		
Fulton's Index	Variable Retained	**	**		0.17
	Assumptions Met	+	-		
	Partial η^2	0.13	0.08		
Quetelet's Index	Variable Retained	**	**		0.50
	Assumptions Met	+	+		
	Partial η^2	0.19	0.40		
Relative Condition	Variable Retained	**	**		0.25
	Assumptions Met	+	+		
	Partial η^2	0.17	0.08		
Residual Index	Variable Retained	**	**		0.21
	Assumptions Met	+	+		
	Partial η^2	0.11	0.17		
Scaled Mass Index	Variable Retained	**			0.14
	Assumptions Met	+			
	Partial η^2	0.14			

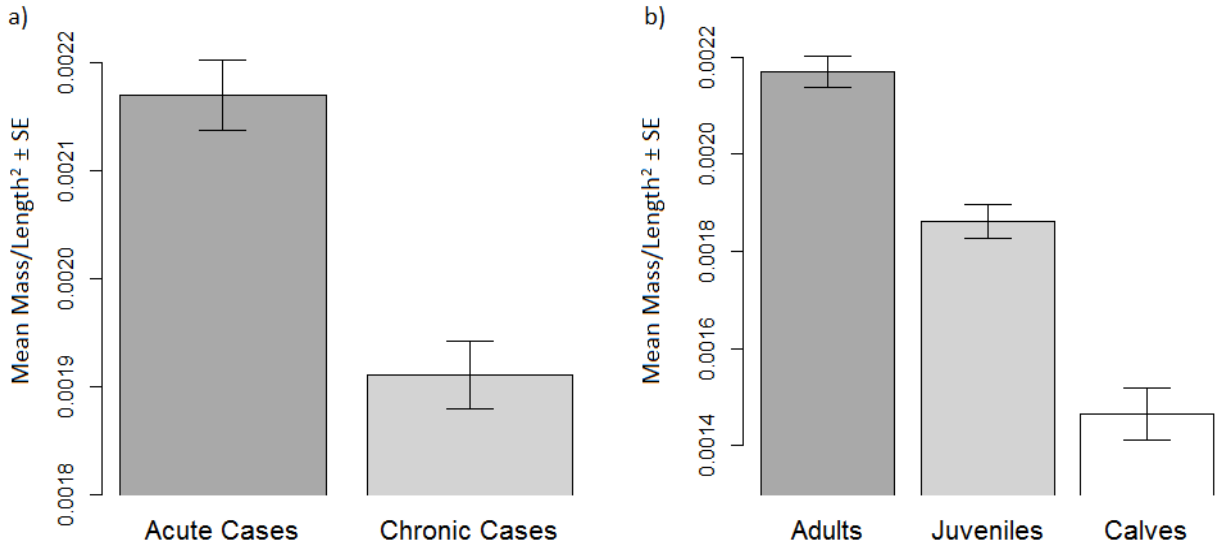

Fig. 2.3. Output from the final mass/length^2 linear regression model for harbour porpoises: lm(Mass/Length^2~as.factor(COD)+as.factor(Age Class)) (n=291). a) The chronic cases had a significantly lower mass/length^2 index than the acute cases (p value < 0.005). b) Adults had the highest mass/length^2 index of all three age classes (p value < 0.005).

4.2 Ziphiids

All final models for each body condition index were poor fits to the data (Table 2.4). As 68% of the animals (15 / 22) were juveniles, the dominance of juveniles in the dataset likely affected covariate selection. All acute cases were juveniles and 67% of the chronic cases were also juveniles. Unfortunately, the cause of death could be not attributed to either an acute or chronic cause for 50% of the animals which were therefore classed as live stranded cases. This small sample size of acute and chronic cases is likely the reason why cause of death was not retained as an important covariate following model selection in the three best fitting models for these species. Only the ventral blubber thickness model retained COD after variable selection, but the effect size was small with a partial η^2 value of 0.10, and this model was the poorest fit to the data (Table 2.4). The best fitting model was the girth/length model although this did not show the expected relationship with age class as while juveniles were not significantly different to the adults, calves were in significantly better condition ($p = 0.04$). The relationship between girth and length was not plotted and assessed for these individuals as the multi-species nature of the data would not produce a realistic scaling relationship of these two measures for these animals, but girth/length as an index appears to be independent of body size (Fig. 2.4). As girth/length explained the most variation in the data with the best fitting model, this was taken to be the most appropriate index of condition for these species even though the expected relationship with age class was not seen. This discrepancy could be the result of the small sample size of adults and calves used for comparison.

Table 2.4 - Summary of the covariates used in the linear regression models for each body condition index, and the results of the model selection for the ziphiids. * Variable retained after model selection. ** Variable retained after model selection and statistically significant ($p < 0.05$). Where ground truthing assumptions were met, the relationships are marked with a '+', while different relationships are marked with '-'. Partial η^2 is the proportion of variance explained by that variable in the final model after excluding variance explained by the other variables. These models are all a poor fit to the data with little to distinguish between them.

Body Condition Index Model		Model Covariates used in Model Selection		Model Adjusted R^2
		COD	Age Class	
Ventral Blubber Thickness	Variable Retained	*		0.001
	Assumptions Met	+		
	Partial η^2	0.10		
Ventral Blubber / Girth	Variable Retained		*	0.03
	Assumptions Met		+	
	Partial η^2		0.13	
Ventral Blubber / Length	Variable Retained		*	0.07
	Assumptions Met		+	
	Partial η^2		0.16	
Girth / Length	Variable Retained		**	0.16
	Assumptions Met		-	
	Partial η^2		0.24	

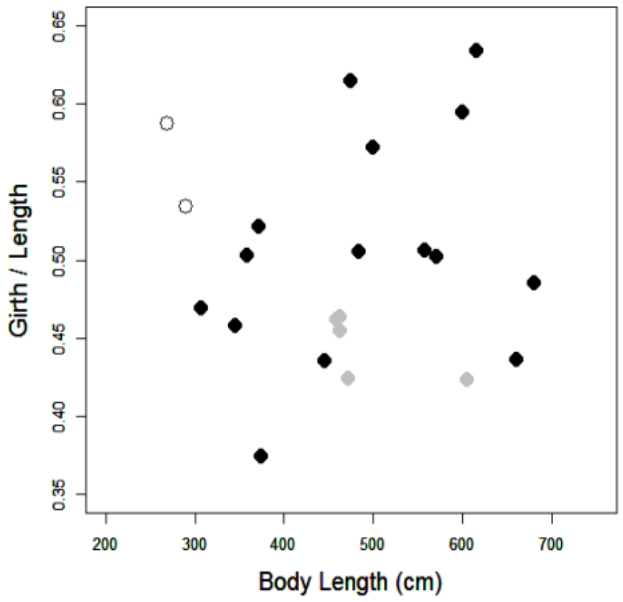

Fig. 2.4. Girth/Length as a condition index against total body length for the ziphiids. There is no relationship between girth/length and total body length, indicating that this is an appropriate index for this species as it is largely independent of body size with calves shown in white, juveniles in black and adults in grey.

4.3 Minke whales

There was an uneven spread of individuals across the age classes as 50% of the minke whale dataset were calves, and 30% were juveniles. The dominant causes of death for these animals were attributed to acute traumas, and the cause of death for just one animal was inconclusive and thus classed as a live stranding case. Even with the dominance of smaller animals and acute causes of death in this dataset, there was significant variation across COD groups and age classes for all body condition indices with the exception of dorsal blubber thickness. The poorest fitting model was the dorsal blubber thickness model which retained only COD as an important explanatory variable, but it was not individually significant (Table 2.5). The two best fitting models were of almost equivalent fit to the data (Table 2.5). The first was the dorsal blubber thickness/girth, but only age class was retained as an important explanatory variable and did not show the expected variation in condition between adults, juveniles and calves (Table 2.5). The second was girth/length which retained both COD and age class, and showed the expected relationships with each covariate (Table 2.5). Importantly, the effect size of the COD explained more of the variation in the data, with a partial η^2 value of 0.71.

A linear model was fitted to the girth and length data instead of a non-linear model used for the mass and length data in harbour porpoises. This was chosen because the dominance of young animals in the dataset means that they likely represent the more exponential phase of a standard growth curve, and there are therefore few data here to model how length and girth may reach an asymptote in larger individuals of this species (Fig. 2.5a). The girth/length condition index appears to be largely independent of body size (Fig. 2.5b), and this together with its good fit to the data and the expected relationships with COD and age class make it the most appropriate condition index assessed here. Specifically, chronic cases had a significantly lower girth/length ratio than acute cases ($p < 0.005$), and both juveniles and calves were in poorer condition than adults (Fig. 2.6).

Table 2.5 - Summary of the covariates used in the linear regression models for each body index, index, and the results of the model selection for male minke whales. * Variable retained after model selection. ** Variable retained after model selection and statistically significant ($p < 0.05$). Where ground truthing assumptions were met, the relationships are marked with a '+', while different relationships are marked with '-'. Partial η^2 is the proportion of variance explained by that variable in the final model after excluding variance explained by the other variables.

Body Condition Index Model		Model Covariates used in Model Selection		Model Adjusted R^2
		COD	Age Class	
Dorsal Blubber Thickness	Variable Retained	*		0.15
	Assumptions Met	+		
	Partial η^2	0.22		
Dorsal Blubber / Girth	Variable Retained		**	0.65
	Assumptions Met		-	
	Partial η^2		0.70	
Dorsal Blubber / Length	Variable Retained	**	**	0.56
	Assumptions Met	+	-	
	Partial η^2	0.45	0.46	
Girth / Length	Variable Retained	**	**	0.64
	Assumptions Met	+	+	
	Partial η^2	0.71	0.37	

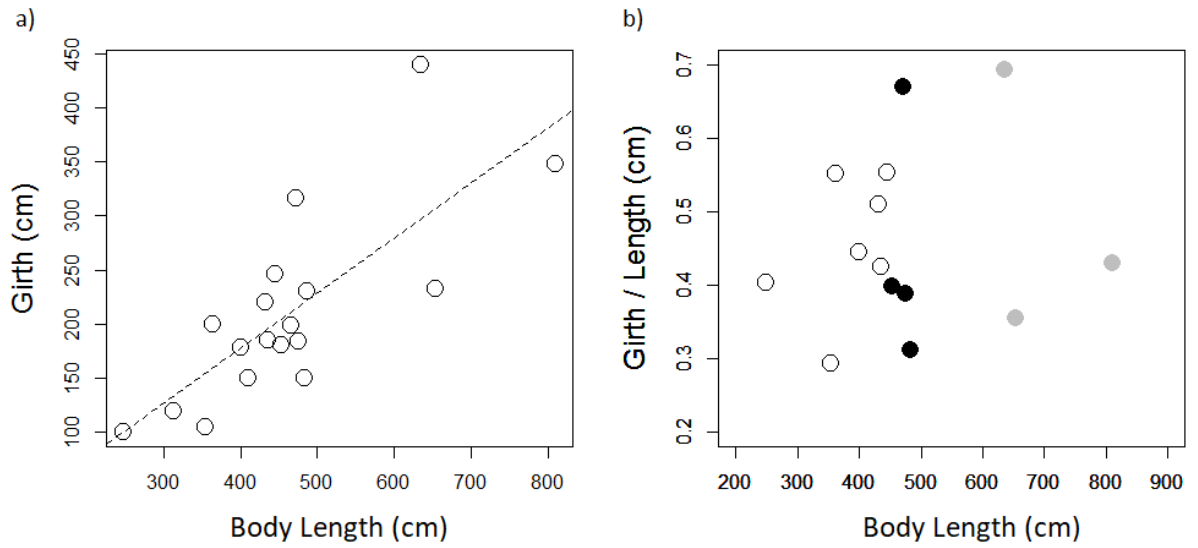

Fig. 2.5. Relationship between girth and length for male minke whales. a) Linear scaling relationship between girth and length for male minke whales where girth = 0.51 x length - 26.4. b) No relationship between girth/length and total body length, indicating that this is an appropriate index for this species as it is largely independent of body size with calves shown in white, juveniles in black and adults in grey.

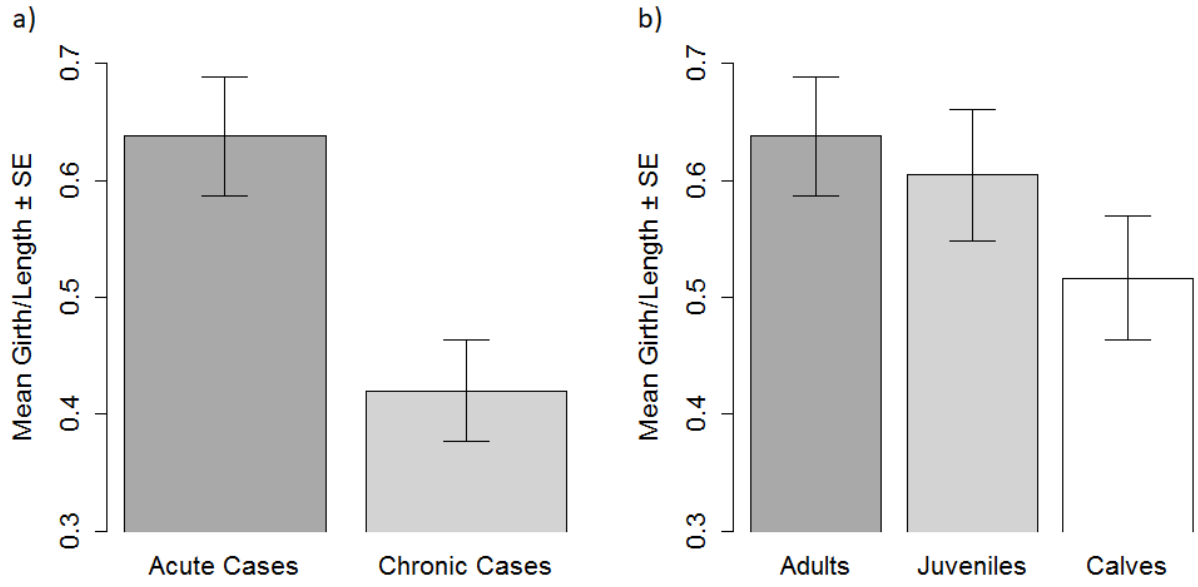

Fig. 2.6. Output from the final girth/length linear regression model for minke whales: lm(girth/length~as.factor(COD)+as.factor(Age Class)) (n=14). a) The chronic cases had a significantly lower girth/length index than the acute cases (p value < 0.005). b) Adults had the highest girth/length index of all three age classes, and were in significantly better condition than calves (p = 0.046).

5. DISCUSSION

Girth Based and Mass Based Indices

Different models for each condition index retained varying combinations of important explanatory variables for each species group. In addition, depending on which index is used, different patterns become significant in the data within a species group. For example, for the harbour porpoises, while some indices suggest that adults are in better condition than both juveniles and calves, others show that adults are in the poorest condition of the three age classes. These results highlight that caution must be taken when interpreting body condition estimates, and identifying the most appropriate one for any species group is important. The identification of the most appropriate condition index in harbour porpoises as mass/length^2 was straightforward given the variation in the data and the large sample size. As a result of the smaller sample size, species-specific differences, and the lack of mass data, the ziphiid models were poor fits to the data, and the identification of the most appropriate index was therefore less clear. While the sample size for the minke whales was also small, the best model for girth/length was a good fit to the data and was identified as an appropriate index for this species in the absence of mass data.

Cetaceans store most of their energy in muscle and adipose tissue (blubber and visceral fat), but a considerable amount may also be stored in internal organs, bones and other tissues (Christiansen et al., 2013; Lockyer, 1986; Lockyer, 1987; Víkingsson, 1995). As such, condition indices based on the mass of non-pregnant individuals are able to capture the extent of these full body energy reserves, and are the most informative as they can provide information on body composition. Of course, for pregnant females, the proportion that foetuses contribute towards the total body mass will increase throughout gestation, so condition indices using mass will therefore be affected. Disparities between the estimated masses and measured masses of the few ziphiid and minke whale individuals used for comparison here demonstrate that the existing relationships of length-mass, and girth-length-mass based on whaling data, are both highly species specific, and likely biased towards larger animals.

For larger animals where mass measurements cannot be taken for logistical reasons, girth measurements may become more important for estimating condition. Girth measurements alone have previously been used as indices of condition in cetaceans as a proxy for energy stores (Best and Rüther, 1992; Lockyer, 1987; Miller et al., 2012a; Víkingsson, 1995), but this metric was not modelled here as girth measurements provide information on both body condition and on changing body size through growth. Here, girth alone was not used so as not to mistake variations in natural growth of the animals for changes in body fat stores. As these changes are not mutually exclusive and it is difficult to tease them apart, the condition indices investigated here were standardised against length.

Girth is measured by the SMASS in front of the dorsal fin because this measurement can be standardised across individuals of different species by using the dorsal fin as a point of reference. Attempts to measure the maximal girth can be more subjective and logistically challenging to assess on large cetaceans because of their size. Although this measurement is

often still the maximum girth for many species, it may not be in all species, particularly large baleen whales where the dorsal fin is positioned further back on the body towards the tail fluke. For this reason, the girth measurements used here may not be a true reflection of an individual's condition as this girth measurement may not accurately capture variation in the energy stores of the individuals depending on where, for a particular species, there is most change in size as a result of variation in fat deposition. A single mass measurement is not subject to the same biases as a result of measurement location, so when standardised against length, will provide a more meaningful estimate of overall body condition.

Blubber Thickness is a Poor Indicator of Condition

Across all three species groups, blubber thickness was a consistently poor indicator of condition, often with the worst fitting models and not retaining the covariates as significant explanatory variables. The two indices of blubber thickness in relation to girth and length were mostly better fits to the data but were still consistently poor indicators. Blubber thickness was also found to be a poor index of condition in a number of other studies (Aguilar et al., 2007; Caon et al., 2007; Evans et al., 2003; Gómez-Campos et al., 2011; Koopman, 2007; Read, 1990; Ruchonnet et al., 2006). This is likely because condition in these animals is not solely a function of blubber reserves, but also of muscle mass as starving animals begin to catabolise muscle as well (Stegall et al., 1999). The reasons for this are likely twofold. Firstly, the blubber must maintain a certain thickness to fulfil its other roles in thermoregulation, streamlining and buoyancy (Iverson, 2009), so the extent to which it can be reduced in thickness during nutritional stress is limited. Secondly, starving animals may begin to catabolise muscle to access vital proteins (Stegall et al., 1999), and for this reason, starved indivduals have likely depleted significant amounts of lean tissue. By using a body condition index that includes mass, other structural body components, like skeletal muscle, are taken into account as these will also be affected by food limitation and are therefore more representative of overall condition.

Species Specific Condition Indices

Harbour porpoises: The predominant cause of death varied by age class as a higher proportion of juveniles and adults died as a result of physical trauma (49.1% and 47.8% respectively) compared to calves (25.0%). This is possibly because juveniles are more likely to be attacked by bottlenose dolphins due to their small size (Patterson et al., 1998), and both juveniles and adults that are actively foraging for themselves are more likely to be caught up in fishing nets in the pursuit of prey than maternally dependent calves. The chronic and acute cases are therefore not equally distributed across each age class. Mass/length^2 was determined to be the most appropriate morphometric index for valid inferences about the condition of harbour porpoises. It showed good fit to the data, was consistent with the hypothesised relationships between both COD and age class, and was more independent of body size than mass/length alone. Correlations with body size are a problem because one main objective of condition indices is often to be able to compare the condition of animals of different sizes. The scaling of mass with length therefore needs to be considered when using indices that attempt to summarise the variation in mass relative to length, and attribute this variation to changes in condition.

There was little difference between the effect size of COD between the different condition indices, showing that it explained similar proportions of the variance in each model. Acute cases were in better body condition because these animals tended to have suffered a sudden critical event which led to their death, in comparison to the chronic cases where a general debilitation and decline in health occurred in individuals that had used up more of their energy reserves. There was more variation explained by age class than COD in the mass/length^2 model. As expected, based on the ground truthing assumptions, adults were in significantly better condition than both juveniles and calves. Juveniles were hypothesised to be in the poorest condition overall because this age group is likely under nutritional stresses after weaning when finding food may be problematic and more energetically demanding (Lockyer et al., 1985). In addition, they would have the smallest fat stores because the energy that they acquire is likely not deposited, but is used for growth as they face the thermoregulatory problems associated with a large surface area to volume ratio (Caon et al., 2007), especially in cold waters. The fact that they were of intermediate condition here may be a result of how the individuals were assigned an age class following the work by Ólafsdóttir and colleagues (2002). Some of the smallest individuals may have only just been weaned, while others were almost at sexual maturity, resulting in a large range in the sizes of individuals in this age group.

Calves were shown to be in the poorest condition, but this would not necessarily imply that the calves are in poorer health. Rather, it more likely demonstrates that caution is required when comparing the condition of calves to the condition of adults using the same mass/length^2 index as even though it is more independent of body size than mass/length in this species, it does not take into account rapid growth processes or the different proportion of mass associated with energy reserves or insulation for these two age classes of significantly different body sizes. This is likely also the case, although to a lesser extent, with smaller juveniles because energy allocation in porpoises has been shown to shift from an emphasis on developing an insulative blubber layer in young animals to preparing the body for annual reproduction at sexual maturity (McLellan et al., 2002). While the Scaled Mass Index appeared to capture the changes in condition between different age classes in small mammals (Peig and Green, 2009), it was not a good fit to these harbour porpoise data. The mass/length^2 index may be better suited to small odontocetes than small mammals previously investigated as they are large with a relatively simple body shape, and their energy reserves in the blubber and muscle constitute a much larger proportion of their entire body mass, between 50-60% (McLellan et al., 2002), compared to energy reserves in terrestrial species.

There were no differences in condition between males during the breeding season and over the rest of the year using the mass/length^2 index. In theory, males could be in poorer condition during the breeding season as a result of the increased energetic demands associated with reproduction. For example, mature individuals are thought to seasonally allocate up to 4% of their total body mass to testicular growth (Neimanis et al., 2000). However, a decrease in condition over the breeding season for sexually mature males has not been reported as it has for females. It is expected that the mass/length^2 index is the most informative body condition index for females as well as males, and differences in the reproductive state of females between resting, pregnant and lactating would be detected (Read, 1990). It is hypothesised that

mass/length^2 is likely also a good condition index for other small and medium sized odontocetes.

Ziphiids: The SMASS have few records of ziphiid strandings because these species typically live offshore in waters more than 200m deep feeding on deep-water fish and squid (MacLeod, 2018). In addition, most species, especially those found around the UK, are mostly solitary or live in small groups (MacLeod and D'Amico, 2006). Their distribution, habitat preferences and social behaviour together with their fewer numbers therefore make stranding events uncommon in the UK, so there is little morphometric data available for analysis. The bias towards juveniles in the dataset could be because younger animals become disorientated more easily and find themselves in shallower waters along coastlines which makes their carcasses more likely to be found.

All models were a very poor fit to the data, likely because of the limited sample sizes in each age class and COD category because the data were dominated by measurements from juveniles and live strandings cases. It is therefore hard to make inferences about the different CODs or age classes. In addition, the mix of three species grouped together, rather than using a single species likely also contributed to the poor fit of the models. These species were grouped together to increase the sample size for analysis. While they are all of similar body shape and ecology, they differ in body size. Were the sample size large enough to split the analysis by species, the model fit to the data may have been improved. It may also have been improved with more available data on size at sexual maturity in order to classify individuals as adults and juveniles more accurately for each species. Finally, a measured mass and more detailed information on cause of death to enable the animals to be classified as acute and chronic cases would likely have improved the model. In the absence of mass data, girth / length is thought to be the most appropriate index of condition for these animals although there appears to be little variation between individuals measured here. Unfortunately, there is little published information with which to compare these results and interpret the lack of variation between individuals. It is possible that this lack of individual variability in energy stores is characteristic of these species if they do not rely on endogenous energy reserves to the same extent as other cetacean species.

Minke whales: As with the Ziphiid data, the individuals measured were biased towards juveniles and especially calves, and acute causes were the most dominant causes of death across all three age classes. Even with these biases in this small dataset, there were significant differences in condition between different CODs and age classes in three of the four indices modelled. The best fitting model that showed the expected relationships with COD and age class was the girth/length model where, based on the effect size, COD explained most of the variation in the data. Balaenopterids are fast-adapted species that are able to annually cycle their fat stores as they move between high latitude feeding grounds and low latitude breeding grounds (Lockyer, 2007). The range in condition of these animals is therefore likely to be greater than the smaller odontocetes that do not experience annual extremes in feeding and fasting periods. This would explain why COD accounted for more variation in the data than age class in these animals compared to the other species groups. As expected, juveniles and calves were in poorer condition than the adults. On their feeding grounds, juvenile minke

whales have been shown to invest most of their excess energy into growth in order to reach size at sexual maturity faster and start reproducing earlier (Christiansen et al., 2013), rather than laying down fat stores. In the absence of better models to estimate mass from the girth and length measurements, using girth/length as an indicator of condition, appears to be an appropriate metric to measure the energy stores of this species.

Conclusions and Future Directions

The morphometric condition indices identified here as the most appropriate to assess the nutritive body condition of these species can be used in comparative studies to assess other potential markers and indices of condition where a 'ground truthing' approach is required to evaluate novel methods. Obtaining morphometric measurements from wild cetaceans is notoriously very difficult, but recent advances in technology and analytical techniques have improved our ability to estimate the size of free-ranging individuals. Photogrammetry involves obtaining accurate measurements from 2D images, and aerial photogrammetry methods have been used in a number of cetacean species since the 1980s to obtain multiple measurements across the body in order to better estimate the size of individuals, investigate growth and assess body condition (Best and Rüther, 1992; Cubbage and Calambokidis, 1987; Durban et al., 2015; Fearnbach et al., 2018; Konishi, 2006; Miller et al., 2012a; Perryman and Lynn, 2002). Aerial photogrammetry using unmanned aerial vehicles (UAVs) specifically, looks like a particularly promising area of research to be able to obtain multiple morphometric measurements from live animals to assess changes in body condition (Christiansen et al., 2016; Christiansen et al., 2018; Durban et al., 2016).

CHAPTER 3: VARIATION IN BLUBBER LIPID CONTENT SUGGESTS TRADE-OFFS BETWEEN ENERGY STORAGE AND OTHER BLUBBER FUNCTIONS ACROSS CETACEAN FAMILIES

The ziphiid and balaenopterid data analysis and discussion part of this chapter are currently in press in a manuscript in *Aquatic Conservation: Marine and Freshwater Ecosystems.*

Kershaw, JL. Brownlow, A. Ramp, CA. Miller, PJO. Hall, AJ. (2019) Assessing cetacean body condition: Is total lipid content in blubber biopsies a useful monitoring tool? *Aquatic Conservation: Marine and Freshwater Ecosystems.*

1. ABSTRACT

Energy reserves in cetaceans are maintained primarily as blubber lipid stores. However, there is little information on what the variation in these stores can tell us about the condition of individuals. The variation in blubber lipid content across three families of cetaceans - delphinids (n = 10; *Lagenorhynchus albirostris*), ziphiids (n = 8; *Mesoplodon bidens*, *Ziphius cavirostris*, *Hyperodon ampullatus*) and balaenopterids (n = 9; *Megaptera novaeangliae*, *Balaenoptera acutorostrata*) was investigated. Full depth, dorsal blubber samples were collected from dead stranded adult and juvenile individuals that died as a result of acute trauma or chronic debilitation. Total lipid was extracted to investigate firstly, variation in blubber lipid content through the blubber depth and, secondly, how blubber lipid content varies with morphometric body condition estimates and other individual covariates. The balaenopterids showed the most variation in blubber lipid content both within and between individuals (2.40 ± 0.3% to 77.55 ± 0.2%), which is consistent with these species' ability to annually cycle their fat stores. Conversely, the ziphiids showed the least variation in blubber lipid content which was consistently high both within and between individuals (51.30 ± 0.4% to 84.06 ± 1.1%). Both the balaenopterids and the delphinids showed significant differences in lipid content between the outer, middle and inner blubber layers, and as a result of this variation, it was concluded that the lipid content measured in shallow depth biopsy samples gives little information on an individual's condition because it provides no information on the layers that are involved in lipid deposition and mobilisation. There were no differences in lipid content with blubber depth in the ziphiids.

Balaenopterids that died as a result of acute trauma had significantly higher blubber lipid content than those that died as a result of chonic debilitation, but this was not the case for either the ziphiids or the delphinids. Full depth blubber lipid content was not correlated with morphometric body condition estimates across any group. This suggests that while measuring full depth blubber lipid content at the dorsal site sampled can provide information on gross differences as a result of different causes of death between balaenopterid individuals, it gives little information on subtler differences in overall body condition in these three cetacean families. The lack of variation both within and between the ziphiid individuals is especially interesting. Ziphiid blubber may play a fundamentally different role compared to other cetacean species. It is known to consist primarily of wax esters that are less easily metabolised, but are less dense than triacylglycerols. Deep divers may benefit from stores of these low density wax esters to remain closer to neutral buoyancy at depth. These species likely have to prioritise maintaining consistent blubber hydrodynamic, thermal and buoyancy parameters which comes at the expense of having variable fat deposits for energy storage. For this reason, caution should be taken when estimating the condition of these animals using any of the standard blubber indices used for other species.

2. INTRODUCTION

The high energy yield of fats, specifically triacylglycerols (esters made up of a glycerol and three fatty acids), relative to carbohydrates or proteins make them the favoured energy storage form in mammals (Coleman and Lee, 2004; Young, 1976), and an appropriate capacity for triacylglycerol storage in adipocytes is important for normal metabolic regulation (Wang et al., 2015). Adipose tissue lipid content has therefore been linked with total fatness in a range of mammalian species (Beck et al., 1993; Shier and Schemmel, 1975; Stirling et al., 2008) such that, when coupled with demographic and ecological data, using lipid content as a body condition index can be a biologically relevant, relatively inexpensive and rapidly assessed marker (McKinney et al., 2014).

Cetaceans have evolved atypical and widespread deposits of lipids as a thick, subcutaneous blubber layer which is biochemically distinct from the fat deposits of terrestrial mammals (Pond, 1978). It is involved in insulation, buoyancy and the maintenance of hydrodynamic shape, and is their most important site of energy storage (Iverson, 2009). Lipids in the blubber are mobilized in times of energetic need and nutritional stress, and then deposited when food is in excess. The biochemical characteristics of blubber lipids have been extensively studied (Ackman et al., 1965; Litchfield et al., 1975; Lockyer et al., 1985; Varanasi and Malins, 1971), and two main lipid classes, triacylglycerols and wax esters, have been identified and shown to vary in relative abundance among cetacean family groups. More recently, the extensive occurrence of positive selection in triacylglycerol metabolism-related genes, involved mainly in triacylglycerol synthesis and lipolysis, has been documented in cetaceans (Endo et al., 2018; Wang et al., 2015). The positive selection of these genes suggests that cetaceans have evolved an enhanced capacity for inhibiting unrestricted lipolysis and are able to finely control the lipid content of their blubber (Wang et al., 2015).

Similarly to other mammal species, the direct quantification of blubber lipid content has been used as a metric of overall energy stores and body condition in mysticetes (Ackman et al., 1975b; Aguilar and Borrell, 1990; Konishi, 2006; Lockyer, 1986) and, to a lesser extent, in odontocetes (Evans et al., 2003; Gómez-Campos et al., 2011; Montie et al., 2008; Read, 1990). Variation in overall body condition and lipid content tend to be more extreme in mysticetes because their seasonality in feeding and reproduction is stronger than in odontocetes (Boness et al., 2002). For example, pregnant female mysticetes typically display the best body condition with the highest blubber lipid content as they accumulate fat reserves for reproductive investment and to ensure adequate energy transfer to the calf during lactation (Aguilar and Borrell, 1990; Lockyer, 1986). However, blubber lipid content also varies between reproductive states in a few delphinid species including striped dolphins (*Stenella coeruleoalba*) (Gómez-Campos et al., 2011) and bottlenose dolphins (*Tursiops truncatus*) (Montie et al., 2008). Another, less well studied group of cetaceans are the deep diving beaked whales and, to date, while the blubber layer of beaked whales has been shown to be highly lipid rich (Koopman, 2007; Singleton et al., 2017) there have been no studies investigating the relationships between blubber lipid content, condition and reproductive status in this less well understood family of cetaceans.

Lipid content analysis is inexpensive and straightforward, and thus applicable on a broad scale. However, the robustness of the method and validation of lipid content relative to multiple accepted condition indices and other indicators of health has not been fully evaluated across different cetacean families, and especially not for beaked whales. For example, how the lipid content varies through blubber depth and across the body of different cetacean species is not well understood (Koopman, 2007). It is important to appreciate the degree of variability in blubber lipid content within an individual in order to correctly interpret the results from blubber samples collected by biopsy dart from live animals, which has become a standard method of collecting tissue samples from live cetaceans (Hunt et al., 2013).

Here, full depth, dorsal blubber samples were collected from dead-stranded individuals across three cetacean families; delphinids, ziphiids and balaenopterids by the Scottish Marine Animal Strandings Scheme. The aim was to assess if blubber lipid content obtained though remote biopsy sampling provides a robust measure of overall body condition across these three families of cetaceans with different life-history strategies that will shape the function, structure and chemical composition of the blubber. Firstly, changes in lipid content through the full blubber depth were investigated to estimate the representativeness of shallow depth biopsies collected from live animals. Secondly, the relationships between blubber lipid content and other individual covariates were assessed. If blubber lipid content is reflective of body condition, it should exhibit certain biological differences. Specifically, here, it should be correlated with morphometric condition indices and the cause of death of these stranded individuals. By comparing the variation in the two better studied families, the delphinids and the balaenopterids, the lipid content variation of the ziphiids can be put into context so as to better interpret estimates of condition in these species.

3. METHODS

3.1 Blubber Biopsies in Context

Over the last 30 years, dart biopsy sampling has become one of the most common collection methods for obtaining biological tissue samples from free-ranging cetaceans, with samples typically taken from the dorsal area of the animals (Hunt et al., 2013). However, as cetacean blubber has been shown to be highly stratified both histologically and chemically (Koopman et al., 1996; Krahn et al., 2004; Samuel and Worthy, 2004; Smith and Worthy, 2006), it is important to understand the extent to which remotely obtained biopsy samples are representative of the variation of various tissue components through the full blubber depth. Dart biopsy samples are typically collected using a crossbow or a pneumatic rifle with modified arrows and dart tips which are usually hollow, thin-walled, surgical grade stainless-steel cylinders, between 4 - 6 mm in diameter and 20 - 60 mm in length. They each have a cutting lead edge and small internal barbs to ensure that the sample is retained within the tip upon collection. The core sample collected from the dart will vary in length, depending on the force of the impact, the angle of impact and the impact site. This core sample consists of both the skin and the underlying blubber tissue which are typically separated and used for different analyses.

The full dorsal blubber thickness and thus the extent to which shallow biopsy samples are able to sample the various components of the blubber layer will vary by species. The data on dorsal blubber thicknesses of all ziphiid species (n = 44) (Blainville's beaked whale (*Mesoplodon densirostris*), Cuvier's beaked whale (*Ziphius cavirostris*), Northern bottlenose whale (*Hyperoodon ampullatus*) and Sowerby's beaked whale (*Mesoplodon bidens*)) as well as all balaenopterid species (n = 66) (fin whale (*Balaenoptera physalus*), humpback whale (*Megaptera novaeangliae*), minke whale (*Balaenoptera acutorostrata*) and sei whale (*Balaenoptera borealis*) sampled by the Scottish Marine Animal Strandings Scheme (SMASS) between 1990 and 2017 were collated to investigate the variation in dorsal blubber thickness of these species from which biopsy samples are routinely taken. These dorsal blubber thickness measurements were taken from adults, juveniles and calves of both sexes and with varying causes of death such that the maximum possible range in blubber thickness could be assessed (Fig. 3.1).

This variation across species was plotted against the measured blubber thicknesses of remote biopsy samples obtained from Northern bottlenose whales and humpback whales (Fig. 3.1). The Northern bottlenose whale samples were collected in Jan Mayen, Norway (79° 0' N, 17° 39' W), in June 2014 and June 2015 (n = 47). Samples were collected using the ARTS system; a pressurised rifle-type tool, using LKDarts (LKARTS-Norway) with a stainless steel tip measuring 6mm in diameter and 60mm in length. The blubber thicknesses of the samples obtained varied considerably from just 5mm to 52mm. The humpback whale samples were collected in the Gulf of St Lawrence, Quebec, Canada (49° 36' N, 64° 20'W) between June and September 2013 and 2015 using a crossbow and a modified arrow with a stainless steel tip measuring 40 mm in length and 8 mm in diameter (n = 33). These samples showed more

consistency in sample size with the blubber thickness ranging between 10mm and 32mm in length (Fig. 3.1).

It can be seen for both species with recorded blubber thicknesses ranging from between 42mm to 74mm and from 44mm to 68mm in Northern bottlenose whales and humpback whales respectively, that the remote biopsy samples are much smaller and thus likely only sample the outer layers of most of the sampled individuals (Fig. 3.1). For this reason, it is of vital importance to investigate the variation in different biomarkers of interest, in this case, lipids, through the blubber layer and put this variation into the context of remotely obtained biopsy samples that only sample the outermost part of the tissue.

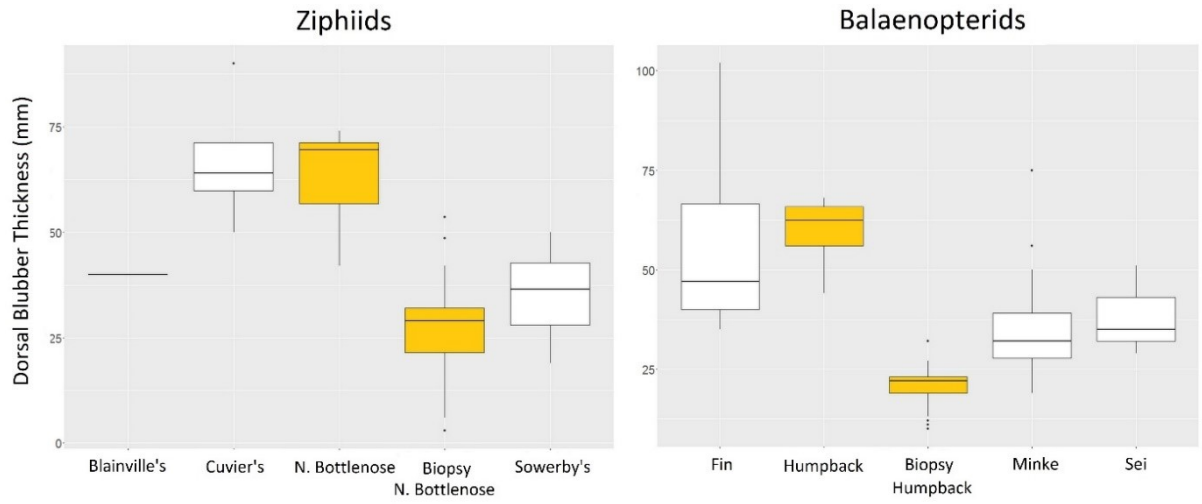

Fig. 3.1. Boxplots of dorsal blubber thicknesses of full depth blubber and biopsy samples recorded for different ziphiid and balaenopterid species. Blubber thicknesses for dead animals were recorded by the SMASS. Blubber thicknesses of remote biopsies taken from live Northern bottlenose whales in Jan Mayen, Norway, and humpback whales in Quebec, Canada, are shaded in yellow and plotted for comparison.

3.2 Sample Collection from Stranded Cetaceans

Dorsal blubber samples collected from a total of 27 individuals, from three cetacean families, were sampled and are summarised in Table 3.1. These were delphinids (n = 10) – white-beaked dolphins (*Lagenorhynchus albirostris*); ziphiids (n = 8) - Cuvier's beaked whales, Sowerby's beaked whales and Northern bottlenose whales; and balaenopterids (n = 9) - minke whales and humpback whales. These were freshly dead individuals that showed minimal signs of decomposition, 25 of which were sampled by the SMASS between 2013 and 2015, and the other two were sampled by the Mingan Island Cetacean Study (MICS) during their 2013 and 2010 field seasons in the Gulf of St Lawrence, Quebec, Canada. For the SMASS sampled animals, as described in Chapter 1, cause of death and morphometric measurements were available. The final two individuals sampled by the MICS were an adult female minke whale and an adult female humpback whale, but no cause of death was confirmed for these animals and morphometric measurements were not taken.

Approximately 2.5 to 5cm^2 blocks of full depth blubber were collected from the dorsal area immediately caudal to the dorsal fin. This area was chosen to emulate the standard biopsy site from live animals. The entire blubber layer was sampled for all animals to include both the epidermis and some underlying muscle so as to recognize the orientation of the sample. The samples were wrapped in aluminium foil, placed individually in plastic containers, and frozen at -20^{0}C before further analysis.

Table 3.1 – Summary of the mysticete and odontocete individuals sampled for full depth blubber samples across three different families. * SMASS (Scottish Marine Animal Strandings Scheme). * MICS (Mingan Island Cetacean Study).

Suborder	Family	Species	Number	Source
Mysticete	Balaenopteridae	Minke whale (*Balenoptera acutorostrata*)	6	SMASS*
				MICS*
		Humpback whale (*Megaptera novaeangliae*)	3	SMASS
				MICS
Odontocete	Ziphiidae	Sowerby's beaked whale (*Mesoplodon bidens*)	4	SMASS
		Cuvier's beaked whale (*Ziphius cavirostris*)	2	SMASS
		Northern bottlenose whale *(Hyperodon ampullatus)*	2	SMASS
	Delphinidae	White-beaked dolphin (*Lagenorhynchus albirostris*)	10	SMASS

3.3 Lipid Extraction

The thickness of the fresh blubber samples was measured to the nearest millimetre by measuring the length from the interface between the skin and the blubber, down to the interface between the blubber and the muscle (Fig. 3.2). Any freezer burnt edges with a dark yellowish colour were removed and discarded. To reduce the loss of lipid while the samples were being prepared, the blubber was subsampled while still partially frozen. A full depth subsample of the original block was cut, and the epidermis and muscle tissue removed. While visible layering was apparent in the blubber of some samples, others did not show visible differences through the blubber depth. For this reason, the blubber was not divided according to visually discernible characteristics, but was subdivided into layers of approximately equal thickness. For balaenopterid and ziphiid samples greater than 30mm in depth, the blubber thickness was sufficient to allow its subdivision into five layers of equal thickness (Fig. 3.2). The inner layer (adjacent to the muscle), the middle layer, and the outer layer (adjacent to the epidermis) were used for lipid extraction while the two transitional layers were discarded (Fig. 3.2). The white-beaked dolphin samples were not of sufficient thickness to follow this same protocol, and for this reason, they were divided into thirds and each layer was used for lipid extraction. Duplicate subsamples, each weighing between 0.15g and 0.3g were taken from the full blubber depth as

well as the inner, middle and outer layers, and lipid was independently extracted from each of the blubber samples using a modified version of a previously published protocol (Folch et al., 1957).

Specifically, the blubber was homogenized with a 2:1 dichloromethane-methanol mixture containing 0.05% BHT (butylhydroxytoluene) with a 1:21 ratio of blubber (g) to solvent (ml). The blubber was ground manually in the solvent with a glass homogenizer until thin and transparent and no longer releasing lipid, and left overnight. The homogenate was then mixed thoroughly with 0.25 its volume of 0.9% potassium chloride (KCl) solution for 10 minutes. The mixture was centrifuged at 900 rcf for 20 minutes to allow it to separate into two phases. The upper phase containing all of the non-lipid substances was discarded. The lower phase, consisting of a solution of tissue lipids, was rinsed twice with anhydrous sodium sulphate to remove any residual non-lipid components at a ratio of 4:1 sodium sulphate to blubber. After rinsing, the lipid-only extract was then transferred to a pre-weighed centrifuge tube. Finally, the solvent was evaporated under dry nitrogen at 30^0C until the solvent had evaporated enough for the lipid residue to become more solid. The centrifuge tube containing the lipid extract was then left open at room temperature for 24 - 48 hours to allow any remaining solvent to evaporate slowly. After complete evaporation of the solvent, the centrifuge tube containing the pure lipid extract was weighed, and the lipid content of the blubber was expressed as a percentage of the wet weight of the sample. This method of lipid extraction is advantageous as it is simple, it can be applied at any scale, there is minimal lipid loss incidental to the washing procedure, and it yields a washed extract which can be taken to dryness without degradation of the lipids (Folch et al., 1957).

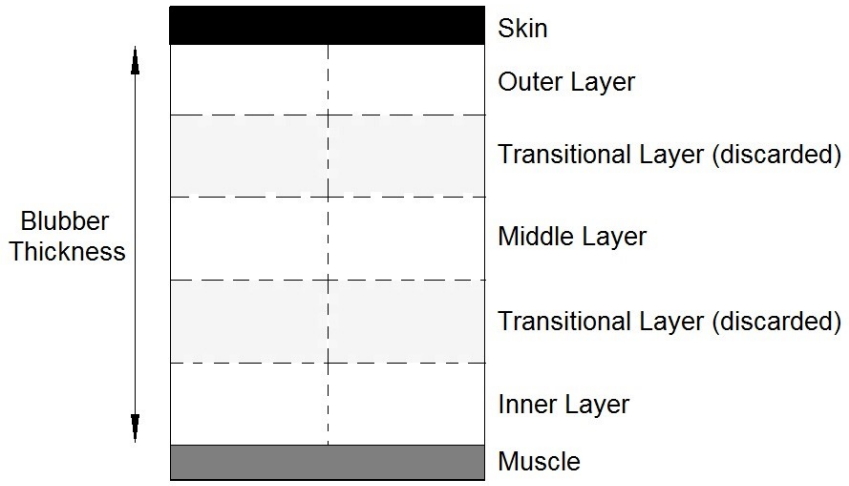

Fig. 3.2. Separation of the full depth blubber samples > 30mm thick into five layers of approximately equal thickness prior to lipid extraction. The vertical dashed line shows where the blubber was divided in half to generate duplicate subsamples. The horizontal dashed lines show where the blubber was divided such that the outer, middle and inner layers were retained for analysis while the two transitional layers were discarded.

3.4 Quality Assurance and Quality Control for Lipid Extraction

All statistical analyses were performed using the statistical package, R, version 3.1.3 (R Core Development Team, 2015). Statistical significance was taken at $p \leq 0.05$.

Quality assurance and quality control tests were performed to assess the limitations and potential sources of error in this lipid extraction method for cetacean blubber samples to better interpret the final results. The limiting factor for the analysis of most remotely obtained biopsy samples is the mass of tissue collected, and for this reason, measurement and extraction errors with sample mass were assessed.

Sample Mass Variability: The effect of sample mass on percentage lipid recovered was assessed, and it was seen that lipid content was not correlated with sample mass using this extraction method (Fig. 3.3a). This indicates no changes in blubber lipid extraction efficiency with samples of varying sizes, and as such, sample masses of between 0.15g and 0.3g are appropriate for extraction to provide robust results.

Extraction Variability: There was no change in the extraction variability, quantified as the measurement standard error between duplicate samples, with increasing lipid content (Fig. 3.3b). There was no difference in the measurement error between the blubber layers (ANOVA; $df = 3$, $F = 1.786$, $p = 0.154$). However, there was significantly higher variation in measurement standard error as well as a higher overall measurement standard error in the white-beaked dolphin samples compared to both the balaenopterids and the ziphiids (ANOVA; $df = 2$, $F = 34.23$, $p < 0.0001$). This could be because the full depth white-beaked dolphin samples were thinner, with an average blubber depth of 17.4 ± 1.28mm (mean ± standard error), than the samples taken from the other two species groups which have a much thicker dorsal blubber layer with means of 53.25 ± 7.56mm and 37.0 ± 5.57mm for the ziphiids and the balaenopterids, respectively. As such, by subsampling a thicker blubber layer, the likelihood of subsampling a more homogenous part of the tissue is higher than when sampling from the thinner layer where the tissue properties may change within the piece that is sampled. As the species that are typically sampled through remote dart biopsy, including ziphiids and balaenopterids, are much larger than white-beaked dolphins, the samples obtained will likely be more homogeneous than from smaller species, so a large standard deviation between duplicate samples is less likely, and this extraction method is robust for such species.

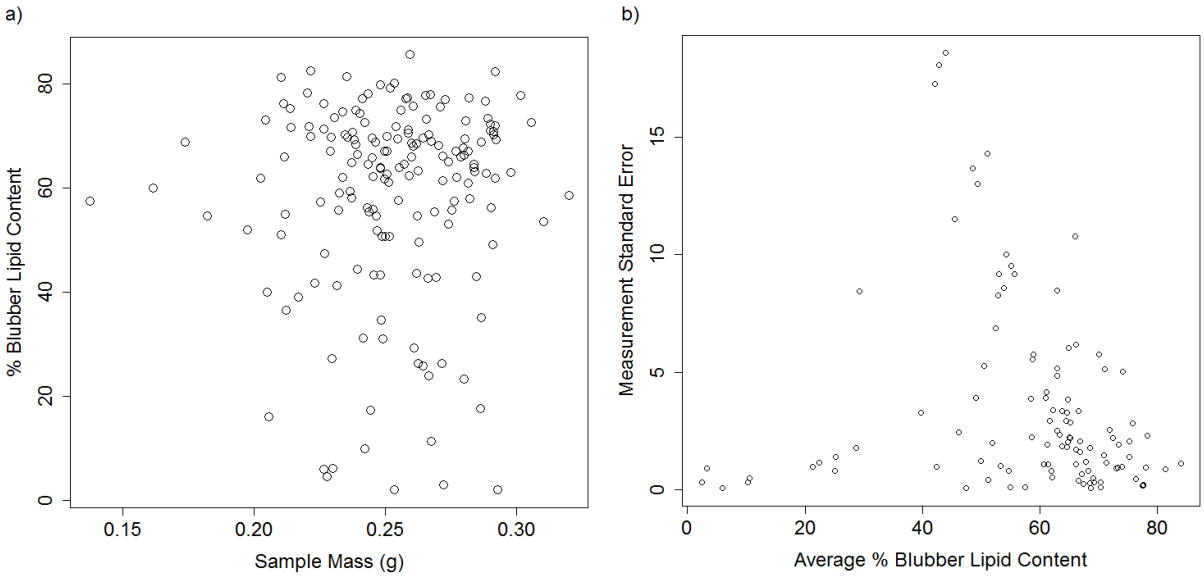

Fig. 3.3. Variation in lipid content and extraction variability with sample mass (n = 108). a) No correlation between lipid content and sample mass suggests no changes in lipid extraction efficiency of tissue samples of between 0.15-0.30g used here. b) No correlation between the measurement standard error and the lipid content suggesting that measurement error does not increase or decrease with increasing lipid content of the samples.

3.5 Statistical Analysis

The data from the white-beaked dolphins, ziphiids and balaenopterids were modelled separately. Two different statistical approaches were used firstly to investigate differences in the distribution of lipids through blubber depth and, secondly, to investigate what other factors may be affecting blubber lipid content including blubber thickness sex, species, age class, cause of death and morphometric body condition. For the white-beaked dolphins, mass/length^2 was used as the most appropriate morphometric index of body condition, and girth/length was used for the ziphiids and the balaenopterids (Chapter 1).

The main challenges for analysing these data were that multiple lipid content measurements came from the same individuals, and these were not normally distributed. The modelling approach used here takes advantage of the statistical structure of generalized linear mixed effect models (GLMMs). GLMMs combine the properties of two statistical frameworks, linear mixed models, which are able to incorporate both fixed and random effects, and generalised linear models which are typically used for non-normal data (Bolker et al., 2008). Random effects can encompass variation among individuals when multiple responses are measured per individual. GLMMs are thus the best tool for analysing non-normal data that involve random effects (Bolker et al., 2008). For this reason, GLMMs were used here to investigate lipid content through the blubber depth whilst accounting for the repeated measurements from the same individuals. A GLMM (glmer function in the package 'lme4') with a gamma distribution, a log link function and each individual treated as a random effect was used to investigate the effect of blubber layer (full, outer, middle, inner) on the lipid content of the samples from each species group.

Generalised Linear Models (GLMs) were then used to investigate the effects of cause of death (COD), blubber thickness, morphometric body condition (either mass/length^2 or girth/length), sex, species (for the balaenopterid and the ziphiid data that were made up of multiple species) and age class on the blubber lipid content of the *full depth* samples from each individual. GLMs with a gamma distribution and a log link function were used to model the non-normal distribution of the lipid content data. All sampled individuals were used for the white-beaked dolphins (n = 10) and for the ziphiids (n = 8). However, for the balaenopterids, the two individuals sampled by the MICS, an adult humpback whale and an adult minke whale, for which COD and morphometric data were not available, were not included in this second part of the analysis (n = 7).

For each species group, a full GLM was generated to include the effects of all explanatory variables. To ensure that the explanatory variables were not correlated, and thus to avoid multicollinearity in the final model, variables were selected for inclusion based on variance inflation factors (VIFs). VIFs are used to determine when two or more predictors in a regression are highly correlated, and measure how much the variance of an estimated regression coefficient increases if the predictors are correlated. Starting with the full model, the VIF for each covariate in the model was calculated (*car* package). The covariate with the highest VIF value was removed from the model in a stepwise fashion until the VIF values for all covariates were below three (Zuur et al., 2010). These covariates were retained for further analyses. The threshold VIF value of 3 was selected based on previous work that showed this to be a conservative cut-off VIF value for the removal of collinear variables (Zuur et al., 2010). When there is collinearity in a model, the standard errors of the parameters are inflated, producing higher VIF values. This results in larger p-values and makes it more difficult to detect an effect (Zuur et al., 2010). It is thought that high, or even moderate, collinearity is especially problematic when ecological signals are weak. When this is the case, VIF cut-off threshold values need to be low, otherwise the inclusion of parameters with high VIF values may cause nonsignificant parameter estimates compared to when there is no collinearity (Zuur et al., 2010).

Then, the 'dredge' model selection function (*MuMIn* package) was used to identify from this subset of uncorrelated variables, which ones best explained the variation in blubber lipid content data, and should be included in the final model. The dredge function is a backwards selection tool that runs models with all possible combinations of the explanatory variables including both the specified interactions between variables and their respective main effects. The goodness of fit of each model was assessed using the AICc (Second-order Akaike Information Criterion which uses a correction for small sample sizes). The models were ranked by their AICc to determine which combination of variables best explains the relationships in the data. The model with the lowest AICc value was used for further interpretation because this contained only the variables and / or interactions of importance. Summary statistics of the model coefficients were used to assess the effect of each covariate on blubber lipid content.

4. RESULTS

4.1 Variation through Blubber Depth

Each species group showed a different pattern in lipid content through blubber depth. The balaenopterids showed the most variation in blubber lipid content both within and between individuals (full dataset range ± standard error from 2.40 ± 0.3% to 77.55 ± 0.2%), followed by the white-beaked dolphins (full dataset range ± standard error from 29.3 ± 8.5% to 74.2 ± 5.0%). The ziphiids showed the least variation (full dataset range from 51.30 ± 0.4% to 84.06 ± 1.1%). For the white-beaked dolphins, the outer layer and the full depth samples both had a higher lipid content than the inner and middle layers (p values both 0.01) that were not significantly different from each other (Fig. 3.4). For the ziphiids, there were no differences in lipid content between layers (Fig. 3.4). There were significant differences between all layers in the balaenopterids, showing that the outer layer had the highest lipid content and the inner layer had the lowest (p values < 0.005) (Fig. 3.4).

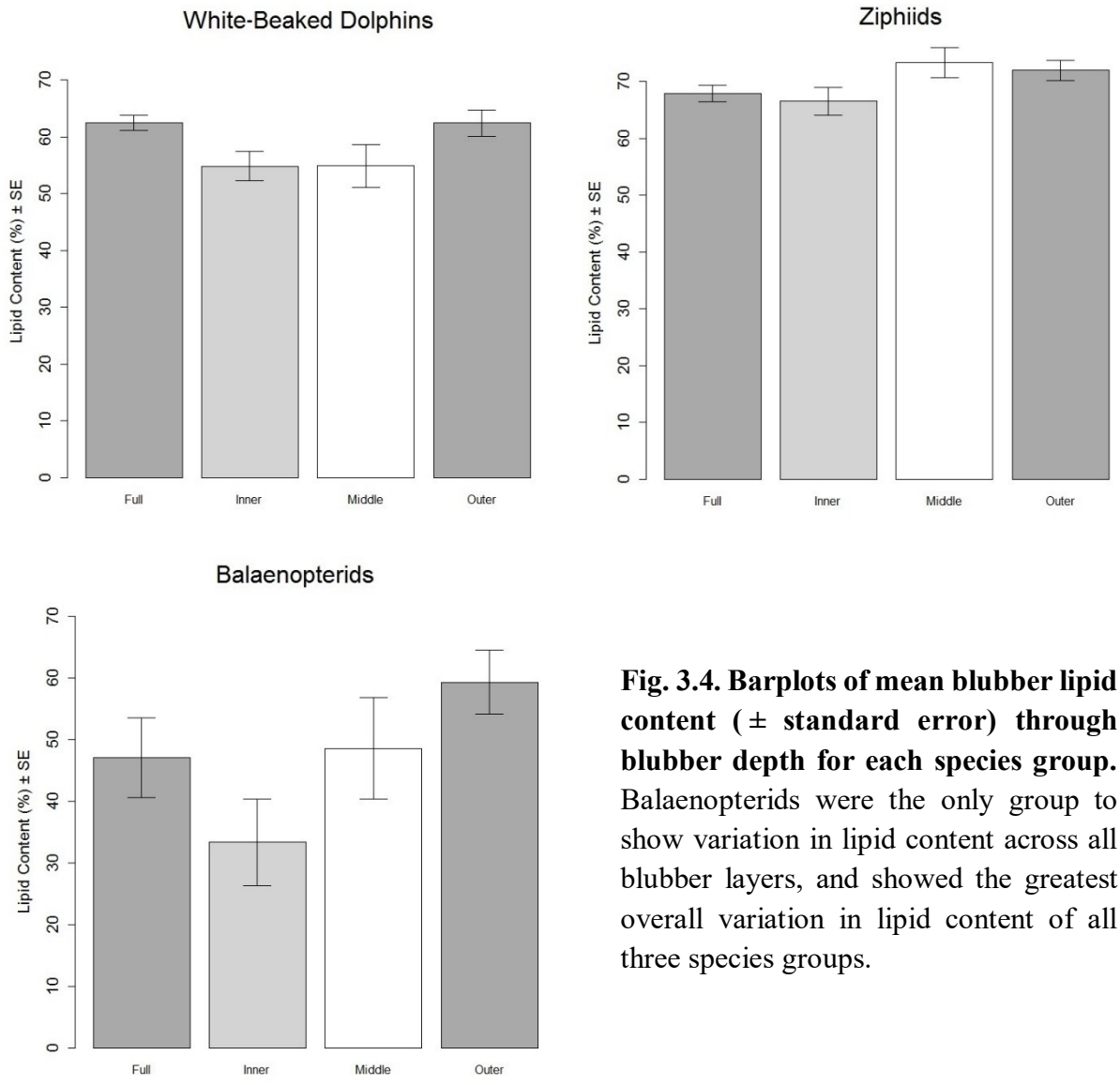

Fig. 3.4. Barplots of mean blubber lipid content (± standard error) through blubber depth for each species group. Balaenopterids were the only group to show variation in lipid content across all blubber layers, and showed the greatest overall variation in lipid content of all three species groups.

4.2 Variation with Other Covariates

The lipid content in the full blubber depth samples was not correlated with blubber thickness for any of the three species groups. No covariates were retained following generalised linear model selection for the white-beaked dolphins or the ziphiids. Sex, age class, cause of death and morphometric body condition therefore did not contribute to the explanation of the variabity in the blubber lipid content of these full depth, dorsal samples. The best fitting GLM for the balaenopterid data included only COD as an important explanatory variable with a significantly lower lipid content in the chronic cases compared to the acute cases ($p = 0.004$) (GLM: $df = 3$, weight = 0.87, Δ AICc of 4.18 to the next best fitting model) (Table 3.2). In fact, individuals that died as a result of an acute trauma had significantly higher blubber lipid content across all blubber layers than those with a chronic cause of death (ANOVA; $F = 44.71$, $df = 1$, $p < 0.0001$) (Fig. 3.5).

Table 3.2 – Summary table of the generalised linear model results investigating the effects of various covariates on blubber lipid content for the three species groups. *VIFs: variance inflation factors measure collinearity between predictor variables. The predictor variables with high VIFs were excluded from the analysis.

Family	Sample Size	Covariates Discarded Based on VIFs*	Covariates Retained following GLM model selection
Delphinids	10	Sex, Age Class	-
Ziphiids	8	Sex, Age Class	-
Balaenopterids	7	Girth / Length	COD

The effect of body condition, either as mass/length^2 for the white-beaked dolphins, or as girth/length for the ziphiids and the balaenopterids, was not retained as an important explanatory variable in the full blubber depth lipid content models. The relationship between the lipid content of each layer and morphometric body condition was then assessed separately. For the balaenopterids, there was a significant positive relationship between lipid content and girth/length in the middle blubber layer alone (linear model; $p = 0.035$, Adjusted $R^2 = 0.4$), which was not seen in the other two groups (Fig. 3.6). Variation in this layer therefore likely drives the differences in full blubber depth lipid content that are associated with different causes of death.

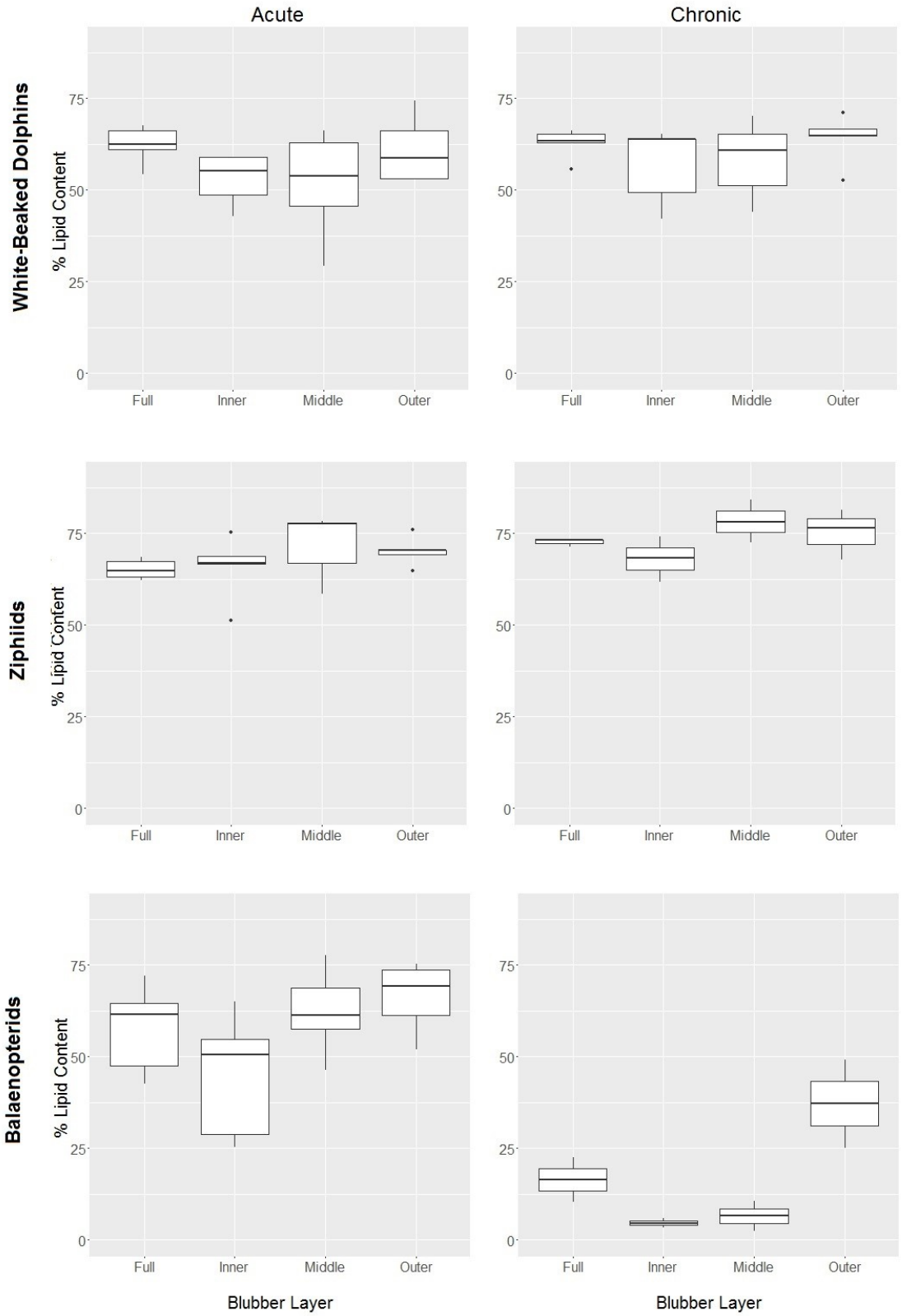

Fig. 3.5. **Boxplots of blubber lipid content across blubber layers between COD categories for the three cetacean families.** The balaenopterids were the only group to show significant differences in blubber lipid content between acute and chronic cases. Blubber lipid content was significantly lower across all blubber layers in the chronic compared to the acute cases (ANOVA; $F = 44.71$, $df = 1$, $p < 0.0001$).

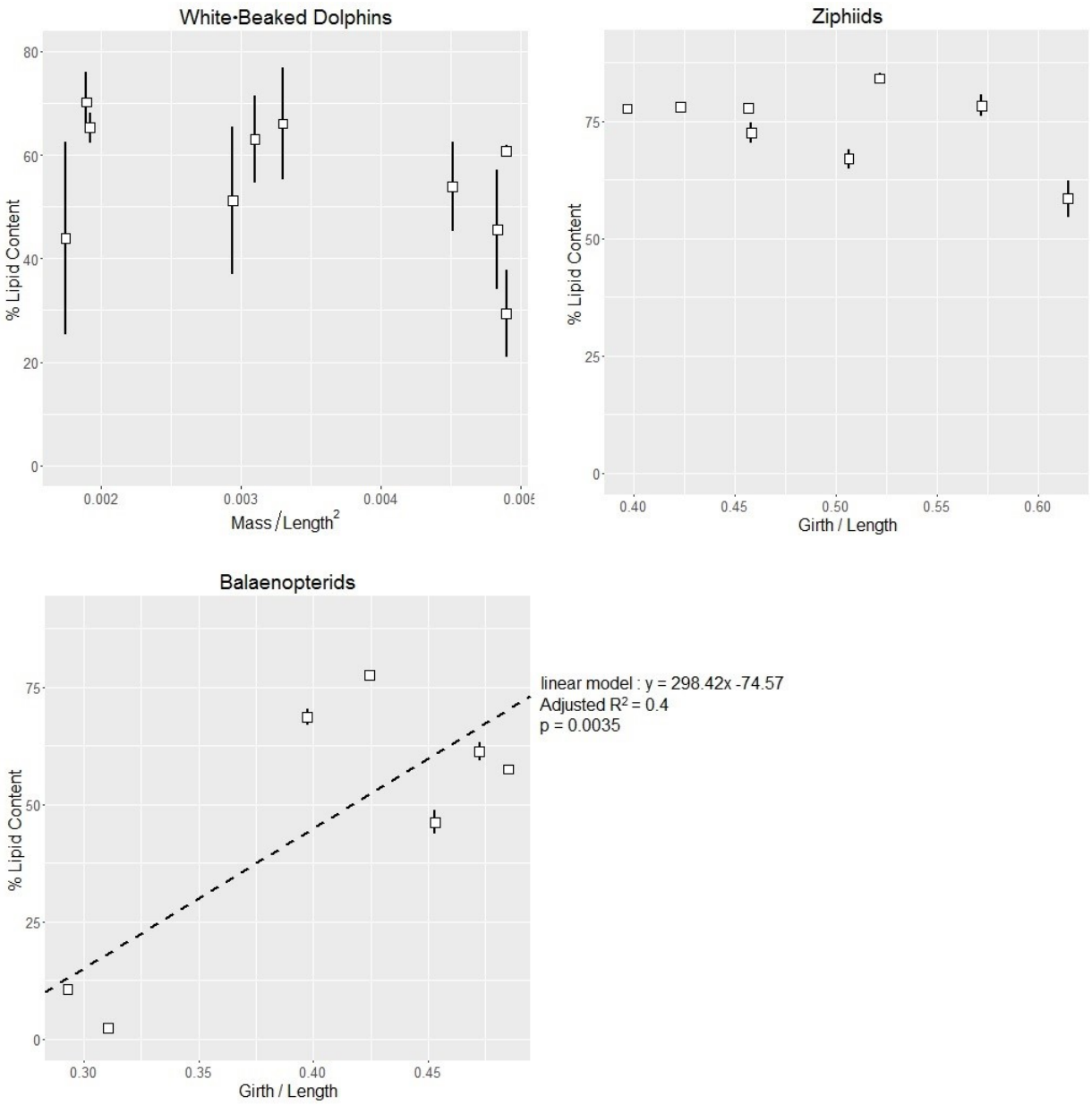

Fig. 3.6. Lipid content in the middle blubber layer plotted against the morphometric body condition estimate for each species group. There was a significant correlation between the lipid content in the middle layer and morphometric body condition in the balaenopterids alone ($p = 0.035$, Adjusted $R^2 = 0.4$).

The lack of a relationship between lipid content and morphometric body condition in the white-beaked dolphins and the ziphiids could have been a result of the small sample size of individuals. It is possible that only a small number of animals in either good or poor condition were sampled here. To investigate this possibility, the range in individual morphometric condition estimates for each species group was assessed using the same data collected by the SMASS used to examine dorsal blubber thickness described above. These data were from 51 balaenopterids from four species (minke, humpback, fin and sei whales), 42 ziphiids from four species (Northern bottlenose whales, Sowerby's, Cuvier's and Blainville's beaked whales

(*Mesoplodon densirostris*)) and 90 white-beaked dolphins. The range in condition estimates varied between each group. There was a four-fold difference between the white-beaked dolphins in the thinnest and the fattest condition (mass/length^2 range between 0.0012 – 0.0049), and a ten-fold difference in the balaenopterids (girth/length range between 0.064 – 0.67). For the ziphiids however, there was less than a factor of two difference between the individuals in the thinnest and the fattest condition (girth/length range between 0.37 – 0.63), indicating a very narrow range in body condition in these species. The individuals sampled for lipid extraction covered a large part of these ranges for the white-beaked dolphins and the ziphiids (Fig. 3.7). Thus the absence of any correlations between blubber lipid content and morphometric condition is likely not a result of the sample size, or a bias in the sampled individuals. The balaenopterids sampled were from only a small range of the potential variation (Fig. 3.7), yet there was still a positive correlation between girth/length and the lipid content in the middle layer, as well as differences between the animals with acute or chronic causes of death.

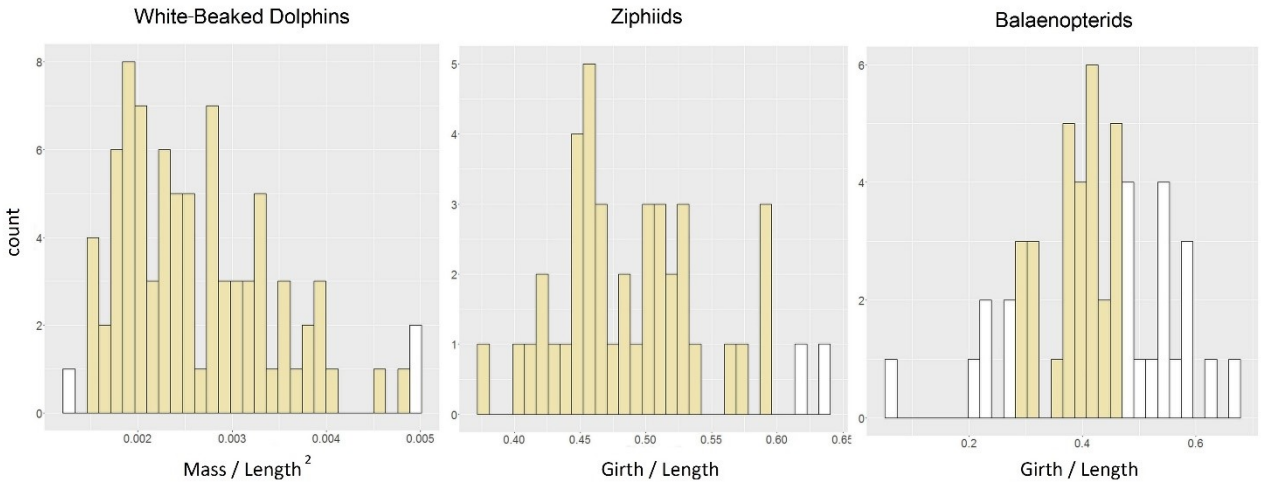

Fig. 3.7. Body condition histograms for the three species groups using morphometric data collected by the SMASS. The shaded bars indicate the range in condition indices of the individuals sampled for blubber lipid extraction.

5. DISCUSSION

Lipid Content Stratification through Blubber Depth

Stratification of lipid content through blubber depth was seen in both the white-beaked dolphins and the balaenopterids. In both of these groups, the middle and inner layers had a lower lipid content than the outer layer closest to the skin, and the inner layer of the balaenopterids had the lowest lipid content overall. The stratification observed here is likely the result of differential metabolism of fatty acids, and thus deposition and mobilisation of lipid stores through the blubber depth (Lockyer et al., 1984; Samuel and Worthy, 2004; Smith and Worthy, 2006). Long-chain polyunsaturated fatty acids of dietary origin are present in higher concentrations in the inner layer compared to the outer layer, and these are preferentially metabolised due to their proximity to the body core (Krahn et al., 2004; Lockyer et al., 1984). This makes the inner and middle layers more metabolically active in terms of lipolysis and lipogenesis compared to the outermost layer which is made up primarily of short chain monosaturated fatty acids (Olsen and Grahl-Nielsen, 2003; Ruchonnet et al., 2006; Samuel and Worthy, 2004). The outer layer therefore likely has a more structural and possibly thermoregulatory role with a more stable composition over time as it is more metabolically inert. Koopman and colleagues (2002) found that the outermost layer of the blubber in starved harbour porpoises (*Phocoena phocoena*) was virtually indistinguishable from normal animals, but throughout the rest of the blubber, the adipocytes shrank and 'disappeared' during starvation.

Here, only the lipid content of the middle layer of the balaenopterids was significantly correlated with morphometric condition. This is consistent with previous reports that the middle layer of fin and sei whale blubber consisted of loose, fatty tissue, and was the most variable in thickness across individuals compared to the other two layers (Lockyer et al., 1985). The lowest lipid content seen in the inner layers of both the delphinids and the balaenopterids is consistent with the findings in other species whereby the inner layer contained proportionally more fibrous tissue than the other two layers (Krahn et al., 2004; Lockyer et al., 1984). Thus, had shallow biopsy samples been taken from these animals, they would provide inflated estimates of blubber lipid content as the outermost layer did not reflect the lipid available for mobilisation in the other layers. In addition, a number of studies have now shown that the lipid content measured in a biopsy sample is not reflective of that of the tissue when sampled at necropsy (Krahn et al., 2004; McKinney et al., 2014; Ryan et al., 2012). The discrepancies are thought to be a result of incomplete sampling of the full blubber depth as well as lipid loss during dart retrieval as the adipocytes are disrupted upon dart impact.

In contrast to the other two families, the ziphiids showed no variation in lipid content through depth. The blubber of ziphiids has been shown to display stratification of fatty acids and wax esters through blubber depth, but the overall lipid content appears to be uniform (Koopman, 2007; Litchfield et al., 1976; Singleton et al., 2017). Ziphiid blubber therefore does not show the same stratified characteristics as seen in other cetacean species in terms of layers of lipid deposition and mobilisation that were seen in the balaenopterids and the white beaked dolphins here. This suggests that the tissue is not being used in the same way for the same energy storage functions as in other cetacean species. Blubber lipids in ziphiids are dominated by wax esters

(Bagge et al., 2012; Koopman, 2007; Litchfield et al., 1976; Singleton et al., 2017), and this different composition of the tissue likely affects the patterns of lipid deposition and mobilisation, as well as tissue function, which will be discussed in detail below.

Lipid Content Variation as an Indicator of Body Condition

The wide variation in lipid content both within and between the balaenopterid samples compared to the other two species groups is likely a result of the life-history strategies of these species whereby they cycle their energy stores during seasonal migrations that link temporally and spatially separated breeding and feeding seasons (Koopman, 2007). As they are adapted to cycle their fat stores, the range in blubber lipid content is likely to vary much more than for species that are not adapted to undertake prolonged fasting periods, and thus live within much narrower limits of stored energy reserves. This was highlighted in the larger dataset of morphometric measures from the SMASS with the 10-fold difference between the girth/length index of individuals in the thinnest and fattest condition. Cause of death was retained following model selection, showing that individuals that died as a result of acute trauma had a higher lipid content across the full blubber depth compared to chronically debilitated animals. This is to be expected because chronically debilitated animals have likely depleted their fat reserves following a more gradual decline in health and perhaps reduced foraging opportunities.

These results from dorsal blubber samples are consistent with previous work on fin and sei whales, where the dorsal posterior area of the body has been shown to be a major site for lipid storage in both the blubber and the muscle, leading to the suggestion that this area may be useful as an indicator of overall body fat condition (Lockyer et al., 1985). While the full blubber depth samples could be used to differentiate between gross differences as a result of different cause of death categories, only the lipid content measured in the middle layer was an appropriate metric to detect subtler differences in condition among the balaenopterid individuals. Neither sex nor age class were retained as important explanatory variables which was surprising given previous evidence of variation in fat stores across sex and reproductive classes in balaenopterids (Aguilar and Borrell, 1990; Lockyer and Smellie, 1985). These data were dominated by juvenile minke whales, and for this reason little can be inferred about the effect of age class on blubber lipid content. Similarly, as the animals had probably not reached sexual maturity, the lack of differences between the sexes could be indicative that sex differences only appear once animals start to deposit and mobilise fat stores associated with the costs of reproduction.

Model selection for the delphinid and the ziphiid data retained no covariates as important explanatory variables. When plotted individually, there were no correlations between the lipid content in any blubber layer and morphometric body condition. One explanation for the lack of variation here could be a consequence of the dorsal site of the blubber sample. Dorsal blubber samples were taken immediately caudal to the dorsal fin in all species in order to investigate the potential use of lipid content as measured in remotely obtained biopsy samples which target this area. However, in small odontocetes, the blubber in the thoracic-abdominal region is hypothesized to play an important role in insulation and energy storage, whereas the region posterior to the dorsal fin is thought to primarily act to maintain hydrodynamic and

locomotory functions and is metabolically inert (Gómez-Campos et al., 2015; Koopman, 1998; Koopman et al., 2002; Tornero et al., 2004). Specifically, harbour porpoises and common dolphins (*Delphinus delphis*) showed the highest blubber lipid content in the anterior-ventral region (Koopman et al., 2002; Tornero et al., 2004), and patterns of lipid distribution in starved harbour porpoises showed that they are mobilised from their ventral girth region but not from other areas of the body (Koopman, 1998). It was hypothesised that altering the structure of the posterior blubber by mobilizing lipids could have serious effects on the locomotory efficiency of the animal as this region of the blubber acts to reduce the costs of locomotion by acting as a biological spring (Koopman et al., 2002).

This pattern of lipid deposition and mobilisation may also extend to other odontocete species such as the white-beaked dolphins and the ziphiids sampled here, which could explain why lipid content at this dorsal sampling site did not correlate with morphometric indices of body condition or cause of death of the individuals. Thus, there are apparent differences in the function of the blubber as an energy store across the bodies of the larger cetaceans, the balaenopterids, and the smaller odontocetes studied to date. These differences are likely due to a combination of the different hydrodynamic body shapes, swimming gaits and manoeuvrability of the species, as well as their life histories and reproductive strategies that determine their reliance on endogenous energy stores. Some areas of the body are therefore more important for energy storage than others, and this variation is not necessarily captured in the dorsal sampling site of biopsy samples for some species.

Implications of the Dominance of Wax Esters in Ziphiids

The apparent absence of variation in lipid content both within and between the ziphiids sampled here could be a result of the different lipid-type composition of the tissue. Ziphiid blubber is made up of between 80% and 100% wax esters (Hooker et al., 2001; Koopman, 2007; Litchfield et al., 1975), and their relative contribution to the tissue changes with different age and reproductive classes (Singleton et al., 2017). It is possible that beaked whale blubber lipid storage properties differ from other species because wax esters have different chemical properties to the triacylglycerols that are more easily and rapidly metabolised (Place, 1992; Pond, 1998). In fact, most mammals are incapable of metabolizing wax esters and thus eliminate them in their faeces. The proportion of the lipid stored in the blubber that is available for mobilisation to provide energy during times of reduced foraging may therefore be much smaller in beaked whales than species of a similar size that preferentially store triacylglycerols. Though more research is needed to understand the capability of cetaceans to mobilize wax ester stores, the preponderance of this less easily metabolised lipid-class in the blubber of these species is a further indication that the tissue is not being used as an energy store in the same way as other cetacean species.

Recent studies on the composition of fresh undigested forestomach and colon contents of minke whale and right whale (*Eubalaena glacialis*) faeces suggested that they may have evolved an unusual metabolic capability, such as specialized enzymes or a particular gut symbiont, which, unlike most other mammals, enables them to utilize the wax esters in their diet (Nordøy, 1995; Swaim et al., 2009). Baleen whales that rely on a wax ester-rich diet from their copepod prey

thus seem to have overcome the digestive limitations of most terrestrial mammals. Interestingly, however, the ingestion of wax esters does not necessarily mean that these compounds will be stored in their adipose tissue. Even though balaenopterids consume large quantities of wax esters, and are apparently able to metabolise them, their blubber is composed almost entirely of triacylglycerols. Given the importance of these lipids in beaked whale blubber, it is likely that these species have also evolved this metabolic capability, but unlike the balaenopterids, beaked whales are also depositing these lipids in the blubber. For example, as beaked whales and sperm whales (*Physeter macrocephalus*) approach adult size, the blubber is increasingly made up of wax esters (Koopman, 2007; Singleton et al., 2017), suggesting that wax ester storage is minimal at birth, and they then develop the ability to synthesize and deposit it.

The differences in the basic structure and common constituents of wax esters and triacylglycerols give them distinct physical properties. Specifically, wax esters have lower densities (specific gravities) than triacylglycerols, such that a unit volume of wax esters will provide more positive buoyancy than the same unit volume of triacylglycerols in sea water (Sargent, 1978). In addition, wax esters are also less sensitive to phase change with changing temperature than triacylglycerols (Nevenzel, 1970) which affects tissue buoyancy as they change from a liquid to a solid. As wax esters are the dominant lipid storage class of calanoid copepods (Sargent et al., 1977), and deep water and vertically migrating fishes (Nevenzel, 1970), it has been suggested that lipids are preferentially stored as wax esters in many marine organisms as they provide greater buoyancy (Sargent, 1978). This may be especially the case for beaked whale species that are particularly deep divers (Tyack et al., 2006), and may use the positive buoyancy provided by wax esters, unaffected by changing temperatures through the water column, to maintain an overall body status close to neutral buoyancy as gas stores compress with depth (Miller et al., 2004; Miller et al., 2016). Neutral buoyancy is thought to minimize locomotion costs both for vertical transits (Adachi et al., 2014; Miller et al., 2012b) and for horizontal swimming (Sato et al., 2013).

Wax esters in the blubber have also been suggested to play a potentially important role in thermoregulation (Bagge et al., 2012; Singleton et al., 2017). The blubber of deep diving species with a higher wax ester content, but with the same overall lipid content as shallower divers, has been shown to be a superior insulating material (Bagge et al., 2012). Deep divers can experience large variations in ambient temperatures during the course of a single dive, and it has been suggested that this increased insulative capacity of the blubber may facilitate deeper, longer dives in these species (Bagge et al., 2012). It was concluded that the function of blubber as an insulator is highly complex and is likely affected by a number of factors including lipid class, stratified composition and dynamic heat storage capabilities as well as currently unmeasured characteristics such as vascularization, collagen content or water content (Bagge et al., 2012). These beaked whale results highlighting the lack of variation in lipid content add to a body of work aiming to understand the selective pressures leading to the presence and abundance of wax esters in the blubber of odontocetes, and their potential physiological functions.

Currently, there is no evidence to suggest that any of the beaked whale species experience substantial or predictable fasting cycles, or periods of food shortage when lipid reserves might be drawn upon (MacLeod, 2018). Species that rely on blubber lipids during fasting may be constrained to storing only triacylglycerols, because this lipid source can be reliably and rapidly mobilized. By extension, perhaps as beaked whales do not experience prolonged fasting periods, they are preferentially able to store blubber lipids that provide some elements of physiological or mechanical advantage during diving instead of energy storage. For these reasons, the role of beaked whale blubber in diving physiology may determine the extent to which it can change in lipid composition because the structural and mechanical properties of the tissue are prioritised at the expense of available energy stores. Small changes in the blubber lipid content may affect their thermoregulatory homeostasis, their hydrodynamic shape required for capture of fast-swimming prey, the nitrogen solubility of the tissue, and possibly most importantly, the maintenance of neutral buoyancy which enables them to forage efficiently at depth. As a result, they maintain a narrow range of blubber lipid content. The very narrow range in the girth/length condition estimates using the larger morphometric dataset from the SMASS further supports the possibility that these species survive within very narrow physiological limits.

Much More than Just an Energy Store

Here, there was no relationship between blubber thickness and blubber lipid content for any of the individuals sampled, regardless of species group. Results of this study therefore support the idea that blubber thickness alone is an inadequate index of fat stores because the lipid content of the tissue varies independently of its thickness (Aguilar et al., 2007; Dunkin et al., 2005; Evans et al., 2003). There are two possible reasons for this lack of a relationship: either the small sample size of this dataset prevented the detection of any correlations between lipid content and blubber thickness, or the other functional roles of the blubber not involved in energy storage also affect blubber thickness. This is a small sample size of 27 individuals, and it is possible that the lack of correlation between lipid content and blubber thickness here could be because there was not a great enough range in blubber thicknesses or lipid content within each species group. However, because the individuals sampled, with the exception of the balaenopterids, covered the range of potential morphometric body condition estimates from the larger SMASS dataset, this is likely not the case.

Various other studies have also shown similar resuls in which blubber thickness was determined to be a poor index of nutritive condition because it was not correlated with changes in overall body fat reserves (Aguilar et al., 2007; Caon et al., 2007; Evans et al., 2003; Gómez-Campos et al., 2015; Koopman, 2007; Read, 1990; Ruchonnet et al., 2006). This is because the extent to which blubber lipids can be mobilised is limited by thermoregulatory and hydrodynamic considerations, and is not simply a function of its energy reserves. It is well recognised that in addition to its role as a depot of energy, cetacean blubber also serves important functions as an insulator, it is involved in active thermoregulation, it streamlines the body, facilitates hydrodynamic locomotion, contributes to water balance, and provides buoyancy (Iverson, 2009). For example, adipocyte fluid content and blubber collagen content also likely dictate blubber thickness. For these reasons, attempts should be made to move away

from using blubber thickness as an indicator of condition, and alternative methods should be investigated across all species, not just beaked whales, as discussed in Chapter 1.

Conclusions and Future Directions

Cetaceans have evolved highly specialized adipose tissues to meet the challenges of their aquatic environment. Varying habitats and life history strategies have shaped different typical blubber functions and composition such that the blubber may serve a fundamentally different purpose across different species. Here, the lipid content of the middle layer in the balaenopterids was correlated with morphometric body condition, in keeping with previous work suggesting that this layer is the most important in terms of lipid deposition and mobilisation. However, it still remains unclear for most cetacean species whether changes in lipid content and blubber thickness in this part of the tissue occur as a result of adipocyte hyperplasia or hypertrophy, or a combination of both. The lipid content measured in the outer part of the tissue for these species, available through remote dart biopsy, thus gives little information on an individual's condition, because it provides no information on the layers that are involved in lipid deposition and mobilisation.

There was a lack of stratification of total lipid through the blubber depth of the ziphiid samples, as well as a lack of variability among individuals. The lack of variability here together with the known high wax ester content of the blubber suggests that its other roles in diving physiology for example, may be of greater importance than its role as an energy depot. The blubber in these species may therefore not be optimized as an energy store as it is in many other cetacean species. The relationship between blubber lipid content and overall energy stores is not straightforward in these animals of different body sizes, life history strategies, foraging ecologies and physiological capabilities. Using the proportions of different saturated / unsaturated fatty acids or the proportion of triaclyglycerols to wax esters, for example, might be a more reliable indicator of condition. Thus, because of the inherent variability in the structure and composition of blubber among families, species, individuals, and even within individuals, extreme caution should be exercised when making inferences and generalizations based on the lipid content of only a small portion of this extremely complex tissue. Further work should prioritise measuring other components of blubber biopsies as potential markers of condition.

CHAPTER 4 : BLUBBER CORTISOL - A NOVEL MARKER OF PHYSIOLOGICAL STATE IN CETACEANS?

Cortisol was extracted and quantified in a subset of the harbour porpoise blubber samples by Meredith Sherrill, and these data were used for her MSc Thesis in 2015.

The harbour porpoise data analysis and discussion part of this chapter has been published in *Ecology and Evolution* (2017).

Kershaw JL, Sherrill M, Davison NJ, Brownlow A, Hall AJ. Evaluating morphometric and metabolic markers of body condition in a small cetacean, the harbor porpoise (*Phocoena phocoena*). *Ecology and Evolution*. 2017;7(10):3494-3506.

1. ABSTRACT

Cortisol is the main glucocorticoid hormone involved in the regulation of lipolysis and overall energy balance in mammals, and circulating concentrations could therefore provide information on physiological state. As a potential metabolic condition marker, blubber cortisol concentrations were investigated across two cetacean species groups with different life-history strategies; firstly, harbour porpoises (*Phocoena phocoena*), and secondly, humpback whales (*Megaptera novaeangliae*) and minke whales (*Balaenoptera acutorostrata*), grouped here as balaenopterids. Blubber cortisol concentrations did not significantly vary around the girth of harbour porpoises (n = 20), but there was significant vertical stratification through the blubber depth with highest concentrations in the innermost layer. Concentrations in the dorsal, outermost layer were representative of concentrations though the full blubber depth, and showed variation by sex, age class and were negatively correlated with a morphometric index of body condition (mass/length^2) in this species. There was no variation in cortisol concentrations between different blubber layers in the balaenopterid samples, so concentrations measured in the outermost layer were representative of those through the full blubber depth (n = 9). The balaenopterid individuals also showed variation between the sexes, and concentrations in the outer blubber layer were negatively correlated with a morphometric index of condition (girth/length). Higher blubber cortisol concentrations measured in animals in poorer condition that are likely metabolising fat stores, rather than depositing them, therefore appear to be consistent in both non-fast adapted and fast-adapted species. Using these species as models for live cetaceans from which blubber biopsy samples are routinely collected, cortisol concentrations in the dorsal, outermost blubber layer could be used as a biomarker of the metabolic state of the tissue and of physiological state in free-ranging animals.

2. INTRODUCTION

Marine mammals encounter a diverse range of both psychological and physiological anthropogenic stressors including habitat alteration and degradation, industrial pollution, noise pollution, incidental entrapment in fishing gear and a reduction in prey abundance, to name just a few (Fair and Becker, 2000). These stressors are likely to induce a physiological response which can be either short-term (acute) or long-term (chronic). This reaction is generally termed the stress response. It is characterised by the release of cortisol, a glucocorticoid, from the adrenal glands, and epinephrine and norepinephrine, the catecholamines, from the adrenal glands and from nerve terminals, which together prepare the individual for the demands of metabolic, physical and psychological stressors (Negrão et al., 2000). Because of the harmful effects of chronic stress, including reproductive failure, immune system suppression, promotion of protein loss from skeletal muscle, neuronal cell death, and suppression of growth (McEwen et al., 1993; Munck et al., 1984; Sapolsky, 1987; Sapolsky et al., 2000), there is increasing interest in measuring the stress response in free-ranging marine mammals.

The adrenal secretion of cortisol in response to stressful psychological stimuli (e.g. anthropogenic disturbance) and physiological stimuli (e.g. nutrient limitation) is controlled by the hypothalamic-pituitary-adrenal (HPA) axis (Liberzon et al., 1997). Cortisol is the major glucocorticoid stress hormone released following stimulation of the HPA axis in many mammals, including cetaceans (Ortiz and Worthy, 2000; St. Aubin et al., 1996) and pinnipeds (Barrell and Montgomery, 1989; Gardiner and Hall, 1997). As a result, cortisol and its metabolites have been measured in various marine mammal matrices including the blood (Atkinson et al., 2011; Barrell and Montgomery, 1989; Thomson and Geraci, 1986), faeces (Hunt et al., 2006; Rolland et al., 2012), saliva (Pedernera-Romano et al., 2006), hair (Bechshøft et al., 2012; Macbeth et al., 2012) skin (Bechshøft et al., 2015), blow expirate (Thompson et al., 2014) and blubber (Champagne et al., 2017; Kellar et al., 2015; Trana et al., 2015, 2016) in order to measure their responses to combinations of environmental and anthropogenic stressors.

However, in terrestrial mammals, the glucocorticoids have also been proposed as long-term regulators of both energy intake and storage (Strack et al., 1995). Glucocorticoids are metabolic hormones that increase in circulation in response to energetic needs, and their concentrations can be interpreted as indicators of allostatic load (Bonier et al., 2009). Cortisol is of particular interest in the regulation of whole body energy stores because it is involved in maintaining the balance between fat storage where triglycerides are deposited, and fat depletion where they are catabolised and released into circulation (McMahon et al., 1988; Peckett et al., 2011). Cortisol is known to play an active role in lipolysis in subcutaneous adipose tissue (Divertie et al., 1991; Djurhuus et al., 2004; Samra et al., 1998), stimulate gluconeogenesis, mobilise amino acids, and increase circulating concentrations of plasma proteins (Bergendahl et al., 1996; Exton et al., 1972). Thus, overall, cortisol is a catabolic hormone, and together these processes act to increase the availability of all fuel substrates by mobilisation of glucose, free fatty-acids and amino acids from endogenous stores (Dinneen et al., 1993; Djurhuus et al., 2002).

Cortisol exerts these effects on target tissues after uptake of free hormone from circulation and binding to intracellular glucocorticoid receptors which are members of the steroid/thyroid receptor hormone superfamily of transcription factors (Arlt and Stewart, 2005). The binding of cortisol to the glucocorticoid receptor in the cytosol results in activation of the steroid receptor complex and its translocation to the nucleus. After translocation to the nucleus, gene transcription is stimulated or repressed following binding of dimerized glucocorticoid receptor/ligand complexes to specific DNA sequences, called glucocorticoid response elements, in the promoter regions of a range of target genes (Arlt and Stewart, 2005). Cortisol therefore plays an important role in the activation of numerous metabolic pathways that control carbohydrate, protein and lipid metabolism (Arlt and Stewart, 2005) among other physiological processes.

To date, investigation of this hormone in marine mammal energy regulation and fasting metabolism has focused on pinnipeds (Guinet et al., 2004; Kershaw and Hall, 2016; Ortiz et al., 2003). Specifically, similar to terrestrial mammals, there is an increase in plasma cortisol concentrations with prolonged fasting events in subantarctic fur seals (*Arctocephalus tropicalis*) and northern elephant seals (*Mirounga angustirostris*) (Guinet et al., 2004; Ortiz et al., 2001). It has been hypothesised that these higher circulating cortisol concentrations may contribute to significantly increased fat oxidation during the fast, and may also serve as a cue to terminate fasting and initiate feeding (Ortiz et al., 2001). Blubber cortisol concentrations have been shown to increase during natural fasting periods of the life cycle of harbour seals (*Phoca vitulina*), particularly during the moult (Kershaw and Hall, 2016), suggesting that cortisol may play a key role in increased fat metabolism in the tissue during highly energetically demanding periods. Blubber cortisol concentrations decreased inversely with proportion blubber lipid content in California sea lions (*Zalophus californianus*) (Beaulieu-McCoy et al., 2017). This hormone could therefore potentially be used to monitor long-term nutritional status.

The aim of this work was to investigate blubber cortisol concentrations as an alternative physiological, endocrine marker of condition in cetacean species with very different life history strategies; firstly, harbour porpoises (*Phocoena phocoena*), and secondly, humpback whales (*Megaptera novaeangliae*) and minke whales (*Balaenoptera acutorostrata*), grouped here as balaenopterids. Harbour porpoises, like most odontocetes are considered as income breeders, because they continue to feed throughout the reproductive cycle, while balaenopterids are considered capital breeders because stored energy reserves acquired on high latitude feeding grounds are depleted to cope with the costs of reproduction on low latitude breeding grounds (Lockyer, 1987). Full depth blubber samples were collected from stranded individuals, and variation in cortisol concentrations by blubber depth, body location, age class, sex and morphometric body condition were explored to investigate the potential use of blubber cortisol concentrations as an informative condition biomarker in dorsal, remotely obtained biopsy samples.

3. METHODS

3.1 Sample Collection

Harbour Porpoises: Full depth skin, blubber and underlying muscle samples were collected from 20 dead harbour porpoise by the Scottish Marine Animal Strandings Scheme (SMASS) between 2013 and 2015. Only freshly dead animals, classified as those that originally stranded alive or had only recently died and thus showed no evidence of bloating, and the meat is considered to be edible (Kuiken and Hartmann, 1991), were sampled for this work in order to prevent erroneous hormone concentration measurements as a result of tissue decomposition after death. As described in Chapter 1, mass, length and girth measurements were taken. Samples were collected from the dorsal, lateral and ventral axes around the girth of the animal, immediately caudal to the dorsal fin. Blubber depth was recorded at each sampling site. Tissue samples were individually wrapped in aluminium foil and stored at -20^0C in plastic vials before hormone analysis. These individuals were both adults (n = 13) and juveniles (n = 7), and males (n = 11) and females (n = 9). The cause of death was determined following post-mortem examination, and classified as either acute (n = 13) or chronic (n = 7), as previously described in Chapter 1. Individual details are shown in Table 4.1.

Balaenopterids: Full depth, dorsal blubber samples were collected from 9 stranded balaenopterids (3 humpback whales and 6 minke whales). Samples were collected by the SMASS (2013-2015), and by the MICS (2010 and 2013) from the dorsal area immediately caudal to the dorsal fin. For the samples collected by the SMASS, information on the cause of death was used to classify each individual as an ‘acute’ or ‘chronic’ case. Morphometric measurements including girth, length and blubber thickness were also collected from the SMASS animals only, and lipid content data were available for all samples from previous analyses in Chapter 2 (Table 4.1).

Table 4.1 – Summary table of the harbour porpoise and balaenopterid individuals used for analysis with the data available for each one. ID codes starting with an 'M' were collected by the SMASS, while codes starting with a 'GSL' were collected by the MICS. Blubber lipid content values taken from Chapter 2. * Body Condition Index: mass / length^2 for harbour porpoises and girth / length for the balaenopterids.

Species Group	Species	ID	Age Class	Sex	COD Class	Body Condition Index*	Blubber Lipid Content
Harbour Porpoise	Harbour Porpoise	M018/13	Adult	Male	Chronic	✓	
		M020/15	Adult	Female	Acute	✓	
		M028/14	Juvenile	Male	Acute	✓	
		M055/14	Adult	Male	Acute	✓	
		M060/13	Adult	Male	Acute	✓	
		M072/13	Juvenile	Male	Chronic	✓	
		M134/14	Adult	Female	Acute	✓	
		M147/14	Adult	Female	Acute	✓	
		M265/13	Adult	Female	Chronic	✓	
		M307/14	Adult	Male	Acute	✓	
		M315/13	Adult	Female	Acute	✓	
		M319/14	Adult	Male	Chronic	✓	
		M343/13	Juvenile	Female	Acute	✓	
		M373/13	Juvenile	Female	Acute	✓	
		M377/13	Adult	Male	Chronic	✓	
		M38.2/14	Juvenile	Male	Acute	✓	
		M396/13	Adult	Female	Acute	✓	
		M040/14	Juvenile	Male	Chronic	✓	
		M061/15	Juvenile	Female	Chronic	✓	
		M068/14	Juvenile	Female	Chronic	✓	
Balaenopterid	Humpback	M159/14	Juvenile	Male	Acute	✓	✓
	Humpback	M163/15	Juvenile	Female	Acute	✓	✓
	Humpback	GSL-2010	Adult	Female			✓
	Minke	M292/13	Juvenile	Female	Acute	✓	✓
	Minke	M297/14	Juvenile	Male	Chronic	✓	✓
	Minke	M180/15	Juvenile	Male	Acute	✓	✓
	Minke	M319/15	Juvenile	Male	Chronic	✓	✓
	Minke	M396/15	Juvenile	Female	Acute	✓	✓
	Minke	GSL-2013	Adult	Female			✓

3.2 Cortisol Extraction and Quantification

3.2.1. Subsample Processing

Harbour Porpoises: Duplicate subsamples (0.1-0.2g) were taken from each sample while still frozen and used for cortisol extraction. Full-depth blubber subsamples were taken from the three sampling sites (dorsal, lateral and ventral) for all 20 harbour porpoises. Subsamples of the outer (adjacent to the epidermis), middle and inner (adjacent to the muscle) blubber layers were taken across these sampling sites for six of the 20 individuals. For thicker blubber samples (depth ≥15 mm), all subsamples were 5mm in depth with the outer layer subsamples taken immediately below the skin and inner layer subsamples taken immediately above the muscle layer. The middle layer subsamples were taken immediately above the inner layer subsamples. For thinner samples (≤15 mm depth), the blubber thickness was divided into three equal parts to give outer, middle and inner layer subsamples of equal thickness. The outer layer only was subsampled from the dorsal site of all 20 individuals (Table 4.2).

Balaenopterids: For samples 20 - 30mm in depth, the blubber was divided into three equal layers; outer, middle and inner. For the thicker samples that were >30mm in depth, the tissue was divided into five equal parts so that the inner layer, the middle layer, and the outer layer were used for processing while the two transitional layers were discarded, as described in Chapter 2. Duplicate subsamples (0.1-0.2g) were taken from each of these layers while still frozen and used for cortisol extraction.

Table 4.2 - Number of sub-samples processed for cortisol extraction and quantification from the harbour porpoise and the balaenopterid full depth blubber samples.

	Blubber Layer	Sampling Location		
		Dorsal	Lateral	Ventral
Harbour Porpoises	Outer Layer	20	6	6
	Middle Layer	6	6	6
	Inner Layer	6	6	6
	Full Depth	20	6	6
Balaenopterids	Outer Layer	9	-	-
	Middle Layer	9	-	-
	Inner Layer	9	-	-

3.2.2. Cortisol Extraction

Given the similarities in the structure and physical properties of the steroid hormones, the same method has been used for the extraction of progesterone and cortisol from human adipose tissue (Newton et al., 1986). For this reason, the extraction method developed by Kellar and colleagues (2006) for reproductive hormones from cetacean blubber samples was used here to extract cortisol. The protocol involves tissue homogenisation followed by tissue debris removal in a series of solvent rinses with recovery of the supernatant each time. The resulting residues are washed to remove any remaining lipid, and the final extract dried down for resuspension and assaying.

Specifically, the 0.1 - 0.2g blubber sub-samples are accurately weighed and manually homogenized in 1000µl ethanol for 2 minutes to standardize the homogenization process. Homogenates are then centrifuged at 3,000 *rcf* for 10 minutes and the supernatants collected into a clean tube. These are then evaporated under nitrogen while incubating at 25^0C. Two milliliters of ethanol : acetone (4:1) are added to the residue and after vortexing and centrifugation at 3,000 *rcf* for another 10 minutes, the solution is again evaporated under nitrogen at 25^0C to leave a new residue. 1ml of diethyl ether is added to this residue, vortexed and centrifuged again at 3,000 *rcf* for 10 minutes. The solution is again evaporated under nitrogen at 25^0C. 1ml of acetonitrile is added, the solution is vortexed and 1ml of hexane is added. The solution is vortexed and centrifuged for 20 minutes at 3,000 *rcf*. The solvents form two immiscible layers with hexane on top. The hexane layer is removed, and the acetonitrile layer re-extracted with another 1ml hexane, centrifuged for 20 minutes at 3,000 *rcf* and the final acetonitrile layer aspirated and evaporated under nitrogen at 25^0C. The final residue is centrifuged briefly and then either frozen before analysis or re-suspended in 500µl phosphate buffered saline (PBS) (pH 7.5) if the cortisol assay is performed immediately.

3.2.3. Cortisol Quantification

Cortisol concentrations were determined using a commercially available Enzyme Linked Immunosorbent Assay (ELISA) kit (DRG International Inc., Marburg, Germany: Cortisol ELISA EIA-1887). This kit has been used to quantify cortisol in blubber biopsies from harbour seals (Kershaw and Hall, 2016). Cortisol concentrations were measured according to the ELISA kit instructions with a standard curve ranging from 0 to 800ng/ml, with a sensitivity of 2.5ng/ml. The hormone concentrations in the samples were calculated from the standard curve using a 4 parameter log-logistic model. All extracts were above the limit of detection, were assayed in duplicate, and the mean hormone concentration was reported in ng/g (cortisol per wet weight of the tissue).

Extracts of varying concentrations, high, medium and low (n=10), were used to calculate inter-assay (between different plates) and intra-assay (within a plate) coefficients of variation (CVs), with mean percentage CVs of <20% and <10% set as the acceptable limits, respectively (Andreasson et al., 2015). The mean inter-assay CV was 10.84%, and the mean intra-assay CV was 5.65%, and were therefore below the acceptable limits of variation. This ELISA has a reported 45% cross-reactivity with corticosterone and a 9% cross-reactivity with progesterone.

3.2.4. Quality Assessment and Quality Control

Quality assurance and quality control tests were performed to validate the use of this cortisol ELISA with cetacean blubber extracts. Limitations and sources of error in the cortisol extraction method were also assessed to help interpret the results and adapt this method for remotely obtained biopsy samples.

Parallelism Assays: For the harbour porpoise and the humpback samples separately, three subsample extracts, from the outer, middle and inner layers were pooled and serially diluted four times using the 0 ng/ml cortisol standard provided by the ELISA kit from the undiluted

sample to a $^1/_{16}$ dilution. The resulting curve of the detection metric (optical density of the sample read at 450nm) as a function of the dilution ratio (1, ½, ¼, $^1/_8$, $^1/_{16}$) was then compared to the standard curve for each species. In order to statistically assess parallelism, two linear regression models for dilution state against optical density, one with and one without an interaction with sample type (ELISA standard or blubber extract), were compared (ANCOVA function in statistical package R, version 3.1.3, R Core Development Team, 2015). If the regression lines for each model do not have significantly different slopes, then these data indicate that the standard curve and the extract dilution curve are parallel. Parallelism of these regression lines supports the assumption that the antigen binding characteristics allow the reliable determination of hormone levels in the diluted blubber extracts (Andreasson et al., 2015), and the ELISA kit is therefore reliably measuring cortisol in the tissue samples.

The comparison between the two linear regression models, one with and one without an interaction with sample type showed that removing the interaction did not significantly affect the fit of the model for the harbour porpoise or the humpback blubber extract dilutions (ANCOVA; $F = 10.78$, $p = 0.1$, and $F = 0.079$, $p = 0.7$ for each species model comparison, respectively). Therefore, it was concluded that the effect on optical density with increasing sample dilutions is the same for the standard curve samples and the blubber extracts, and that the regression lines are parallel (Fig. 4.1). This ELISA is therefore suitable for the quantification of harbour porpoise and humpback whale cortisol.

Matrix Effect Test: Successful immunologic assays require an optimal pH and ionic strength that promotes specific antibody–antigen complexes while reducing the nonspecific binding of other proteins in the samples that increase assay interference. As the ELISA kit used here was designed for use with serum or plasma samples, the compatibility of the kit with extracts resuspended in PBS was assessed. Equal volumes of each standard were spiked with PBS and assayed in tandem with the unspiked standard curve. The known and the apparent concentrations in the spiked samples should show a positive linear relationship with a slope of approximately 1.0 (Hunt et al., 2014). The matrix effect test was successful with a slope of 1.08 (a slope of between 0.8-1.2 was considered acceptable) (Fig. 4.2). It was concluded that matrix effects are minimal and this sample diluent is therefore compatible with the immunoassay.

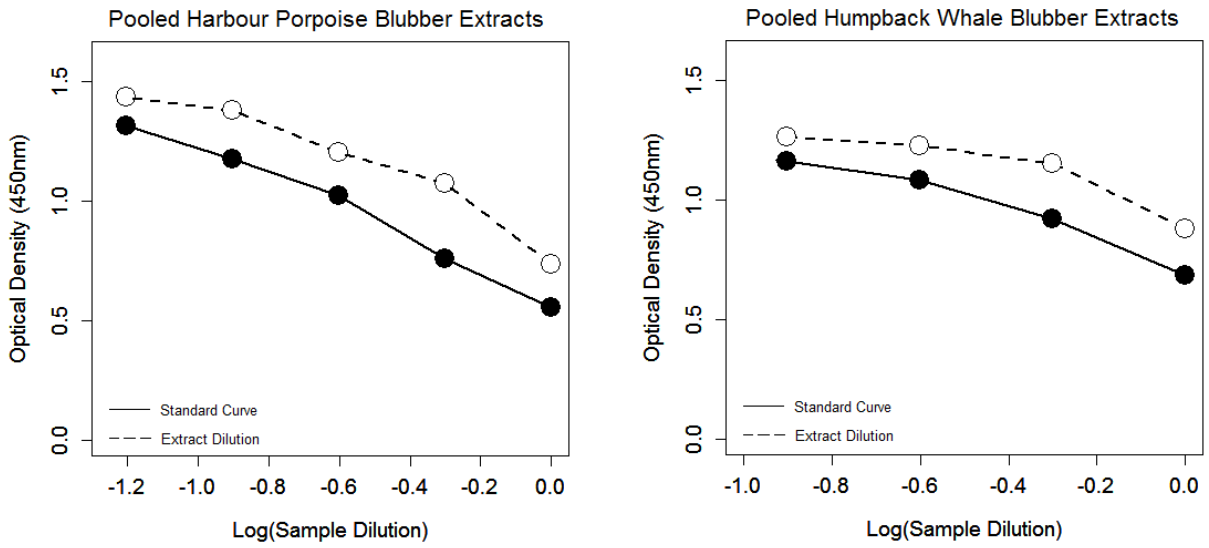

Fig. 4.1. Linearity assessment for the cortisol ELISA with harbour porpoise and humpback whale blubber tissue extracts. Serial dilutions of three pooled extracts for each species show parallelism with the ELISA standard curve (ANCOVA: $F = 10.783$, $p = 0.103$ and $F = 0.079$, $p = 0.7$ for each species linear model comparison respectively).

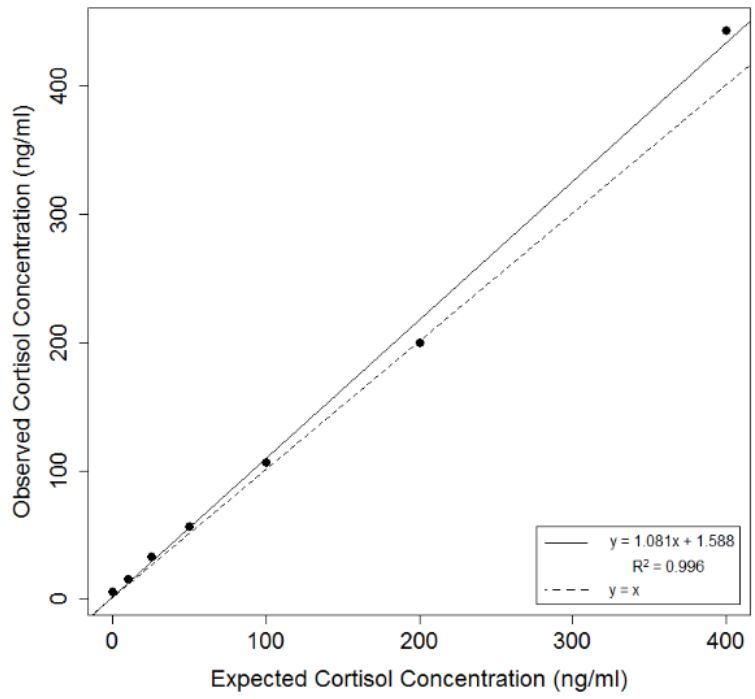

Fig. 4.2. Matrix Effect Test Validation. The linear regression lines for the expected against the observed concentrations are shown for the expected 1:1 relationship and the measured relationship from the assay results. The assay results show a positive linear relationship with a slope of 1.08, thus confirming that PBS is an acceptable sample diluent compatible with this cortisol ELISA kit.

Extraction Efficiency: In order to assess the extraction efficiency of this method, cortisol recovery from spiked samples was measured. As blubber biopsies from live animals vary in size, it was necessary to determine the extraction efficiency of this method across tissue samples of different masses. Samples ranging between 0.1 and 0.3g, the typical range of masses of biopsy samples obtained from free-ranging cetaceans, were used for analysis. One full depth

blubber sample from a harbour porpoise was divided into masses of 0.05g, 0.1g, 0.15g, 0.2g, 0.25g, and 0.3g (all ±0.025g), each one in triplicate, such that one sample of each mass was unspiked while the other two were spiked with 100ng of cortisol. Cortisol was then extracted and measured as outlined above, and the average percent recovery calculated for each sample.

The extraction efficiencies ranged from an average of 88.4% for the 0.05g samples, down to an average of 75.7% for the 0.3g samples. However, the relationship between the extraction efficiency and sample mass was not linear (Fig. 4.3a). For this reason, a 4 parameter log-logistic model was fitted to these data to model the effect of tissue mass on extraction efficiency (Fig. 4.3a). This model was used to calculate the expected extraction efficiencies for all the blubber subsamples processed in this study based on their mass. These extraction efficiencies were then used to correct the measured cortisol concentrations in each sample to give a final cortisol concentration used for statistical analysis.

Minimum Sample Mass and Correction Factors: Remotely obtained blubber biopsy samples are often used for multiple analyses in order to maximize the potential information gained from the tissue. Subsampling remotely obtained biopsies for multiple analyses can therefore result in small amounts of tissue available for processing, and the minimum amount of tissue needed to obtain robust, replicable measurements of cortisol concentrations in the blubber samples using this extraction method should be established. Here, multiple extractions from the same piece of tissue of differing masses were assayed. Again, full depth, duplicate subsamples of different masses between 0.05 and 0.3g (all ±0.025g) at 0.05g intervals were taken from the same blubber sample. Cortisol was extracted and measured in these samples in tandem, and the mean cortisol concentration calculated for each one (Fig. 4.3b).

As the sample mass increased, both the cortisol concentration measured and the variation between the duplicate subsamples decreased (Fig. 4.3b). Only the 0.15g, 0.2g and 0.25g subsamples overlapped in their measured cortisol concentrations (Fig. 4.3b). Thus, based on the decrease in extraction efficiency (Fig. 4.3a) and increase in measurement variability (Fig. 4.3b), it is recommended for maximum extraction efficiency and measurement replicability, that blubber samples for extraction should be between 0.15 and 0.2g. For this method to be applicable to remotely obtained biopsy samples that are sometimes smaller than this recommended 0.15 - 0.2g mass range, a ‘correction factor’ model was built using these data. The ‘true’ concentration was taken as the average concentration measured in the 0.15g and the 0.2g samples. The concentrations measured in the samples of other masses were divided by this ‘true’ concentration to give a correction factor. A 4 parameter log-logistic model was fit to these data to model the effect of tissue mass on the correction factor required (Fig. 4.3c). As sample mass decreases from the average recommended 0.175g, the correction factor increases, indicating that smaller sample masses give inflated cortisol concentration estimates (Fig. 4.3c). This approach could be used to correct cortisol measurements from remotely obtained biopsy samples to avoid overestimating cortisol concentrations as an artefact of a small biopsy sample mass analysed.

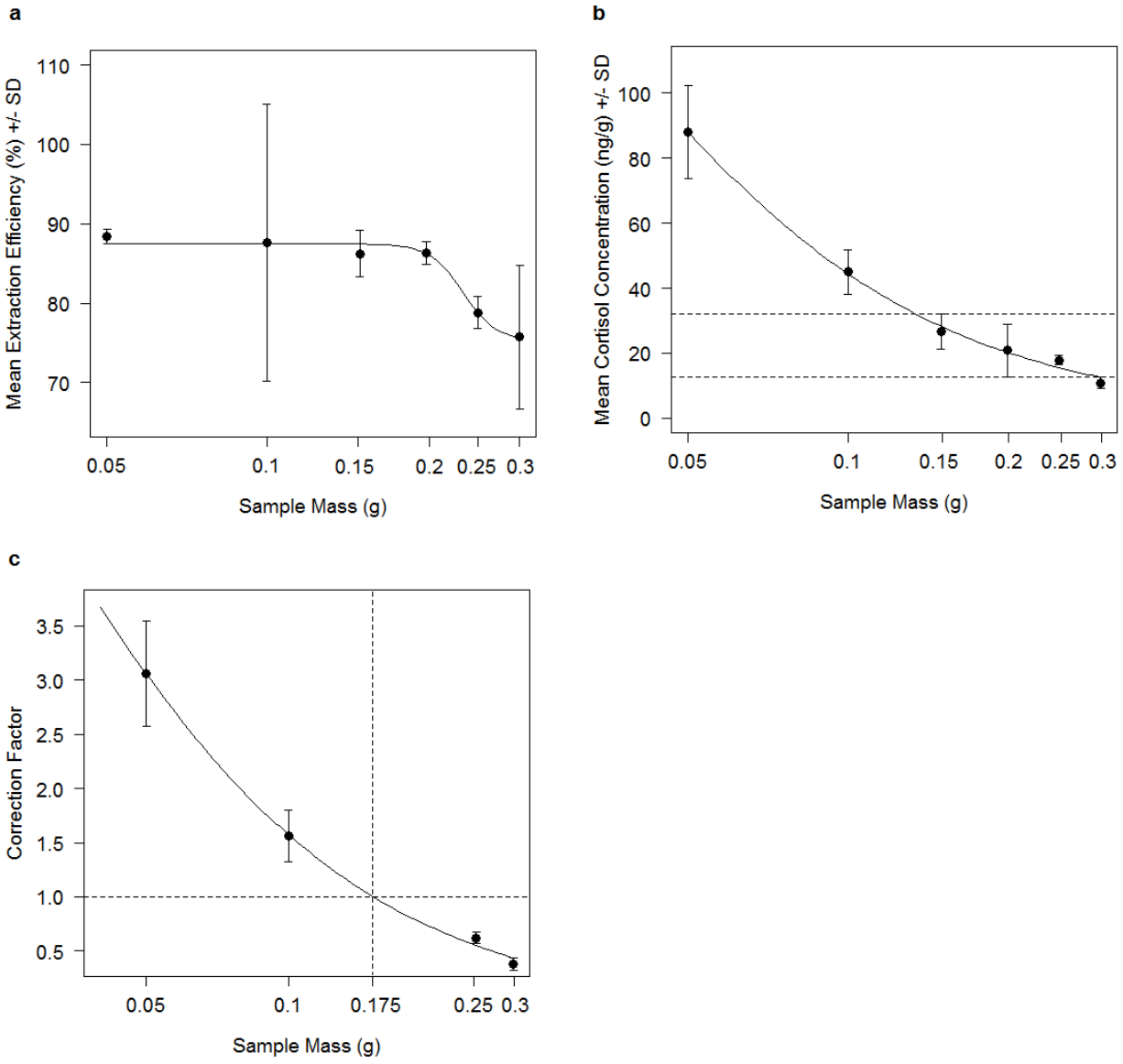

Fig. 4.3. Cortisol extraction efficiency and quantification errors based on sample mass. a) Extraction efficiency decreases with increasing blubber sample mass in a non-linear fashion. b) Mean cortisol concentration in duplicate sub-samples decreases with increasing sample mass in a non-linear fashion. Measured concentrations overlap in sample masses between 0.15g and 0.25g. c)Smaller samples masses than the ideal sample mass recommended as between 0.15-0.2g give inflated cortisol concentrations and therefore a larger correction factor is applied to measured cortisol concentrations to compensate for this bias.

3.3 Statistical Analysis

All statistical analyses were performed using the statistical package, R, version 3.1.3 (R Core Development Team, 2015).

3.3.1. Harbour Porpoises

Two different modelling approaches were used to assess firstly, the effects of both sampling site and sampling depth on blubber cortisol concentration and, secondly, the effects of other explanatory covariates (age class, sex, COD and morphometric body condition). A summary of each set of subsamples used for the various models is given in Table 4.3.

Body Location and Blubber Layer: Generalised Linear Mixed Effect Models (GLMMs) were used to investigate cortisol concentrations both across body locations and through the blubber depth whilst accounting for the repeated measurements from the same individuals that were considered as random effects (Bolker et al., 2008). Two GLMMs (*glmer* function in the R package *lme4*) with a gamma distribution to model the right skew in the cortisol concentration data, a log link function, and each individual treated as a random effect were used to investigate firstly, the effect of body location on cortisol concentration in full depth subsamples (n = 20 individuals with three samples each, Table 4.3), and secondly, the effect of body location *and* blubber layer (n = 6 individuals consisting of three females and three males, four acute cases and two chronic cases with 12 samples each, Table 4.3). An interaction between body condition (mass/length^2) and location, as well as between body condition and layer were also included to consider the possibility that animals in varying condition may show differences in their cortisol distribution though the blubber depth and across their bodies as a result of differential energy store mobilisation. Backwards model selection using the *dredge* function was used to identify the variables and/or interactions that best explain the variation in hormone concentrations and thus to include in the final models. The goodness of fit of each model was assessed using the AICc. The model with the lowest AICc value was used for further interpretation.

Individual Covariate Analysis: Generalised Linear Models (GLMs) were used to assess the effects of other variables on the cortisol concentrations measured in the outer layer of the dorsal samples. The outer layer, dorsal subsamples are representative of biopsies taken from live animals (n = 20, Table 4.3). GLMs were used to model the right skew in hormone concentrations using a gamma distribution and a log link function. The full GLM including all the explanatory variables (age class, sex, COD category as either acute or chronic, and mass/length^2) was generated. Again, backwards model selection using the *dredge* function was used to identify the variables that best explain the variation in hormone concentrations, and thus to include in the final model based on the smallest AICc. Visual inspections of the model residual plots and fitted values were used to check the fit and assumptions of the final model.

3.3.2. Balaenopterids

As with the harbour porpoise data, two different modelling approaches were used to assess firstly, the effect of sampling depth on blubber cortisol concentration and, secondly, the effects of other explanatory covariates (sex, COD, body condition and lipid content). A summary of each set of subsamples used for the various models is given in Table 4.3.

Blubber Layer: As with the harbour porpoise data, GLMMs were used to investigate cortisol concentrations through blubber depth whilst accounting for the repeated measurements from the same individuals. Firstly, a GLMM with a gamma distribution, a log link function, and each

individual treated as a random effect was used to investigate variation in cortisol concentration by blubber depth (n = 9 individuals with three samples each, Table 4.3). An interaction between body condition (girth/length) and layer was also included to consider the possibility that animals in varying condition may show differences in their cortisol distribution though the blubber depth.

Individual Covariate Analysis: The cortisol concentrations in the outer blubber layer were modelled using a GLM including sex, body condition (girth/length), outer blubber lipid content and COD as other explanatory variables to investigate variation in cortisol concentrations. For this second model, only the samples collected by the SMASS where COD and morphometric measurements had been recorded were used, thus reducing the sample size down to just seven animals (Table 4.3). As previously described, the largest model was built first, and backwards model selection using the *dredge* function was used to identify the variables that best explain the variation in hormone concentrations, and thus to include in the final model based on the smallest AICc. Visual inspections of the model residual plots and fitted values from the final model gave confidence in the results and allowed interpretation of the model coefficients to assess the effects of each covariate.

Table 4.3 - Summary of each harbour porpoise and balaenopterid sample subset used for different models to assess cortisol variation by body location, blubber layer and other individual covariates. *GLMM: Generalised Linear Mixed Effect Model. **GLM: Generalised Linear Model.

Species Group	Number of Individuals	Body Location	Blubber Layer	Model
Harbour porpoises	20	Dorsal, Lateral, Ventral	Full	GLMM* of Body Location
	6	Dorsal, Lateral Ventral	Full, Outer, Middle, Inner	GLMM of Body Location and Layer together
	20	Dorsal	Full, Outer	GLM** of Individual Covariates
Balaenopterids	9	Dorsal	Outer, Middle, Inner	GLMM of Blubber Layer
	7	Dorsal	Outer	GLM of Individual Covariates

4. RESULTS

4.1 Harbour Porpoises

Body Location and Blubber Layer: Blubber cortisol concentrations ranged between 3.65 - 759.51ng/g. The final GLMMs following variable selection did not retain either body location or the interaction with body location and body condition as significant explanatory variables. Mean concentrations of 69.09 ± 31.30ng/g, 90.48 ± 52.22ng/g and 83.22 ± 59.95ng/g were measured in the full depth dorsal, lateral and ventral samples, respectively. Thus, there were no significant differences in blubber cortisol concentrations across these three different sampling locations (Fig. 4.4a). When blubber layer was considered together with body location and condition, the final GLMM following variable selection retained only blubber layer as an important explanatory variable. This suggests that the pattern of cortisol distribution through blubber depth does not change with sampling site, or animal condition. The highest concentrations were measured in the inner and middle layers (means of 180.02 ± 177.06ng/g and 156.28 ± 167.02ng/g, respectively) (p values < 0.01) (Fig 4.4b), and there was no significant difference between the concentrations in the full depth samples compared to the outer layer alone (means of 88.65 ± 68.38ng/g and 77.84 ± 48.32ng/g, respectively) (Fig. 4.4b).

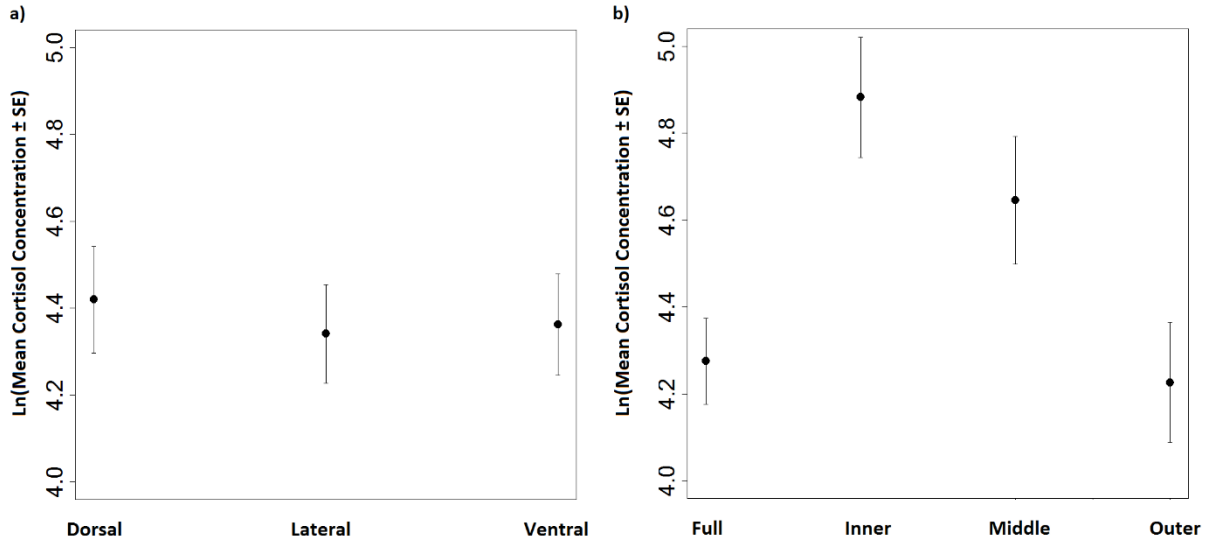

Fig. 4.4. Final GLMM outputs for cortisol concentrations across the body and through the blubber layer for the harbour porpoises. a) GLMM output for blubber cortisol concentrations around the girth. There were no significant differences in cortisol concentration in full depth blubber samples from three different sampling locations. b) GLMM output following variable selection for blubber cortisol concentrations with both location and blubber depth. The inner and middle layers had significantly higher cortisol concentrations than the full depth and outer layers (p values < 0.01), while the outer layer was not significantly different to the full depth sample overall.

Individual Covariates: Variable selection for the GLM of cortisol concentration in the dorsal, outer layer samples revealed that there were less than two points difference in the AICc between the two best fitting models, indicating that they were of equivalent fit to the data (Table 4.4). These models retained both age class and sex, and body condition and sex as important explanatory variables (Table 4.4). Juveniles had higher cortisol concentrations than adults ($p = 0.05$), and there was a negative relationship between mass/length^2 and cortisol concentration which was significant at the 10% level ($p = 0.1$). Overall, females had significantly higher cortisol concentrations than males with means of 111.37 ± 59.86ng/g compared to 66.77 ± 29.73ng/g ($p = 0.02$) (Fig. 4.5). This could be as a result of some reported cross-reactivity of the ELISA kit with progesterone (< 9%). However, one adult female that died as a result of dystocia (classed here as an acute case) did not have an elevated blubber cortisol concentration (53.90ng/g) as would be expected if there were high levels of blubber progesterone associated with pregnancy (Trego et al., 2013). Nonetheless, to investigate the potential confounding effect of this cross-reactivity, the male and female data were modelled separately with the same covariates and model selection process. For the male dataset, again, the final model selection showed that there was less than a two point difference in the AICc between the two best fitting models including both age class and mass/length^2 although neither was individually significant. For the female dataset, variable selection excluded all covariates, and these results are likely a result of the small sample size used for analysis when the data are split by sex. COD was not retained in the final model likely because it is tightly linked to the body condition of the individuals as the acute cases were generally in better condition, and showed a smaller range in mass/length^2 than the chronic cases with mean mass/length^2 values of 0.0022 ± 0.0035 and 0.0018 ± 0.00050 for each group respectively. The body condition of the animals therefore explained more of the variation in the data than the COD.

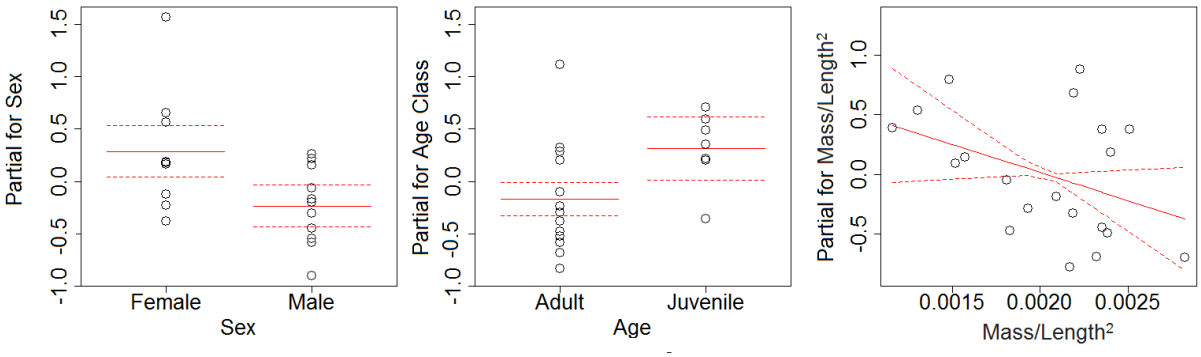

Fig. 4.5. Partial termplots for the covariates retained in the final two best fitting GLMs following variable selection for the harbour porpoise data: glm(Outer.Cort ~ Sex + Age.Class, family=Gamma(link="log")) and glm(Outer.Cort ~ Sex + Mass/Length^2, family=Gamma(link="log")) (n=20). Termplots plot regression terms against their predictors with the associated standard errors, while holding other predictors at their mean values. Significantly higher cortisol concentrations were measured in females than males ($p = 0.02$), and in juveniles than adults ($p=0.05$). There was a weak negative relationship between mass/length^2 and blubber cortisol concentration ($p = 0.1$).

4.2. Balaenopterids

Blubber Layer: Cortisol concentrations showed less variation in the balaenopterids than in the harbour porpoises with a range of between 53.89 – 154.95 ng/g. Model selection for the GLMM which investigated variation in cortisol concentration through blubber depth showed that there were no significant differences in cortisol concentration between the inner, middle and outer layers (means of 93.7 ± 10.0ng/g, 98.3 ± 5.3ng/g and 89.5 ± 9.9ng/g, respectively) (Fig. 4.6). The interaction between body condition and blubber layer was not retained in the final model either.

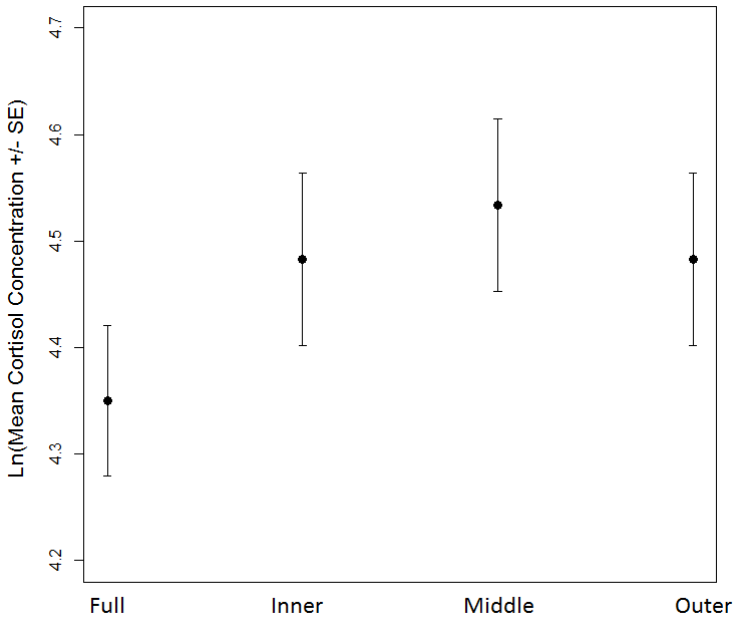

Fig. 4.6. Final GLMM output for cortisol concentrations through the blubber depth for the balaenopterids. Model selection did not retain layer as an important explanatory variable as there were no significant differences in cortisol concentration between the inner, middle and outer layers.

Individual Covariates: Variable selection for the GLM which investigated other factors contributing to the variation in measured cortisol concentrations in the outermost layer of the balaenopterid samples retained girth/length and sex as important explanatory variables (Table 4.4). There was a strong negative correlation with girth/length ($p < 0.001$), such that individuals in poor condition had higher blubber cortisol concentrations than those in better condition (Fig. 4.7). As with the harbour porpoises, females had a higher blubber cortisol concentration than males ($p = 0.002$) with mean concentrations of 91.4 ± 8.86ng/g and 87.9 ± 15.88ng/g, respectively, although the highest concentration was measured in a male. There was no difference in cortisol concentrations between animals with acute or chronic CODs. So, similarly to the harbour porpoise data, it appears that body condition explains more variation in cortisol concentrations than COD. Finally, lipid content was not retained following model selection either. In fact, cortisol concentrations were not correlated with lipid content across any of the samples ($n = 27$) collected from all nine individuals (Linear regression model: Adjusted $R^2 = 0.04$, $p = 0.93$).

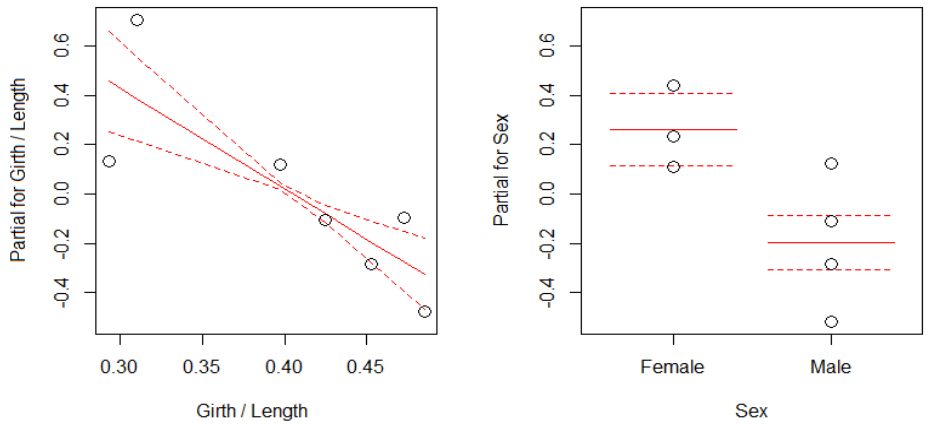

Fig. 4.7. Partial termplots for the covariates retained in the final best fitting GLM following variable selection for the balaenopterid data: glm(Outer.Cort ~ Girth/Length + Sex, family=Gamma(link="log")) (n=7). There was a significant negative relationship between girth/length and blubber cortisol concentration ($p < 0.001$), and significantly higher cortisol concentrations were measured in females than males ($p = 0.002$).

Table 4.4 - Results of GLM model selection for outer layer blubber cortisol concentrations for harbour porpoises (n=20) and balaenopterids (n = 7) showing the three best-fitting models with the lowest AICc values. Harbour porpoise models 1 and 2 are of equivalent fit to the data.

Species	Model	Covariates Retained	*df*	*AICc*	*ΔAICc*	*weight*
Harbour Porpoises	1	Cortisol ~ Sex + Age Class	4	209.4	0.0	0.29
	2	Cortisol ~ Sex + Mass/Length^2	4	210.9	1.5	0.14
	3	Cortisol ~Age class	3	211.9	2.5	0.08
Balaenopterids	1	Cortisol ~ Girth/Length + Sex	4	190.4	0.0	0.36
	2	Cortisol ~ Girth/Length + Sex + Lipid	5	192.6	2.2	0.12
	3	Cortisol ~ Girth/Length + Sex + COD	5	193.9	3.5	0.06

5. DISCUSSION

Quality Assurance – Quality Control

Based on the extraction efficiency and variability, an optimum sample mass of between 0.15 and 0.2g of tissue was identified for cortisol extraction using these methods. Larger samples have poorer extraction efficiency as both the homogenisation of the tissue becomes less efficient with larger pieces, and the mass:solvent ratio is no longer optimal for efficient cortisol extraction. Smaller sample masses will produce inflated cortisol concentration estimations as a result of the error associated with small measurements, and then the multiplication effect as sample mass is used to estimate the concentration in ng/g of tissue. It is recommended that biopsy samples obtained from live animals should therefore be within this mass range for reliable cortisol quantification using this extraction method.

Cortisol concentrations are reported as per gram of blubber tissue, which includes non-lipid components of the blubber, such as proteins and water. Other studies have reported blubber cortisol concentrations as nanograms of cortisol per gram of lipid extracted from the tissue (Champagne et al., 2017; Champagne et al. 2018) because lipid mass was thought to be a more stable reference than wet tissue mass. For this work, cortisol concentrations were not normalised by lipid content for two reasons. Firstly, as the effects of cortisol on tissue metabolism are of interest in terms of increased lipolysis or lipogenesis, normalising by lipid content would not allow the investigation of a potential correlative relationship between hormone concentrations and lipid content of the tissue. Here, there was no correlation between lipid content and cortisol concentrations in any of the balaenopterid samples. Secondly, when measuring blubber cortisol concentrations, these are likely a combination of circulatory cortisol within the extra-cellular matrix, cytoplasmic cortisol within the adipocytes, and cortisol associated directly with the adipocyte lipid droplets themselves as a result of its lipophilic nature. Variability in cortisol measurements is therefore likely the result of variability in these different sources of 'tissue' cortisol, and is not solely a function of the lipid content within the lipid droplets inside the adipocytes. Had cortisol concentrations been normalised to lipid content, there may have been greater individual variation in hormone concentrations, especially for the balaenopterid samples where total lipid content of the tissue showed marked differences between individuals (Chapter 3) compared to the wet tissue mass which was standardised as much as possible. The sources of this individual variability would then require some careful interpretation for the reasons outlined above.

Variation Across Sampling Sites and through Blubber Depth

Blubber cortisol concentrations measured in harbour porpoises, between 3.65 - 759.51ng/g, and in the balaenopterids, between 53.89 – 154.95ng/g, were higher than those previously reported in common dolphins (*Delphinus delphis*) (Kellar et al. 2015) and belugas (*Delphinapterus leucas*) (Trana et al., 2016) with reported average ranges of between 3.99-24.3ng/g and 0.26-1.76ng/g for each species, respectively. The order of magnitude differences between the maximum concentrations measured in each of these studies compared to this one could be a result of species-specific differences in hormone concentrations, as well as small method alterations in the extraction and quantification processes between studies.

Here, no significant variation in blubber cortisol concentrations was seen across the three different sampling locations on harbour porpoises. Dorsal sampling through the collection of remote biopsies could therefore provide information on typical blubber cortisol concentrations across this area of the body. These results are consistent with the notion that while harbour porpoises and other small odontocetes may selectively mobilise lipids unevenly across different areas (Koopman et al., 2002), blubber composition remains relatively consistent across the body (Koopman et al., 2002).

Evidence of stratification in cortisol concentration was seen through the blubber depth in harbour porpoises with highest concentrations measured in the middle and inner layers. Overall, the highest concentrations were measured in the inner layer, which was also found in belugas (Trana et al., 2015). This is perhaps because the inner layers are more highly vascularised in shallow diving odontocetes compared to the superficial blubber layers closer to the skin (McClelland et al., 2012). Inner layers are also closer to larger blood vessels that supply the underlying muscle tissue. Together, these likely result in a higher concentration of cortisol from the circulatory system in this part of the blubber.

This stratification in cortisol concentration is unsurprising given that cetacean blubber is stratified into three layers that can be differentiated visually, histologically and biochemically in many cetacean species (Olsen and Grahl-Nielsen, 2003; Smith and Worthy, 2006). In harbour porpoises specifically, the inner and middle blubber layers are more dynamic, and are likely used for lipid deposition and mobilization at a much faster rate than the outermost layer next to the skin (Koopman et al., 1996). Given the differences in the rate of turnover of the tissue, the cortisol concentrations measured in the full depth blubber samples represent the integration of the hormone through the tissue over a longer time period than either the middle or inner layers alone. There were no significant differences in the concentrations measured in full depth blubber samples compared to the outer layer. Thus, these superficial samples could be representative of the longer term presence of cortisol in the blubber that is not subject to strong fluctuations as a result of short term changes in the blood flow through the tissue to the skin during thermoregulation (Barbieri et al., 2010; Noren et al., 2009). Cortisol concentrations in the outer blubber layer available through remote dart biopsy could thus be used as a potential indicator of longer-term physiological changes.

The cortisol concentrations measured in the balaenopterid samples were both lower, on average, and showed less variation than those measured in the harbour porpoises. Concentrations in the outer layer are representative of concentrations measured though the full blubber depth as there was no variation in cortisol concentrations through the different blubber layers in these samples. This was surprising given previous findings of stratification of this hormone through the blubber depth in belugas (Trana et al., 2015), and the variation in concentrations measured in harbour porpoises here. It is also surprising given the documented differences in tissue structure, lipid content and fatty acid signatures of the different blubber layers in mysticetes (Ackman et al., 1975a; Lockyer et al., 1984, 1985) which demonstrate the different potential functions of the layers in terms of nutrient transport, lipid storage and mobilisation, and structural support. It seems logical therefore, that hormone concentrations would also show similar variation.

Currently, the only published data on steroid hormone concentrations in mysticete blubber are progesterone concentrations measured in dead minke whales and bowhead whales (*Balaena mysticetus*) (Kellar et al., 2013; Mansour et al., 2002) and shallow depth biopsy samples from free-ranging humpback whales (Clark et al., 2016; Pallin et al., 2018a; Pallin et al., 2018b). There was no stratification in progesterone concentrations though blubber depth in minke whales (Mansour et al., 2002), and concentrations through the blubber depth were indicative of pregnancy status. Stratification through blubber depth was not investigated in the bowhead whale samples, but concentrations measured in the superficial blubber tissue next to the epidermis were able to distinguish between pregnant and non-pregnant females (Kellar et al., 2013). Superficial blubber biopsy samples were also able to distinguish between confirmed pregnant and non-pregnant humpback whales (Pallin et al., 2018a). The lack of stratification in cortisol concentrations seen here could be comparable to the lack of stratification recorded for progesterone in these species.

While obtaining good sample sizes of individuals for comparison is easier for the smaller odontocete species, the dephinids and porpoises for example, good quality samples collected from stranded mysticetes are harder to come by. There is therefore a huge gap in our understanding of both the physical and chemical properties of large cetacean blubber tissue since studies conducted using whaled samples. Consequently, nothing is known of the vascularisation of mysticete blubber, and it may be that there is reduced blood flow through the thicker blubber layer of these animals compared to smaller odontocete species. Thus, without fluctuations in perfusion of the blubber, cortisol, as a small steroid hormone, could diffuse passively into the tissue until it is equally distributed, and the time scales over which these concentrations change compared to the smaller species may be much longer. In addition, the extent to which these species may also be able to control a peripheral shut down of blood flow to the blubber is completely unknown. If there is a strong pressure to reduce blood flow to the tissue in these animals during certain periods of their life cycle, or perhaps even during certain activities, deep diving for example, this could explain why there was no increase in cortisol concentration in the inner-most blubber layer as would be expected. Finally, the small sample size of just nine individuals used for analysis needs to be acknowledged. It is possible that had a large number of animals been sampled, greater variation in blubber cortisol concentrations may have been observed.

Cortisol Concentrations as a Potential Marker of Condition

Covariate analyses revealed that, even with a small sample size of just 20 individuals for the harbour porpoises, and seven individuals for the balaenopterids, as well as high levels of individual variation, cortisol concentrations in dorsal, outer layer samples were negatively correlated with morphometric indices of body condition, and females showed higher concentrations than males. In terrestrial mammals (Castellini and Rea, 1992), humans (Bergendahl et al., 1996) and pinnipeds (Champagne et al., 2012; Champagne et al., 2006; Guinet et al., 2004), extended periods of food restriction are associated with an increase in the circulating concentrations of cortisol. During these periods of reduced food intake or fasting, cortisol increases lipolysis to provide energy (Bergendahl et al., 1996), and is involved in the maintenance of circulating glucose concentrations through increased gluconeogenesis (Exton

et al., 1972). It is likely that the same principles apply to cetaceans, and as such, increased cortisol concentrations in the blubber of harbour porpoises and balaenopterids in poorer condition could be a result of increased mobilisation of fat reserves in these individuals. Similarly, higher concentrations measured in the blubber of juvenile harbour porpoises could be as a result of these individuals mobilising energy reserves to maximise growth to achieve size at sexual maturity (McLellan et al., 2002) instead of depositing lipid stores. Juveniles are likely also under both more nutritional and physiological stress than adults associated with foraging independently for the first time and being immunologically challenged by new pathogens, again, leading to the mobilisation rather than the deposition of fat stores. The small sample size of balaenopterid individuals with accompanying morphometric data and cause of death information was confounded by both species (dominated here by minke whales) and age class (all individuals were juveniles), and for these reasons, it was not possible to assess age-class variation or potential species-specific differences in blubber cortisol concentrations.

Interestingly, the COD category was not retained as an important explanatory variable following model selection for either species group, demonstrating that the body condition of individuals explained more variation than the way in which an animal died. Previous work on short beaked common dolphins and California sea lions showed that animals that died as a result of fishery bycatch had significantly lower blubber cortisol concentrations than stranded individuals (Beaulieu-McCoy et al., 2017; Kellar et al., 2015). These differences were attributed to the average time to death for these two groups of animals, with the conclusion that the stranded animals had a longer perception of the threat-to-self until death which resulted in a measureable increase in blubber cortisol concentrations as a result of elevated circulating concentrations in both species (Beaulieu-McCoy et al., 2017; Kellar et al., 2015). However, it is possible that for acute causes of death by entrapment in fishing gear, an equally large, circulating, cortisol stress response would have taken place over the course of minutes before death. But, because of the potential for profound vasoconstriction during the dive response in a drowning animal, this cortisol increase was not measurable in the blubber. This is likely true for the short beaked common dolphin and California sea lion studies, as well as the porpoise and balaenopterid individuals sampled here. There are therefore other factors related to changes in peripheral circulation and the dive response that need to be considered when interpreting the variability in blubber cortisol concentrations of stranded animals.

With these additional sources of variability in mind, the results here, for both species groups, highlight the need to investigate the effect of the condition and physiological state of individuals before concluding that cortisol concentrations measured in the blubber are the result of heightened psychological stress alone. These data suggest that the relationship between blubber cortisol concentrations and overall body condition is similar in these two groups of non-fast-adapted and fast-adapted species with different life-history strategies as well as differences in blubber composition and morphology.

Total cortisol concentrations measured here show the same relationship with overall morphometric condition indices in the fast-adapted and non-fast adapted species. However, there is no information from these data about the rate at which the increased glucocorticoid response to reduced food intake or fasting occurs in the two species groups. The rate at which

cortisol increases to facilitate lipolysis during periods of food deprivation will likely differ in fast-adapted and non-fast-adapted species. For example, there may be a slower ramp-up in cortisol concentrations in balaenopterids compared to spikes in cortisol over much shorter periods in porpoises. Then, these cortisol spikes may be cleared quickly upon resumption of feeding in porpoises, but concentrations may remain high in the blubber of balaenopterids as the tissue is typically required to maintain a lipolytic state for much longer. Different rates in the glucocorticoid increase in response to fasting are likely also coupled with increases in protective mechanisms that prevent tissue damage caused by rapid lipid mobilisation with the production of reactive oxygen species, for example. Despite un-answered questions regarding the rate of change of tissue cortisol concentrations between species with different life-history strategies, given the current understanding of the pleiotropic role of cortisol in mammalian fat and protein metabolism, it is expected that concentrations in this tissue, in these species, may be used to infer an animals' physiological state.

Future Directions

Turnover Time Scales?

The greatest challenge for robust interpretation of cortisol concentrations in this 'new' matrix, is how to quantify the time frame captured by this single hormone measurement. The time frame over which steroid hormones, like cortisol, and their metabolites are distributed around the body in the blood and are then excreted in the faeces, or sequestered in the blubber for example, will vary greatly. As such, there is little known about how they may be stored and mobilised in the blubber, and at what rates. Currently, there is still conflicting evidence as to the time scales over which circulating steroid hormones are thought to be reflected in the blubber. For example, no significant relationship was seen between serum and blubber progesterone concentrations in non-pregnant harvested bowhead whales (Kellar et al., 2013). It was therefore suggested that progesterone concentrations are mirrored in these two sample types over longer periods (e.g. over weeks to months, the time frame of reproductive changes) but not shorter periods (e.g. over hours to days, the time frame of daily fluctuations). In addition, in wild caught harbour seals, blubber cortisol concentrations were shown not to be significantly affected by capture time, and thus were likely not driven by a stress response to the capture event, but were indicative of longer term physiological changes (Kershaw and Hall, 2016). In bottlenose dolphins however, blubber cortisol was found to qualitatively reflect circulating cortisol concentrations after oral administration of hydrocortisone (Champagne et al., 2017). As a result, there remains some disagreement of the interpretation of cortisol concentrations in this tissue as an indicator of a shorter term stress response (Champagne et al., 2018), or a longer term indicator of overall physiological state, uncoupled from short term fluctuations in the circulating concentrations of the hormone.

While the turnover of cortisol in the blubber of marine mammals remains unknown, steroids are known to diffuse passively from the blood into human adipose tissue (Deslypere et al., 1985), but the turnover in adipose tissue is slow (Hughes et al., 2010). Here, there was no relationship between blubber lipid content and cortisol concentration in the balaenopterid samples, suggesting that the presence of cortisol in the tissue is not just a function of the lipophilic nature of the hormone. Blubber cortisol concentrations thus probably provide

information on both the stress response of an individual, and a physiological response to energetic requirements, but these are most likely over longer term time scales than those apparent in circulation. As the blubber of shallow diving cetaceans has been shown to be more highly vascularised than the adipose tissue typical of terrestrial species (McClelland et al., 2012), a greater perfusion of the tissue could result in the high concentrations of cortisol measured in both species groups here, but it is still unknown to what extent steroid hormones are in dynamic equilibrium between the blubber and the circulatory system. As such, further work is urgently needed to establish the relationships between hormone levels in different tissues and excreta, how these concentrations relate to each other, and what this means in terms of the physiology of the animal, the stimulation of the HPA axis and the overall impact on the individual.

Localised Cortisol Production?

Adipose tissue in mammals is known to be a major site for the metabolism of glucocorticoids and other steroids as it has been shown to express a range of enzymes involved in the activation, interconversion and inactivation of both the steroid sex hormones, and the glucocorticoids (Bélanger et al., 2002; Meseguer et al., 2002; Seckl and Walker, 2001; Stulnig and Waldhäusl, 2004). The metabolism of glucocorticoids in adipose tissue is thought to be mediated primarily by one enzyme, 11β-hydroxysteroid dehydrogenase type 1 (11 β-HSD1), which catalyses the conversion of hormonally inactive precursors (cortisone and dehydroxycorticosterone) to their hormonally active metabolites (cortisol and corticosterone) (Seckl and Walker, 2001; Stimson et al., 2009; Stulnig and Waldhäusl, 2004). Therefore, in terrestrial mammals, as well as its release following the activation of the HPA axis, cortisol is also produced within the adipose tissue and the liver from its inert precursor, cortisone. However, while 11 β-HSD1 amplifies local concentrations of glucocorticoids in the adipose tissue, it does not contribute significantly to systemic glucocorticoid concentrations, as has been shown with transgenic mice (Masuzaki et al., 2001). Specifically, overexpression of 11 β-HSD1 in the adipocytes of transgenic mice results in normal serum concentrations of glucocorticoids and normal HPA axis function, but highly elevated levels of glucocorticoids locally in adipose tissue (Masuzaki et al., 2001).

In vitro studies have recently shown that bottlenose dolphin blubber microsomes exhibit the ability to metabolize cortisol to cortisone, and potentially the reverse reaction as well, cortisone to cortisol, presumably through the activity of 11βHSD (Galligan et al., 2018). As such, the blubber may act as an important site for both the metabolism and production of glucocorticoids, and it is possible that blubber could act as an independent endocrine organ, and is not merely a storage tissue reflective of the past or present circulating concentrations of glucocorticoid hormones. While it is unknown how much of the cortisol pool in cetacean blubber may be derived from local production, the cortisol concentrations measured in the blubber here likely reflect a combination of both circulating concentrations of this hormone through passive diffusion from the blood, and the local production of the hormone in the tissue itself. For these reasons, care should therefore be taken when interpreting glucocorticoid hormone levels in blubber biopsies and associating them with a response to chronic stress until the metabolic activities of blubber are better understood (Galligan et al., 2018).

Blubber cortisol concentrations should therefore be interpreted with the multi-functional roles of cortisol in the regulation of the distribution of fat deposits, adipogenesis and adipose metabolic and endocrine function in mind (Lee et al., 2014), rather than as a passive hormonal store with no metabolic effect on the tissue itself. Given the mass of adipose tissue as blubber in marine mammals, it is possible that the relative contribution of the blubber to an individual's steroid hormone metabolism is quite significant, and the endocrinological functions of blubber in terms of glucocorticoid metabolism should thus be further investigated. Efforts to extract and quantify cortisol's inactive precursor, cortisone, from the tissue, and investigate the equilibrium between the two should be prioritised. Attempts were made to measure cortisone concentrations in the blubber extracts using a commercially available ELISA but the results were not successfully reproducible here. The enzyme activity of 11 β-HSD1 during periods of fasting and feeding in these species would help us to understand how much of the cortisol within the blubber tissue originates from the adipocytes themselves during energetically demanding periods.

Conclusions

While very little is known about how cortisol may be produced, stored and mobilised in marine mammal blubber, and at what rates, the evidence of involvement in the regulation of physiological state and its presence in adipose tissue, make it a good candidate biomarker of condition worth investigation in cetaceans. Determining the dynamics of blubber cortisol throughout the life cycle of these species would be a key step towards understanding the endocrine control of energy regulation in marine mammals. Of key importance now is a better understanding of species specific concentrations in order to establish a baseline library of endocrine information for comparison with future studies. If these results showing a negative correlation between blubber cortisol concentrations and morphometric indices of body condition persist across other cetacean species, the analysis of cortisol concentrations in remotely obtained blubber biopsies, the metabolic site of action of this hormone, has the potential to be a valuable tool for studying physiology and body condition particularly in free-ranging cetaceans. More generally, given the importance of cortisol in the regulation of lipolysis, concentrations in the adipose tissue of other mammalian species for which morphometric measurements cannot be obtained could also be of use as an informative indicator of the metabolic state of the tissue and whether the animal is likely to be laying down or mobilising fat.

CHAPTER 5 : APPLYING ENCOCRINE BIOMARKERS TO INVESTIGATE VARIATION IN REPRODUCTION AND BODY CONDITION IN A NORTH ATLANTIC POPULATION OF HUMPBACK WHALES (*MEGAPTERA NOVAEANGLIAE*)

I collected the aerial photogrammetry video footage of humpback whales using a Phantom 4 UAV. Images were extracted from the video footage and processed for the calculation of the Length Standardised Surface Area Index by Charlotte Bellot and Professor Patrick Miller. These data were used for Charlotte Bellot's MPhil Thesis in 2018, and are also presented here.

1. ABSTRACT

The Gulf of St Lawrence, Quebec, Canada, is a major summer feeding area for humpback whales (*Megaptera novaeangliae*) and other baleen whales in the North Atlantic. Annual surveys conducted by the Mingan Island Cetacean Study during the summer since the 1980s have resulted in a well-established catalogue of photo-identified individuals and an extensive archive of tissue samples. Blubber biopsy samples (n = 185) collected from female humpback whales between 2004 and 2017 were analysed to investigate variation in annual reproductive and physiological state through the quantification of progesterone and cortisol concentrations, respectively. Annual pregnancy rates varied from 0% to 33%, which were lower than previously published for other humpback whale populations, but are in-keeping with the low calving rates seen in this population. In addition, unlike for other populations, there was no evidence for annual reproduction in either the blubber progesterone data or sightings data. These low pregnancy rates could be an artefact of the small number of mature females sampled every year, or they could indicate a low fecundity rate leading to slow population growth.

Blubber cortisol concentrations were not correlated with total disturbance time, used as a proxy for an acute stress response to the research vessel. Blubber cortisol concentrations are therefore thought to reflect variation as a result of changes in physiological condition and metabolic state rather than an acute stress response. Linear mixed model selection showed that cortisol concentrations varied across sampling years, and there was a negative relationship with pregnancy rates. Overall, as cortisol concentrations decreased from year to year, the proportion of pregnant females increased, and vice versa. There was no change in blubber cortisol concentrations across the feeding season. The expected seasonal variation was probably masked by individual variation. Cortisol concentrations did not vary by reproductive state (pregnant, lactating, resting, immature), but there was some evidence that juveniles and calves had higher measured concentrations than adults. This is thought to be a result of these two age classes mobilising their energy reserves for growth and development rather than laying down fat reserves. Finally, for a subset of females and males, cortisol concentrations were positively, but not significantly, correlated with a morphometric body condition index, the Length Standardised Surface Area Index (LSSAI), calculated from photogrammetry aerial images collected using an unmanned aerial vehicle (UAV). Overall, linear model selection showed that LSSAI varied between the sexes, with males having larger fat stores than females. The LSSAI also varied between reproductive classes with lactating females in the poorest body condition and pregnant females in the best condition. When combined with endocrine profiling to determine reproductive status, aerial photogrammetry could be a valuable tool to measure population health. A better understanding of baleen whale body condition and its link to reproductive status and success is fundamental to appreciate how populations recovering from over exploitation though whaling will be affected by changing environments in the coming decades.

2. INTRODUCTION

The Gulf of St Lawrence, off the east coast of Canada, is a major feeding area for humpback whales (*Megaptera novaeangliae*) and other baleen whales in the North Atlantic. Humpback whales are top marine predators, and the population that feeds in the Gulf shows strong site fidelity to this summer feeding area (Ramp et al., 2010a). The Mingan Island Cetacean Study has been conducting annual surveys of parts of the Gulf during the summer feeding season since the 1980s, which has resulted in a well-established catalogue of photo-identified individuals and an extensive archive of tissue samples. The retrospective analysis of these long-term data sets has enabled the investigation of patterns in persistent organic pollutant concentrations (Metcalfe et al., 2004), habitat use and distribution (Doniol-Valcroze et al., 2007), sex specific survival (Ramp et al., 2010a) and social structure (Ramp et al., 2010b).

Long term datasets of photo-identified individuals allow for population monitoring over time, which is particularly valuable as we aim to assess the potential impacts of anthropogenically altered habitats. For example, analysis of the temporal variation in the occurrence of humpback whales and fin whales in the Gulf has shown an approximate 2-week difference in their arrival between 1984 and 2010 (Ramp et al., 2015). This difference was strongly related to earlier ice break-up in the Gulf as a result of rising sea surface temperature, which likely triggers earlier primary production (Ramp et al., 2015). The observed changes in phenology in response to ocean warming are a remarkable example of the behavioural plasticity of these species (Ramp et al., 2015), but whether the observed rate of change in timing is maintained, and how this change will affect the health of the populations are unknown.

In addition to long term survey effort and observational studies in this area, the collection of biopsy samples between 1992 and 2010 has enabled studies on trophic niche partitioning within the four species of rorqual whales in the Gulf (minke whales (*Balaenoptera acutorostrata*), humpback whales, fin whales (*Balaenoptera physalus*) and blue whales (*Balaenoptera musculus*)) (Gavrilchuck et al., 2014) following the collapse of the several commercial groundfish populations in the early 1990s (Hutchings and Myers, 1995; Myers et al., 1996). There has been an increase in feeding on higher trophic-level prey, such as small pelagic fish, particularly for humpback and fin whales since the collapse of the groundfish populations (Gavrilchuck et al., 2014). This work provides evidence for differential resource use following ecosystem change in this area, further demonstrating the behavioural plasticity of these species. However, how these changes in diet may affect population health and thus vital rates are unknown. The next step to contribute to a better understanding of this apparent population resilience to environmental change is to use inter-disciplinary, newly developed methods to investigate variation in reproduction and body condition that can have population-level effects by affecting fecundity and survival.

Knowledge of population pregnancy rates and calving rates provides information on both population health and potential for growth. Historically, the pregnancy status of cetaceans was assessed by examining carcasses taken in commercial whale hunts (Chittleborough, 1965). More recently, endocrinological techniques have shown that blubber progesterone concentrations are indicative of pregnancy in both mysticetes (Kellar et al., 2013; Mansour et

al., 2002) and odontocetes (Kellar et al., 2006; Perez et al., 2011; Trego et al., 2013). Progesterone is a lipophilic steroid hormone that is produced by the corpus luteum, and is the primary regulator of oestrous cycling and pregnancy in mammals (Pineda, 2003). Its lipophilic properties mean that increases in circulating concentrations are measurable in the blubber. Pregnancy was confirmed in these studies by physical examination of the stranded carcasses and recording the number of corpora, corpus lutea and size and/or length of the foetus, if present.

The collection and analysis of blubber biopsy samples is therefore a readily obtainable and non-lethal way of assigning pregnancy in free-ranging cetaceans, and has been applied to free-ranging bottlenose dolphins (*Tursiops truncatus*) (Perez et al., 2011), long-finned pilot whales (*Globicephala melas*) (Perez et al., 2011) and humpback whales (Clark et al., 2016; Pallin et al., 2018a; Pallin et al., 2018b). From these previous studies, it appears that there may be species-specific blubber progesterone concentration thresholds that are indicative of pregnancy. Each investigation therefore requires progesterone threshold validation for the sample processing and hormone quantification methods employed.

Comparison of trends in body condition between different reproductive classes (pregnant, resting and immature females) can provide valuable insights into relative rates of energy acquisition and expenditure, and how these may affect the reproductive cycle. As previously discussed in Chapter 3, blubber cortisol concentrations could be used as a marker of overall physiological state in cetaceans. However, there is evidence that blubber cortisol concentrations in bottlenose dolphins are correlated with circulating concentrations after oral administration of hydrocortisone to simulate an artificial stress response (Champagne et al., 2017). As such, it was suggested that blubber samples from remote sampling may be useful to detect stress loads in this species (Champagne et al., 2017). If this is the case in larger cetaceans as well, the interactions of whales with the research vessels that follow and approach them for biopsy sampling could result in a stress response that is detectable in the blubber sample collected. This potential stress response would be similar to that seen when animal handling cannot be avoided for the collection of physiological samples from wildlife. Increases in circulating cortisol concentrations are characteristic of a stress response to such handling and physical restraint procedures in pinnipeds (Champagne et al., 2012; Engelhard et al., 2002; Harcourt et al., 2010) and in small cetaceans (Schwacke et al., 2014; Champagne et al., 2018). It is therefore very important to appreciate if the magnitude and duration of this potential stress response to the research vessels may compromise the specific aims of the study by masking any underlying variation in hormone concentrations. This issue was addressed for the first time here in baleen whales by recording the total duration of the research vessel's interaction with a subset of the sampled individuals. The relationship between the duration of the interaction and the cortisol concentrations measured in the blubber was investigated.

As well as using endocrine profiling to estimate the physiological state of individuals, morphometric measurements are valuable for body condition estimates. Due to the challenge of obtaining direct morphometric measurements in free-ranging cetaceans, the use of photographic images to make these measurements, a process called photogrammetry, has become increasingly popular. Photogrammetry techniques employed to date include laser

photogrammetry, which only captures part of the animal's body (Durban and Parsons, 2006), stereo-photogrammetry, which uses known morphological dimensions of the species to determine unknown dimensions by scaling (Bräger and Chong, 1999), and under-water videography, which is limited by clarity of the water and close approaches of cetaceans to swimmers (Spitz et al., 2000). Aerial photogrammetry studies have previously used fixed-winged aircraft or helicopters to obtain full body aerial images of individuals (Miller et al., 2012a; Perryman and Lynn, 2002), and increasingly now use unmanned aerial vehicles (UAVs) to obtain images. The use of UAVs has made these techniques much more accessible (Durban et al., 2015; Durban et al., 2016).

To date, however, only a handful of studies using UAVs have obtained data at a high enough resolution and with sufficient sample sizes to link morphometric measurements to body condition. Specifically, changes in the body condition of four reproductive classes of humpback whales across a breeding season were investigated to infer the relative energetic cost that each class faces over this period (Christiansen et al., 2016). In another study of baleen whales on their breeding grounds, photogrammetric measurements were used to determine the relationship between calf growth rate and maternal rate of energy store loss in Southern right whales (*Eubalaena australis*) using repeated measurements of body volume (Christiansen et al., 2018). Most recently, changes in the body condition of endangered, southern resident killer whales (*Orcinus orca*) were assessed using high-resolution images from UAVs to show a decline in the body condition of mature females over a 5-year period (Fearnbach et al., 2018). Thus, by combining long-term information of known individuals with new and accessible UAV technology, these studies can provide data on trends in body condition and the costs associated with reproduction.

A dataset of archived blubber biopsy samples from female humpback whales collected by the MICS is used to investigate: Firstly, variation in pregnancy rates over a 13-year time period. Secondly, if blubber cortisol concentrations are related to a potential acute stress response as a result of interactions with the research vessel. Thirdly, the relationship between reproductive state and blubber cortisol concentrations as an indicator of physiological state over time. Fourthly, how blubber cortisol concentrations are related to photogrammetric measures of body condition obtained using images collected by a UAV. By combining new methods in endocrine profiling and aerial photogrammetry, we can start to measure the health of individuals and develop tools for integration into long term monitoring protocols of populations.

3. METHODS

3.1. Blubber Biopsy Sample Collection with Life History Data

3.1.1. Female Samples Collected by the MICS between 2004 and 2017

A long-term dataset of 185 remotely obtained, shallow, blubber biopsy samples was collected from 114 female humpback whales between 2004 and 2017, excluding 2014, by the Mingan Island Cetacean Study (MICS). Sampling occurred from May to October in the Gulf of St. Lawrence, Quebec, Canada (Fig. 5.1). Biopsies were collected from rigid-hulled, inflatable boats using a crossbow and arrow system with hollow-tipped, stainless-steel biopsy darts (40 mm in length and 8 mm in diameter) (Gavrilchuck et al., 2014) from the dorsal and flank areas of the individuals. Samples were stored either in aluminium foil, plastic vials, or glass vials on ice immediately after collection, and were subsequently frozen at −20°C until analysed. The sex of these individuals was determined through genetic analysis of the skin (Palsbøll et al., 1992).

Annual surveys of humpback whales in the Gulf of St Lawrence (Fig. 5.1) by the MICS since 1982 have generated a catalogue of 432 known individuals. Individuals were photo-identified using natural markings, with particular emphasis on the pigmentation on the ventral face of the fluke and the size and shape of the dorsal fin (Katona and Whitehead, 1981). Demographic data were obtained for the sampled individuals using this catalogue and sightings database. Detailed sightings histories of these individuals have been recorded throughout a season where they are sighted multiple times in the area between May and October, and also between years. Female humpbacks typically reach maturity at 4–5 years of age (Chittleborough, 1965), thus to be conservative, individuals were considered as adults when they were sighted for the first time with a calf, or they were known for at least 5 years (Ramp et al., 2010a). Individuals were considered as juveniles when they were known for less than 5 years or if they were first sighted as a calf, and their exact age was therefore known (Ramp et al., 2010a). Lactating females were defined as large individuals that were accompanied consistently by a smaller one (approximately 1/3 of its size) during an entire feeding season (Ramp et al., 2010a). Calves were considered as these smaller individuals that were closely associated with the adult during an entire season. Finally, confirmed pregnant females were defined as those that were seen in the subsequent year with a calf.

3.1.2. Male and Female Samples with Accompanying Photogrammetry Data

A second set of 22 blubber biopsy samples from both males and females were obtained as a collaborative project between the University of St Andrews and the MICS during the summer field seasons of 2016 and 2017. These samples were collected as part of a study funded by the Strategic Environmental Research and Development Program (SERDP) to investigate methods of quantifying the body condition of free ranging cetaceans. The field protocol for this study involved first tagging the animals with bio-loggers equipped with suction cups for attachment, obtaining aerial footage for photogrammetric analyses of body size using unmanned aerial vehicles (UAVs), and finally, taking biopsy samples as described above. Where samples were

taken from unknown animals, these were also sexed though genetic analysis of the skin. The samples from females were also included in the larger, long-term dataset described above.

Fig. 5.1. Map of the Gulf of St Lawrence study area adapted from the Department of Fisheries and Oceans Canada website (http://www.dfo-mpo.gc.ca). The first main study area stretches along the northern shore of the Gulf between Pointe-des-Monts and Pointe-Parent, with particular focus on the area between Mingan and Anticosti Island. This study area is known as 'Mingan'. The second main study area is along the Gaspé penninsula from Percé to Grande-Vallée. This study area is known as 'Gaspé'. The whole of the Gulf is an important summer foraging ground for baleen whales in the North Atlantic.

3.2. Blubber Endocrine Profiling

3.2.1. Cortisol and Progesterone Extraction

The same steroid extraction protocol developed by Kellar and colleagues (2006), and previously described in Chapter 3, was used to simultaneously extract cortisol and progesterone from the biopsy samples. Hormones were either extracted from the whole sample, or where tissue mass was great enough, longitudinal sub-samples of the biopsies were used for analysis.

3.2.2. Cortisol and Progesterone Quantification

Cortisol: Cortisol concentrations were determined using the same commercially available ELISA kit (DRG International Inc., Marburg, Germany: Cortisol ELISA EIA-1887), as described and validated in Chapter 3. Cortisol concentrations are expressed as ng/g of wet tissue. This kit has been used to quantify cortisol concentrations in blubber biopsies from harbour seals (Kershaw and Hall, 2016). Extracts of varying concentrations (n = 18), were used to calculate inter-assay and intra-assay coefficients of variation (CVs). Mean percentage CVs of <20% and <10% were set as the acceptable limits for inter-assay and intra-assay variability respectively (Andreasson et al., 2015). The mean and inter-assay CV was 15.4% (with a range of between 3.1% and 27.4%) and the mean intra-assay CV was 7.2% (with a range of between 0.1% and 14.7%), and were therefore below the acceptable limits of variation.

Progesterone: A commercially available ELISA (DRG International Inc. Progesterone ELISA EIA-1561) was used for the quantification of progesterone in the biopsy sample extracts. The concentrations were measured according to the ELISA kit instructions with a standard curve ranging between 0 and 40ng/ml with a sensitivity of 0.045ng/ml. As with the cortisol concentrations, the progesterone concentrations in the samples were determined using a 4 parameter log-logistic model based on the standard curve, and final concentrations expressed as ng/g of wet tissue. All samples were assayed in duplicate and the mean hormone concentration reported as progesterone per wet weight of the blubber sample in ng/g. Extracts of varying concentrations were used to calculate inter-assay (n = 22) and intra-assay (n = 12) CVs. The mean inter-assay CV was 14.7% (with a range between 0.2% and 25.5%), and the mean intra-assay CV was 5.2% (with a range between 0.2% and 14.1%), and were therefore below the acceptable limits of variation. This ELISA reports a 0.2% cross-reactivity with corticosterone and < 0.02% cross-reactivity with cortisol.

3.2.3. Quality Assurance and Quality Control

Quality assurance and quality control tests were performed to validate the use of this cortisol ELISA with cetacean blubber extracts in Chapter 3. Here, the same quality assurance and quality control tests were performed to validate the use of this progesterone ELISA with cetacean blubber extracts.

Parallelism Assays: Three biopsy extracts from females were pooled and serially diluted four times using the 0 ng/ml standard provided by the ELISA kit. The resulting curve of the detection metric (optical density of the sample read at 450nm) as a function of the dilution state (1, ½, ¼, $^1/_8$, $^1/_{16}$) was then compared to the standard curve. As described in Chapter 3, two linear regression models for dilution state against optical density were compared (ANCOVA function in statistical package R, version 3.1.3, R Core Development Team, 2015) to assess parallelism between the two curves. There were no significant differences between either model (ANCOVA; $F=0.81$, $p = 0.4$). Therefore, we can conclude that the effect on optical density with increasing sample dilutions is the same for the standard curve samples and the blubber extracts, and that the regression lines are parallel (Fig. 5.2a). This ELISA is therefore suitable for the quantification of humpback whale progesterone.

Matrix Effect Test: As the progesterone ELISA kit was designed for use with serum or plasma samples, the compatibility of the kit with extracts resuspended in PBS was assessed. As described in Chapter 3, equal volumes of each standard were spiked with PBS and assayed in tandem with the unspiked standard curve. The known and the apparent concentrations in the spiked samples should show a positive linear relationship with a slope of approximately 1.0 (Hunt et al., 2014). The matrix effect test was successful with a slope of 0.98 (a slope of between 0.8-1.2 was considered acceptable). It was concluded that matrix effects are minimal and this sample diluent is therefore compatible with the immunoassay (Fig. 5.2b).

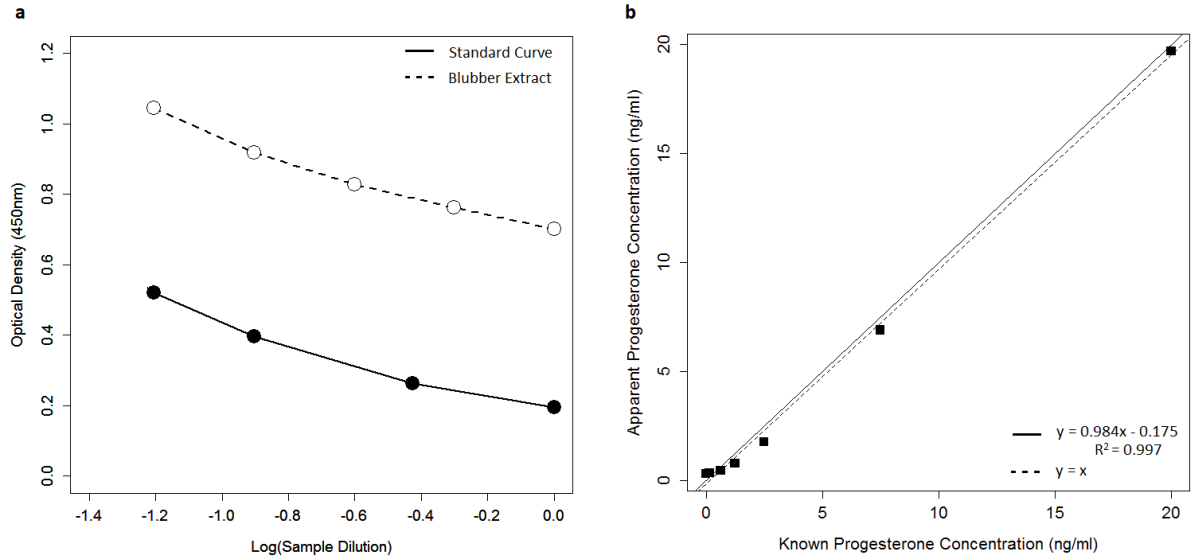

Fig. 5.2. a) Linearity assessment for the progesterone ELISA with humpback whale blubber extracts. Serial dilutions of three pooled extracts for each species show parallelism with the ELISA standard curve. **b) Matrix Effect Test validation for the progesterone ELISA kit with samples re-suspended in PBS.** The linear regression lines for the expected against the observed concentrations are shown for the expected 1:1 relationship and the measured relationship from the assay results. The assay results show a positive linear relationship with a slope of 0.98, thus confirming that PBS is an acceptable sample diluent compatible with this ELISA kit.

Extraction Efficiency and Correction Factors: Given the similarities in structure and physical properties of the steroid hormones, the same extraction efficiency values with increasing sample mass calculated in Chapter 3 for cortisol were used for progesterone. In addition, the effect of decreasing sample mass on inflated progesterone concentrations was assumed to be the same as measured for cortisol in Chapter 3. Thus, the previously calculated extraction efficiencies and sample mass correction factors were used to correct both the measured cortisol and progesterone concentrations in each sample to give the final hormone concentrations used for statistical analysis.

3.2.4. Statistical Analysis

3.2.4.1. Blubber Progesterone Concentrations as an Indicator of Pregnancy Status

In the full sample set of 185 females, there were 14 confirmed pregnant individuals. A mixture of immature females (known to be less than 5 years old) and calves (n = 31) were used as non-pregnant controls. An additional set of nine biopsy samples from adult and immature males collected as part of the 2016 - 2017 SERDP funded project were added to the 31 non-pregnant controls. The progesterone concentrations measured in these 40 control samples are shown in Fig. 5.3a. Using these two datasets of progesterone concentrations from individuals of known pregnancy status, an empirical cumulative distribution function (ECDF) modelling approach was used to estimate empirical probabilities (proportion of observations) that a certain measured progesterone value is above or below those of the confirmed pregnant or non-pregnant animals.

An ECDF is a probability model. It is a non-parametric estimator of the underlying cumulative distribution function of a random variable, x (blubber progesterone concentration here). It assigns a probability of $^1/_n$ (where n is the sample size) to each data point, orders the data from smallest to largest in value, and calculates the sum of the assigned probabilities up to and including each data point (Fig. 5.3b). The ECDF can then give the fraction of sample observations less than or equal to a particular value of x. Here, two ECDFs were modelled for the progesterone concentrations in the confirmed pregnant and non-pregnant animals. A probability of being pregnant and non-pregnant was then assigned to all of the unknown animals using the *stat_ecdf* function in the *ggplot2* package in R. If the probability of being pregnant was > 0.05, the individual was classified as pregnant, and if the probability of being not-pregnant was > 0.05, the individual was classified as not pregnant. If any individuals were assigned an intermediate probability of either being pregnant or not pregnant, these animals were classified as 'inconclusive'.

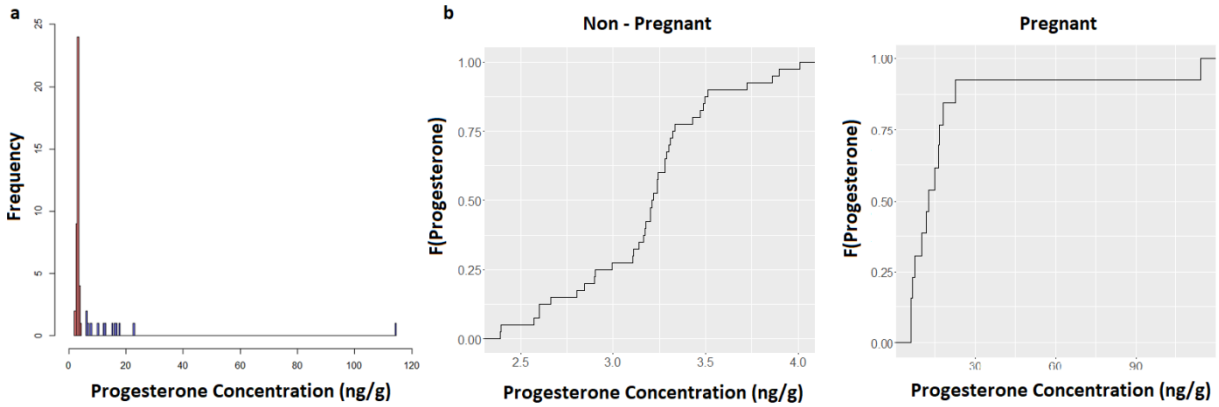

Fig. 5.3. a) Histogram of blubber progesterone concentrations measured in 53 humpback whale biopsies of known reproductive status collected between 2004 and 2017. The concentrations measured in the confirmed pregnant females are shown in blue while the confirmed non-pregnant individuals are shown in red. **b) ECDF models for the pregnant and non-pregnant animals built based on the progesterone concentrations shown in a).** These models were then used to assign a pregnancy status to the other females of unknown reproductive status based on their blubber progesterone concentrations.

3.2.4.2. Blubber Cortisol and an Acute Stress Response

In order to investigate the possibility that the measured blubber cortisol concentrations are reflective of an acute stress response to the approaches and interactions with the research vessel, the total 'disturbance time' was recorded for whales that were biopsied in the 2016 and 2017 field seasons as part of the SERDP funded project (n = 27). The disturbance time was taken as the time from when tagging approaches were started on either a group of animals or an individual, to when the biopsy sample was taken (Fig. 5.4). This time ranged from between just 16 minutes (an individual that was approached only for biopsy sampling as it had been tagged on a previous day), to 4 hours and 48 minutes (an individual that was tagged, followed for UAV photogrammetry footage and then biopsied on the same day). This time therefore encompasses the entire duration of the potential stressor event from initial approach by the research vessel to sampling, and is used as a proxy for the magnitude of the potential acute stress response. If this stress response is captured in the blubber cortisol concentration measurement, we would expect a positive correlation between disturbance time and cortisol concentration.

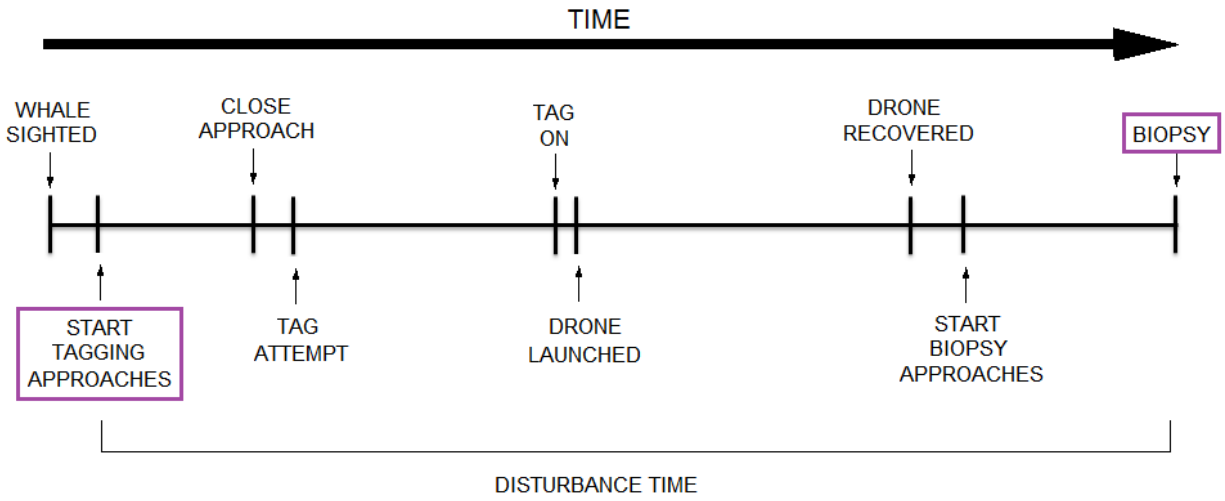

Fig. 5.4. Typical time line for the total 'disturbance time' spent interacting with humpback whales during fieldwork as part of the SERDP funded project in 2016 and 2017. This varied by individual depending on the weather conditions (suitable/ unsuitable for flying the UAV) or their behaviour making them easier / harder to approach which shortened or lengthened the total disturbance time.

3.2.4.3. Blubber Cortisol Variation within the Population

The cortisol concentrations measured in all the humpback whale biopsies were modelled using a linear mixed model (LMM) using individual as a random effect to take into account that cortisol concentrations were measured in 38 of the 114 females multiple times (up to 5 for some individuals) both within the same year and across different years. The cortisol concentrations were normally distributed, so the data were not transformed. A LMM with a Gaussian distribution and an 'identity' link function was fitted to the data using the *lmer* function in the *lme4* package in R (R version 3.4.3, 2017). A global model was generated including the following explanatory variables: year, reproductive status (as determined though blubber progesterone quantification: resting, pregnant, lactating, immature), age class (adult, juvenile, calf), annual pregnancy rate (% of pregnant females of the total number of adult females sampled), Julian day, an interaction between Julian day and reproductive status, study area within the Gulf (Mingan or Gaspé) and time in storage (years between the sample being collected and being analysed). An interaction between Julian day and reproductive status was included in order to consider a potential changing relationship across the feeding season between the reproductive classes.

As previously described, backwards model selection using the *dredge* function was used to identify the variables that best explain the variation in hormone concentrations, and thus to include in the final model based on the smallest AIC. Backwards model selection showed that two models were of equal fit to the data as they showed an AIC within two units of each other. This subset of models was then used for model averaging using the *model.avg* function in the *MuMIn* package in R. Model averaging is used to average regression coefficients across multiple models in order to capture the overall effects of the different variables (Banner and

Higgs, 2017). Model averaging is particularly useful when there is little to differentiate between a set of candidate models, and avoids the justification of a single, final model. The relative importance of each variable was calculated, which reflects both its prevalence in the candidate set of models, and its influence on the likelihood of the model it was included in. The model-averaged coefficients were calculated based on full averages, which sets the coefficients of absent variables to zero, rather than excluding them from the average (conditional averages). The full average ensures that the presence of variables does not bias the model-averaged estimate away from zero. Interpretations of the averaged model coefficients were used to assess the effects of each covariate.

3.3. Aerial Photogrammetry using an Unmanned Aerial Vehicle

3.3.1. Image Collection

To obtain overhead photogrammetry images of humpback whales, a DJI Phantom 4 UAV was flown above the individuals in wind speeds of less than 15 km/hr and in sea conditions less than Beaufort 3. The DJI Phantom 4 is fitted with bottom-side landing gear which was used as handles for safe launch and recovery from the research vessel. It was flown using the DJI GO App as the interface through an android tablet. By communicating with the controller, this provides a live feed from the camera on-board the UAV, and details of its status and settings. Video footage at a 4K resolution was recorded continuously when the UAV was airborne. This ensured that the whole surfacing sequence of a whale was captured, and the 'best' position of the whale at the surface when the body was fully extended could then be extracted.

The UAV flew at a target elevation of between 15 and 20m above the whales while the research vessel followed the targeted individual or group of individuals at a slow speed of less than 5 knots, roughly 200m away so as to minimise disturbance. Target whales were carefully observed throughout all UAV flights, and no response (startle, avoidance, trumpeting or shallow diving) was observed during any of the flights. Typically, UAV flights were made after a suction-cup tag had been deployed on a study animal, and that animal was the primary target for imaging. Once the UAV was airborne, any animal in the same groups as the tagged, target individual was also followed for imaging.

Table 5.1 - Total photogrammetry records with and without an accompanying biopsy. *Unknown animals are adult animals where the sex was unknown so their reproductive status could not be confirmed.

Sex / Reproductive Status	Total	Total Biopsy / Photogrammetry
Calf (males and females)	5	0
Immature (males and females)	3	3
Mature Males	5	5
Resting Females	9	7
Lactating Females	5	2
Pregnant Females	5	5
Unknown*	5	0
	37	**22**

3.3.2. Calculation of the Length Standardised Surface Area Index

Video footage was edited using VLC media player 3.0.2. Only sequences of interest with whales at or near the surface and clearly visible in the frame were retained for analysis. From these sequences individual frames were extracted using a freely available software called Free Video to JPG Converter 5.0.101.201. From the images extracted, the best quality frames were selected using the following criteria: 1) The depth and the angle of the individual relative to the UAV's position meant that the full body was visible from directly above. 2) The posture of the individual meant that the full dorsal surface was visible. 3) The elevation of the drone meant that the whale made up most of the frame. 4) The brightness of the image meant that the outline of the body was clearly visible. Examples of poor and good images are shown in Fig. 5.5.

Fig. 5.5. Examples of images extracted from the 4K video sequences recorded with the DJI Phantom 4. In the left panel are unusable images. From top to bottom: curved posture of the whale, the UAV is too high relative to the water's surface, poor light conditions create too much glare on the surface. In the right panel are images of sufficient quality to be used for photogrammetry analysis.

Frames judged to be of usable quality to measure relative body dimensions were measured in R studio (version 1.1.447, 2017), using image processing and analysis software protocols as described by Christiansen and colleagues (2016), and modified for use here. Briefly, the positions of the tip of the rostrum and the notch in the flukes are marked on the image, and this is considered to be the total length of the whale in pixels. The software then automatically calculates the body axis and creates lines perpendicular to the body axis, dividing the length (from the rostrum tip to the fluke notch) into 20 equal sections (Fig. 5.6a). Then, the external boundary of the whale is marked manually to create an outline of the whale's body shape enabling measurement of the width of the whale, in pixels, at different points (Fig. 5.6a). The software used by Christiansen and colleagues (2016) was specifically modified to enable detailed zoom to mark the edges of the whale. The outline measurements of each whale were used to calculate a length-standardized surface area index (LSSAI). The length-standardized surface area of each of the 20 sections of each whale's body was calculated using the trigonometric equation for the area of a trapezoid as:

$$\boldsymbol{A = (B/L + b/L) * (\frac{h}{L})/2}$$

Where *A* is surface area, *B* and *b* are the widths of the top and bottom of each trapezoid region of the whale's body in pixels, and *h* is the height of each trapezoid region in pixels (see blue trapezoid example shown in Fig. 5.6a). To standardize the area by length, each dimension is divided by total length of the whale (*L*), also in pixels. *A* was then summed over the regions most likely to reflect changes in body condition of individual whales, identified as those in the middle of the body that showed the most inter-individual variation (Fig. 5.6b). Specifically, these were the body sections between lines 7 and 17, representing 10 body sections across the middle of the body (Fig. 5.6b). These regions excluded the areas nearest the rostrum and flukes that also varied but are not expected to vary as the animals deplete lipid stores, but are more likely to change as growth occurs.

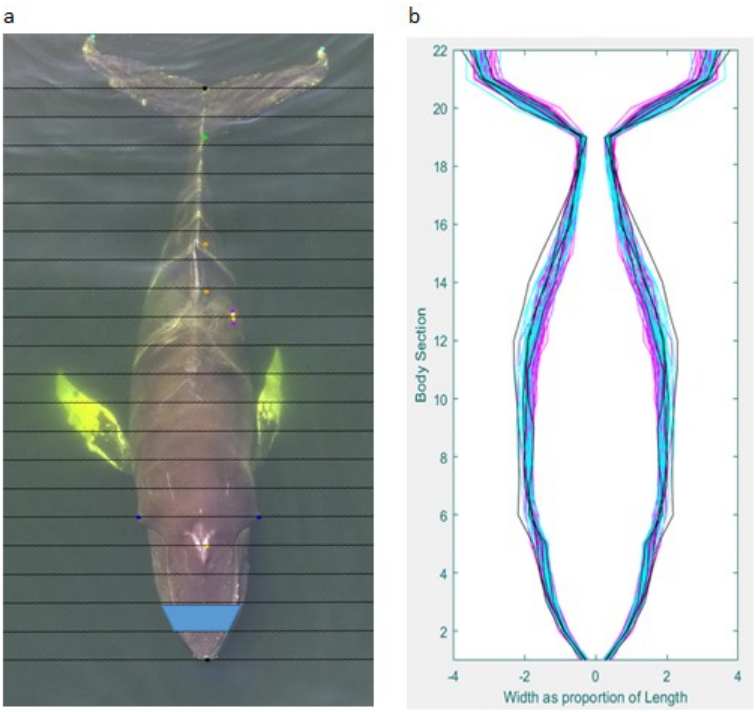

Fig. 5.6. a) Example image of a whale selected for analysis. Black dots mark the tip of the rostrum and fluke notch representing total length. The software divides the whale into 20 equal length sections shown as horizontal black lines. Each section of the whale's body can then be represented as a trapezoid for calculation of surface area (example shown in blue). **b) Length-standardized width measurements of different whales.** Each whale is represented by a different coloured line. The width measurements across the body demonstrate that the central regions of the whales' bodies show the highest inter-individual variability. These regions were used for calculation of the LSSAI as a metric of body condition.

3.3.3. Statistical Analysis

Linear regression models were used to determine which factors best explained the variation in the LSSAI. The LSSAI data showed a normal distribution. Covariates of interest to explain the variation in the LSSAI data were: blubber cortisol concentration, Julian day, sex, reproductive status (calves, immature males and females, resting females, pregnant females and lactating females) and age class (adults and juveniles). A full model including all explanatory variables was generated, and the *stepAIC* function in the *MASS* package was used for backward and forward stepwise model selection by AIC by specifying *direction = "both"*. The final model containing only the covariates that contributed to the explanation of the variation in the data was the one with the smallest AIC of more than two points compared to the next best fitting model. This model was chosen for further interpretation. Model validation tests were used to identify potential violations of the assumptions of the linear regression model. Specifically, scatter plots of residuals versus fitted values as well as the residuals against each explanatory variable showed equal variances in the final model used for interpretation. Additionally, normality of residuals was interpreted from a quantile–quantile plot which showed no deviation from normality. Influential points and outliers were also assessed using leverage and Cook's distance, but no such values were identified. Finally, the *gvlma* function in the *gvlma* package was used to perform a global validation that linear model assumptions had been met for the model, thus confirming the applicability of this modelling approach.

4. RESULTS

4.1. Endocrine Profiling

4.1.1. Identifying Pregnant Females

Of the 185 biopsies from females, 15 were lactating, 14 were confirmed pregnant, an additional 18 were identified as pregnant based on measured progesterone concentrations, and 11 had 'inconclusive' progesterone concentrations, so their reproductive status was undetermined. Non-pregnant females had an average blubber progesterone concentration of 2.77 ± 0.059 ng/g, while pregnant females had an average blubber progesterone concentration of 21.3 ± 3.82 ng/g. One lactating female had an 'inconclusive' progesterone concentration, so was classed as lactating here. None of the other lactating females showed elevated progesterone concentrations indicative that they were simultaneously lactating and pregnant. Over the 13-year period, nine females were sampled twice in one season. These were four resting females, two pregnant females, two juveniles and a calf. Progesterone concentrations extracted in the duplicate samples from each of these females classified them into the same reproductive class each time, thus giving confidence in the consistency of this method and these results.

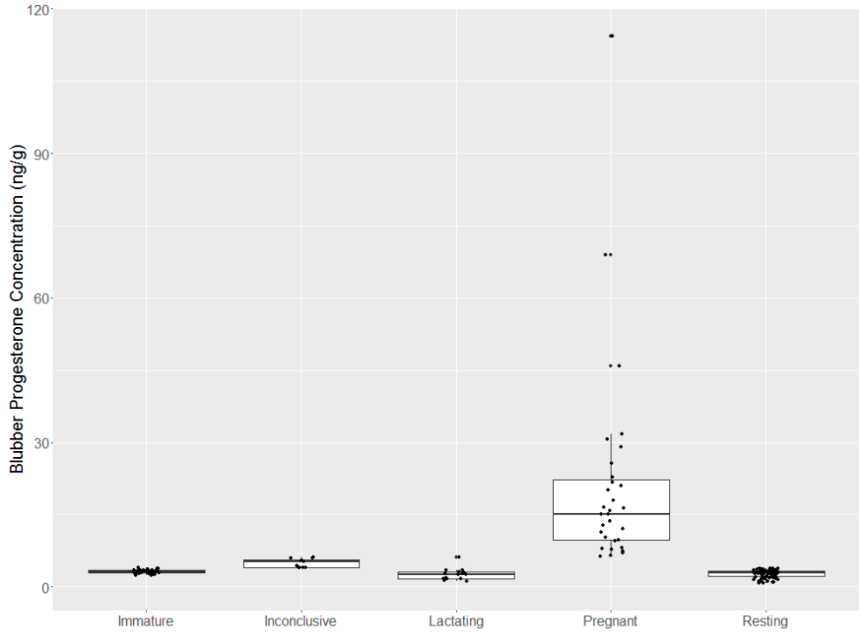

Fig. 5.7. Blubber progesterone concentration by reproductive status. Pregnant females had significantly higher blubber progesterone concentrations than the other reproductive classes (ANOVA; df = 4, F = 28, $p < 0.001$), that were not significantly different from each other.

4.1.2. Variation in Pregnancy Rates and Reproductive Success

The percentage of pregnant females in each year was calculated as the number of pregnant females divided by the number of adult (and therefore reproductively mature) females also sampled in that year. The percentage of pregnant females ranged from 0% in 2005 and 2011

up to 33% in 2008 and 2010 although sample sizes were small (between one and six pregnant females across years) (Fig. 5.7). Based on the detailed sightings histories of individuals recorded by the MICS, the percentage of successful pregnancies could be calculated for 2004, 2007 and 2016 because all pregnant females were re-sighted the following year either with or without a calf. The percentage of successful pregnancies was calculated for these three years as the number of pregnant females seen the following year with a calf, divided by the total number of pregnant females, and ranged from 67% in 2016 to 100% in both 2004 and 2007 (Fig. 5.8).

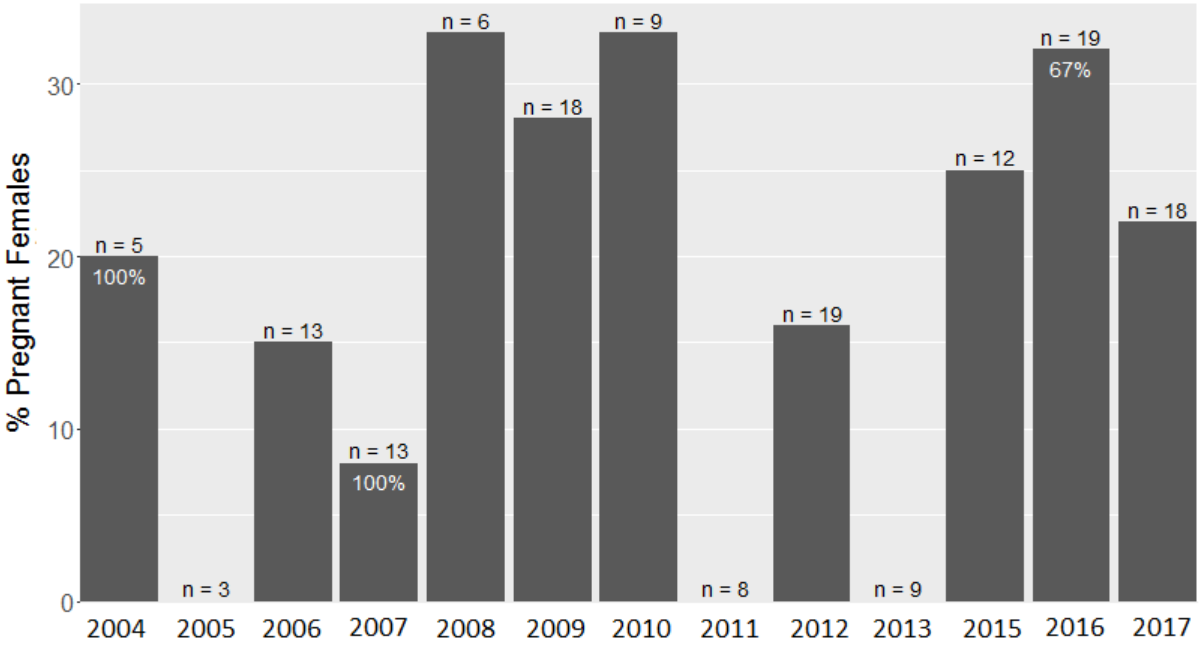

Fig. 5.8. Variation in pregnancy rates between 2004 and 2017. The percentage of successful pregnancies is indicated in white inside the bars for 2004, 2007 and 2016 as all pregnant females were re-sighted in the following years. In 2005, 2011 and 2013, no pregnant females were sampled. The total number of mature females sampled (n) is indicated for each year.

4.1.3. Blubber Cortisol and the Acute Stress Response

Blubber cortisol concentrations were not significantly correlated with total disturbance time measured for 27 individuals (linear regression model: F-statistic 1.49, Adjusted $R^2 = 0.02$, $p = 0.23$) (Fig. 5.9). If a stress response is captured in the blubber cortisol concentration measurement, we would expect a positive correlation between disturbance time and cortisol concentration. We are confident that measured blubber cortisol concentrations in this species, over this time period of approximately 5 hours, are not indicative of an acute stress response to our approaches on the animals. In order to assess this potential acute stress response, cortisol concentrations in another matrix should be measured.

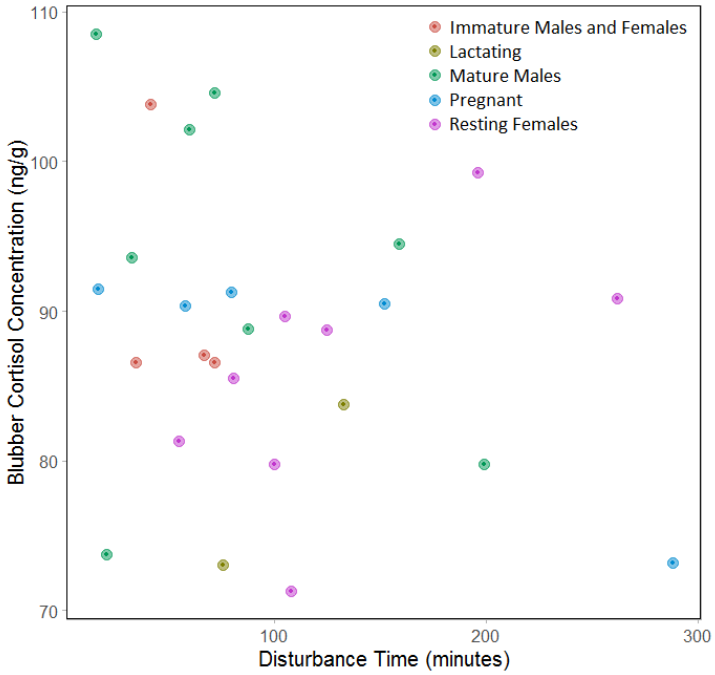

Fig. 5.9. Blubber cortisol concentrations are not correlated with the disturbance time by the research vessel. The disturbance time was recorded for 27 whales that were both tagged and biopsy sampled during the summer field seasons in 2016 and 2017. Individuals are coloured by reproductive state.

4.1.4. Blubber Cortisol Variation within a Population

There was considerable variation seen in cortisol concentrations ranging from 49.09ng/g to 237.79ng/g. LMM variable selection revealed that the two best fitting models, within two AIC points of each other included combinations of year, age class and annual pregnancy rate as important explanatory variables. Results from the model averaging of these two best-fitting LMMs showed that year alone was individually statistically significant. Specifically, 2007 and 2017 showed significantly higher and lower cortisol concentrations compared to a number of other years (2008, 2009, 2011, 2012, 2013 and 2015) (p values all < 0.01) (Fig. 5.10). Year showed the highest relative variable importance with a value of 1.0 because it was included in both of the models used for averaging. While age class was not individually significant (p value > 0.05), and had a relative variable importance of 0.32, it's inclusion in the model showed that overall, calves and juveniles had higher blubber cortisol concentrations than adults.

Annual pregnancy rate had a relative variable importance of 0.50 but, as with age class, was not individually significant (p value > 0.05). Model averaging results showed that there was an overall negative relationship between annual pregnancy rate and blubber cortisol concentrations. Higher pregnancy rates were seen in years with lower measured cortisol concentrations. Qualitatively, of the 12 consecutive year comparisons, this relationship between higher cortisol concentrations and lower pregnancy rates was consistent for eight of them. For example, between 2007 and 2008, and 2011 and 2012, there was an overall decrease in cortisol concentrations with an increase in pregnancy rate (Fig. 5.10). Conversely, between

2010 and 2011 there was an increase in cortisol concentrations and a decrease in pregnancy rate (Fig. 5.10). Pregnancy rate the year *after* the samples were taken was not retained as an important explanatory variable following model selection, indicating that there doesn't appear to be a time lag in the correlation.

Reproductive state (immature, resting, pregnant, lactating) was not retained following variable selection, so the variability in the cortisol concentrations therefore appears to be explained more by differences between the age classes. In addition, Julian day was not retained as an important explanatory variable either. Individual variation appears to mask any variation linked to changes over the May – October feeding season. Of the nine females that were sampled twice within a season, the average difference in cortisol concentrations measured in the samples was +14.0% of the first sample extracted, with a difference range of between -21.9% and +66.5%. Therefore, for these nine females that were sampled longitudinally, it appears that there was an overall increase in blubber cortisol concentrations measured over the feeding season. Importantly, storage time was not retained following variable selection so, measurement artefacts as a result of sample degradation through prolonged freezing do not appear to be a problem here.

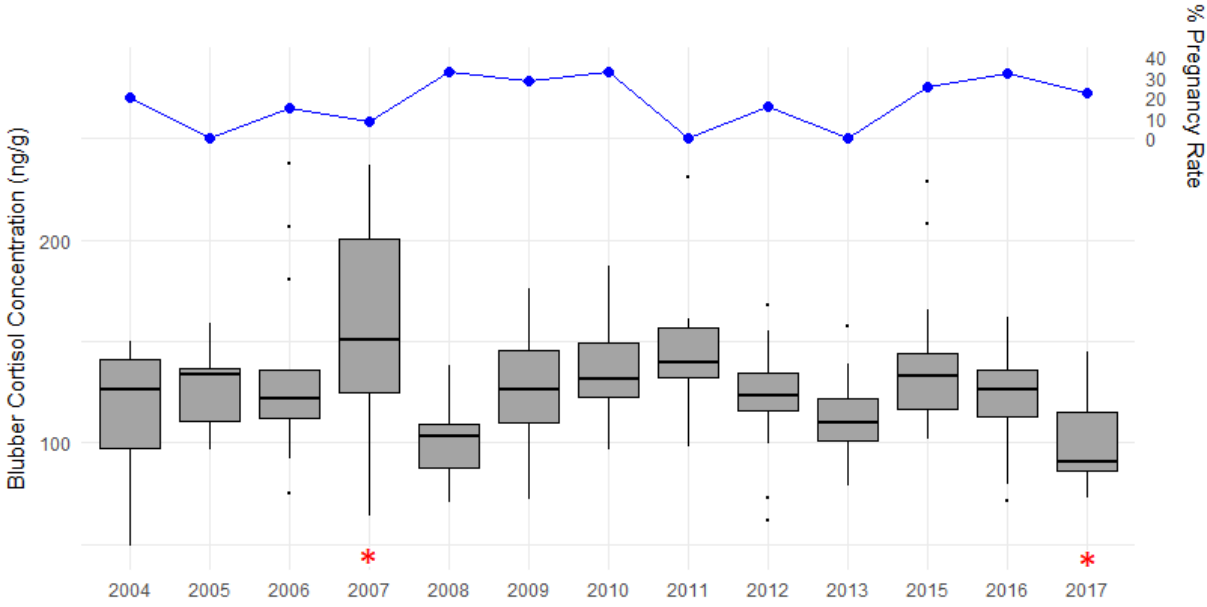

Fig. 5.10. Annual variation in blubber cortisol concentrations and pregnancy rates. Blubber cortisol concentrations are shown using the boxplots that indicate the median, inter-quartile ranges and outliers. Years that showed significant differences in measured concentrations compared to other years are indicated by the red asterisks. Variation in the percentage of pregnant females across years is shown by the blue line.

4.2. Aerial Photogrammetry

Stepwise model selection showed that the best fitting model included sex, reproductive status and blubber cortisol concentration as important explanatory variables (ΔAIC 3.1 to the next best fitting model), although no relationships were individually significant (p values all > 0.05). Overall, males had a larger LSSAI than females and there was a weak positive relationship with blubber cortisol. Pregnant females had the largest LSSAI while lactating females had the smallest, but there was considerable overlap between all reproductive classes (Fig. 5.11). As no calves were biopsied, their relationship between LSSAI and cortisol concentration could not be assessed. Julian day was not included in the final model likely because of the 37 LSSAI measurements, 32 of them were from individuals imaged in the early part of the feeding season, in June and July, while only five of them were imaged in later in the feeding season, in September. Age class was also excluded from the final model.

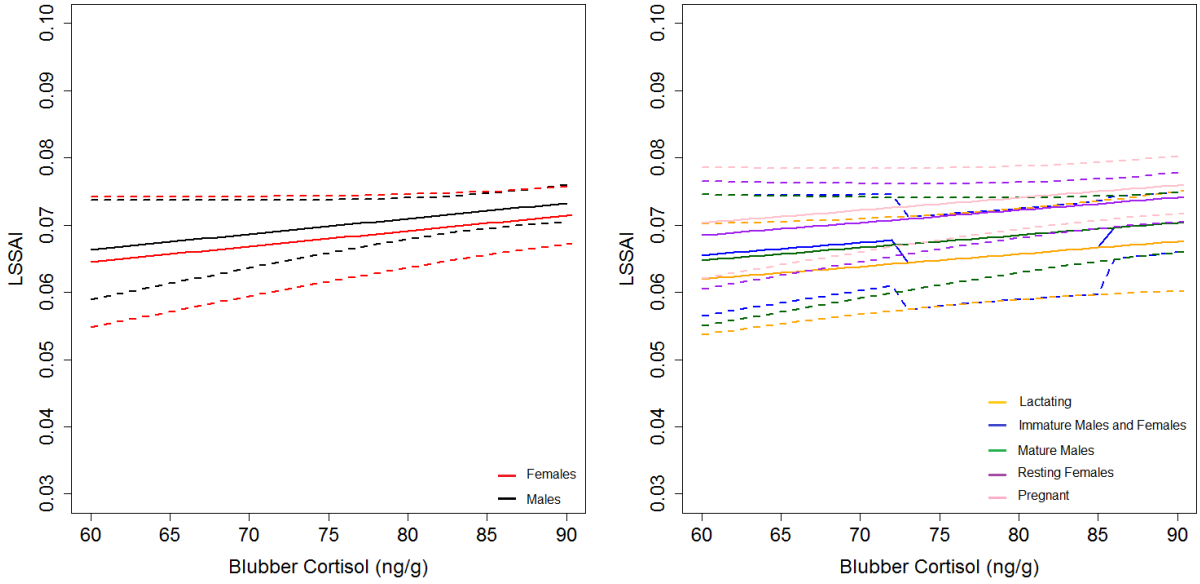

Fig. 5.11. Prediction plots for the final linear regression model for LSSAI: lm(LSSAI~Cortisol+as.factor(Sex)+as.factor(Reproductive_State). The relationships are shown by the solid lines with the associated 95% confidence intervals indicated by the dashed lines. Overall, there was a weak positive correlation between LSSAI and blubber cortisol but this was not significant, nor was the difference between males and females. There was some variation in LSSAI by reproductive status, but differences between groups were not significant.

5. DISCUSSION

Variation in Pregnancy Rates

The results of the progesterone extraction and quantification further support the use of this method as a tool to identify pregnancy in humpback whales. Results showed previously unobserved trends in pregnancy rates in this population over the 13-year period of this study. However, the difference in blubber progesterone concentrations between pregnant and non-pregnant females was smaller than in previously published studies on baleen whales (Clark et al., 2016; Kellar et al., 2013; Mansour et al., 2002; Pallin et al., 2018a; Pallin et al., 2018b). This resulted in the pregnancy status of some individuals being inconclusive. Serum progesterone concentrations of bottlenose dolphins and killer whales (*Orcinus orca*) remain high in the early and middle stages of pregnancy, then decline in the later stages shortly before parturition (Bergfelt et al., 2011; Robeck et al., 2016). Similarly, progesterone concentrations measured in the baleen of bowhead whales showed that concentrations remained stable throughout pregnancy and then decreased at or near parturition (Hunt et al., 2014). While not the same sampling matrix (serum or baleen), the general trend appears to be that circulating concentrations of progesterone decrease towards the end of pregnancy. The exact timing of calving is unknown for the Gulf of St Lawrence humpback whales. It is possible that lower concentrations measured in the blubber of females later in the feeding season could be the result of this decrease in concentrations at the end of gestation, and these females were therefore classified as 'inconclusive' when they were in fact pregnant. More longitudinal sampling of individual females across a season would provide a better understanding of changes in hormone concentrations during pregnancy.

Alternatively, pseudopregnancies could have resulted in intermediate progesterone concentrations. Pseudopregnancy occurs in many mammal species and is the process whereby the longevity of the corpus luteum and the duration of elevated progesterone concentrations are prolonged in the absence of fertilisation (Robeck and O'Brien, 2018). Captive studies on false killer whales (*Pseudorca crassidens*) and Pacific white-sided dolphins (*Lagenorhynchus obliquidens*), for example, have shown that they can exhibit elevated progesterone concentrations during ovulation and for extended periods (up to 103 days for one individual, for example) subsequent to ovulation (Atkinson et al., 1999; Robeck et al., 2009). While this has been documented in captive cetaceans, the rate at which this anomaly might occur in wild populations remains unknown (Pallin et al., 2018a) and therefore cannot be tested or controlled for here.

The most conservative estimates of annual pregnancy rates are presented, and ranged from 0% to 33% across different sampling years. These rates are very low compared to the pregnancy rates previously published for other humpback whale populations. For example, variation in pregnancy rates over a 6-year period between 2010 and 2016 for a population on its summer feeding grounds around the Western Antarctic Peninsula were between 36% and 86% (n = 268) (Pallin et al., 2018b). This population also showed significant increases in the proportion of pregnant females from the start of the feeding season to the end, consistent with demographic

variation in migratory timing (Pallin et al., 2018b). Sample sizes of pregnant females were too small here to assess variation across the feeding season in the Gulf of St Lawrence.

Estimated annual pregnancy rates of between 18.5% and 48.4% in 58 females feeding in Monterey Bay, California, in 2010 and 2011 (Clark et al., 2016) were lower than in the Antarctic, but higher than in the Gulf of St Lawrence. The pregnancy rates calculated here were also lower than the estimated 37.2% generated by Chittleborough (1965) from whaling data. While examination of the reproductive tracts of humpback whales killed during whaling operations indicated an average ovulation rate of approximately once per year (Chittleborough, 1965), it is thought that on average, female humpback whales have a longer, 2–3 year reproductive cycle (Steiger and Calambokidis, 2000). This cycle consists of a one year gestation period, approximately one year for lactation and weaning and, for some whales, one resting year before subsequent reproduction (Steiger and Calambokidis, 2000). In the recent study of Antarctic peninsula humpback whales however, there was strong evidence of annual reproduction as 54.5% of lactating females were also pregnant (Pallin et al., 2018b). These high pregnancy rates combined with the prevalence of females that were both pregnant and lactating were thought to be consistent with a growing population recovering from past exploitation, and suggests favourable environmental conditions (Pallin et al., 2018b). Consecutive year calving has also been documented using sightings data alone from photo-identified humpback whales, but was observed at only 2% every year in the Gulf of Maine population (Robbins, 2007). There was no evidence for annual reproduction in the Gulf of St Lawrence humpbacks sampled here through either the blubber progesterone data or sightings records.

Even with the 13-year dataset of samples used for analysis here, the sample sizes of reproductively mature females every year was low. This is because the individuals often targeted for sampling were unknown animals where the priority was to obtain a skin sample for genetic sexing in order to add these individuals to the MICS catalogue of photo-identified and sexed animals. Many of the samples were therefore from juveniles and calves. Had more routine sampling been performed of known, sexually mature, adult females, the annual pregnancy estimates may have been higher. This having been said, the low pregnancy rates measured here are in keeping with the low numbers of calves sighted every year in the Gulf of St Lawrence (Ramp, C. personal communication).

In order to understand whether the low calving rates in the population are as a result of the apparent low pregnancy rates, foetal loss, perinatal mortality and / or calf mortality on the breeding grounds or on migration, information on pregnancy success rates would be important. Reproductive success rates (calculated here for only three of the study years because not all pregnant females were re-sighted the following year) ranged from 67% to 100%. Reproductive failure, defined as identified pregnancies failing to produce a calf, or failing to produce a calf that survives long enough to be observed in the following feeding season, was estimated at 13.3% for the Gulf of Maine population (Pallin et al., 2018a). Such longitudinal studies of well-known individuals with detailed sightings histories allow reproductive success to be investigated over time.

Responses in the demography of baleen whales to climate change have already been documented (Leaper et al., 2006; MacLeod, 2009), and long-term studies are invaluable for such responses to be detected. Thus, while this population of humpback whales has shown behavioural plasticity to cope with environmental changes (Gavrilchuck et al., 2014; Ramp et al., 2015), the low pregnancy rates measured here could be evidence that this population does not show as much resilience to change as previously thought, and population health could be affected.

A meta-analysis of published humpback whale population growth rates suggests that, globally, they are increasing, but growth rates in the Northern Hemisphere are predicted to be slower than those in the Southern Hemisphere (Wedekin et al., 2017). To date, there are no published growth rates for the Gulf of St Lawrence population specifically, but the most recent estimate from another population in the North West Atlantic is an annual increase of 9.4% (SE = 0.01) (Heide-Jørgensen et al., 2012). Moving forward, estimating population growth rates for the Gulf of St Lawrence would help to determine if apparent low fecundity rates are counteracted by high calf survival, for example, or if this population shows signs of reduced population growth.

Measuring Acute Stress or an Integrative Physiological Marker?

Studying natural variation in glucocorticoid hormone concentrations in wild populations is inherently very difficult because a stress response to the temporary tracking, approach, capture and restraint necessary for sampling of many species may alter these physiological parameters of interest (Ortiz and Worthy, 2000; Schwacke et al., 2014; St. Aubin et al., 2013). The collection of biopsy samples from large cetaceans typically involves three stages: sighting the individuals to be targeted, tracking the targets over multiple surfacings between dives, and final close approaches (between 10 – 40m depending on the biopsy equipment to be used) for sampling. These encounters with the research vessel could elicit a stress response in the target animals. It is therefore very important to appreciate if the magnitude and duration of this potential stress response to the interaction with the research vessel could compromise the specific aims of different studies by masking any underlying variation in blubber cortisol concentrations.

The relationship between the disturbance time, as a proxy for an acute stress response, and blubber cortisol concentrations was assessed for the first time in a baleen whale. There was no correlation between the two. Within the 5-hour time window measured here, the blubber is an appropriate tissue to sample in order to investigate natural, physiological variation rather than short term changes in circulating concentrations as a result of activation of the hypothalamic-pituitary-adrenal (HPA) axis in response to an acute stressor. Currently, however, there is conflicting evidence as to the rate at which circulating cortisol concentrations are taken up into blubber.

Plasma cortisol concentrations in wild caught harbour seals were positively correlated with capture time, suggesting that they were largely driven by a stress response to the capture event

(Kershaw and Hall, 2016). However, blubber cortisol concentrations taken at the same time were shown not to be significantly affected (Kershaw and Hall, 2016). In a study on captive bottlenose dolphins, individuals received oral hydrocortisone every six hours to elevate circulating cortisol concentrations over a 5-day period (Champagne et al., 2017). There was an initial increase in blubber cortisol concentrations between days 1 and 3. In four of the five study animals, blubber cortisol concentrations then decreased from day 3 to day 5 despite persistent elevations in serum cortisol concentrations (Champagne et al., 2017). There therefore appears to be at least a partial de-coupling of circulating concentrations and blubber concentrations in these two studies.

During a standardised 'out of water' stress test in captive bottlenose dolphins, blubber cortisol concentrations increased significantly over the two-hour sampling period (Champagne et al., 2018). Blubber concentrations were correlated with concentrations measured in simultaneously collected serum samples (Champagne et al., 2018). However, these dolphins were stationed out of the water on mats during the acute stress test to facilitate the simultaneous sampling of both blood and blubber. This sampling protocol out of water is likely associated with significant impacts on overall physiology because individuals experience the action of gravity on their body mass as well as the thermoregulatory challenges of keeping cool in air. Peripheral perfusion of the blubber is likely to have increased significantly in order to compensate for a reduced ability by the dolphins to dissipate body heat. Circulating cortisol was therefore likely more available for blubber uptake, so the extrapolation of these results to wild animals may not be appropriate.

Uptake and turnover of cortisol by the blubber is most likely linked to the extent and rate of perfusion of the tissue. Perfusion is mediated firstly by the microvasculature in the blubber and secondly, by the blood flow through the vasculature. Microvasculature of mysticete blubber has yet to be investigated, but odontocete blubber is more highly vascularised than porcine adipose tissue (McClelland et al., 2012). This suggests that there is a possibility for the tissue to be highly perfused. Differences in blood flow through this microvasculature as a result of vasoconstriction and vasodilation will contribute to changes in blood flow through the tissue. Thermal-imaging studies have shown that marine mammals are able to finely control and dramatically alter blood flow to the skin in order to lose heat (Barbieri et al., 2010) and to facilitate hair re-growth (Paterson et al., 2012). There will also be differences in blood flow through the tissue as a result of cardiac output. When diving, the heart rate is dramatically reduced in cetaceans, and then increases again during surface intervals (Williams et al., 2017; Williams et al., 2015). Measured changes in blood flow through the blubber to the skin over these time periods have yet to be published, but these periods of bradycardia and tachycardia will likely result in changes in perfusion to the periphery. These perfusion patterns may be even more pronounced in animals experiencing an acute stress response (Williams et al., 2017). Immediately following release from net entanglement, narwhals (*Monodon monoceros*) showed a more pronounced bradycardia compared to other dives (Williams et al., 2017). By extension, a more pronounced bradycardia as a result of a fight or flight acute stress response would result in a reduction of blood flow to the periphery, and therefore a reduced availability of circulatory cortisol for uptake in the blubber.

The high variability in blubber cortisol concentrations among individuals was not surprising because concentrations may be influenced by a number of factors that were not measured here. These include, for example, changes in cortisol carrier protein concentrations that will affect free hormone availability. Over 90% of circulating cortisol, in most mammals studied to date, is bound to carrier proteins (e.g., corticosteroid-binding globulin and serum albumin), and it is thought that only free, unbound, cortisol is incorporated into body tissues (for a review see Breuner and Orchinik, 2002). In addition, recent evidence suggests that the conversion of cortisol to cortisone occurs in the blubber, speculatively through the activity of the enzyme 11β-Hydroxysteroid dehydrogenase (11βHSD) (Galligan et al., 2018). The conversion of cortisone to cortisol may occur as well (Galligan et al., 2018). These results provide preliminary evidence that blubber tissue is able to metabolise steroid hormones, in keeping with what has previously been described and recognised as an important function of adipose tissue in other mammals (for a review see Ahima, 2006). Concentrations measured here are therefore likely both circulatory in origin and from the blubber itself. In addition, as with blood sampling, these measurements represent one cross sectional sample at a single point in time which may in itself produce variable results depending on the metabolic state of the tissue at the time of sampling. These complications therefore need to be considered before using tissue concentrations to characterise central endocrine function as a result of HPA axis activation and the acute stress-response alone.

Variation in Blubber Cortisol Concentrations

Glucocorticoid variability in cetacean populations has been associated with poor environmental conditions and changes in prey availability (Ayres et al., 2012), anthropogenic disturbance (Ayres et al., 2012; Rolland et al., 2012) and chronically contaminated environments (Fair et al., 2017). Here, there was significant variation in blubber cortisol concentrations between different sampling years. The highest concentrations were measured in 2007 and the lowest concentrations measured in 2017. Inter-annual differences in blubber cortisol concentrations explained most of the variability in the data, and could be indicative of annual variation in environmental conditions that affected the foraging and therefore physiological state of individuals. Lower concentrations may have been measured in more favourable years with increased prey availability for example. An important next step for this work is to add time-series environmental data to these results in order to investigate links with inter-annual variation in ecosystem productivity.

Unexpectedly, the reproductive status of females was not retained following model selection. Age class was retained, and showed that adults had lower blubber cortisol concentrations than both calves and juveniles. It was hypothesised that the variation by reproductive status would be more pronounced than variation by age class as lactating females undergo extreme lipolysis to meet the energetic demands of lactation (Christiansen et al., 2016). Conversely, it was hypothesised that pregnant females would have the lowest blubber cortisol concentrations. It has been estimated that they must increase their body mass by up to 65% to meet the energetic costs of reproduction for the following year (Lockyer, 1981), and would therefore be depositing rather than mobilising fat stores to a greater extent than other reproductive classes. Specifically,

pregnant female baleen whales acquired an estimated extra 10–15% of fat reserves compared to resting females over a feeding season (Lockyer, 1984). The lack of variation by reproductive state but the inclusion of age class in the final model suggests that the variation in cortisol concentrations is perhaps more strongly explained by the energetic demands of growth in juveniles and calves. This is in keeping with the blubber cortisol results presented in Chapter 3 whereby higher cortisol concentrations were measured in juvenile harbour porpoises compared to adults. This could be because humpback whale juveniles and calves are mobilising rather than depositing energy reserves in order to maximise growth. This would, firstly, reduce their surface area to volume ratio which will affect their thermoregulatory requirements in cold water. Secondly, it would reduce their predation risk from killer whales. Thirdly, it would help to achieve size at sexual maturity faster.

While not individually significant, annual pregnancy rate was retained in the final model. Overall, years with higher cortisol concentrations showed a lower pregnancy rate, and this relationship was consistent when the majority of consecutive sampling years were compared. Qualitatively, an increase in cortisol concentrations from one year to the next led to a decrease in the pregnancy rate, and a decrease in cortisol concentrations correlated with an increase in pregnancy rate. Interestingly, there was no such correlation between the pregnancy rates and blubber cortisol concentrations in the *previous* year, suggesting that there does not appear to be a time-lag between the two.

Embryonic/foetal resorption or early termination of pregnancy during periods of stress, including disease and nutritional stress, for example, occurs in a number of mammals (Conaway et al., 1960; Huck et al., 1988). This foetal resorption may be an adaptive mechanism in response to poor environmental conditions. Such a mechanism has not been directly documented in baleen whales, but, female right whales (*Eubalaena australis*) (Seyboth et al., 2016) and killer whales (Ward et al., 2009) have been shown to not become pregnant in years of low food availability. Reduced reproductive success has also been shown in fin whales in response to a reduction in prey abundance (Williams et al., 2013). So, if such a foetal reabsorption mechanism does occur, it could allow pregnant female humpback whales to respond to unpredictable environmental conditions on the feeding grounds. This would allow them to maximise reproductive success in years when foraging conditions were favourable and only experience the minimal costs associated with the early stages of pregnancy in poorer years. This process could explain the link between annual pregnancy rates and blubber cortisol concentrations if high blubber cortisol concentrations measured on the feeding grounds are indicative of generally reduced foraging success, and therefore poorer environmental conditions.

There was no variation by Julian day, which was also unexpected as individuals sampled at the beginning of the feeding season were hypothesised to have higher cortisol concentrations than those at the end of the season as a result of increased lipogenesis. It appears that the expected seasonal variation was masked by individual variation, possibly as a result of the cross sectional nature of the sampling, as discussed above. The lack of any seasonal relationship could be the result of different timings of the migration of individuals which affects how long they have been fasting or foraging for. Demographic segregation of migration was observed for

humpback whales during the whaling era (Chittleborough, 1965), and it is possible that individual differences determine migratory timing as well. For example, the individuals sampled later in the season could in fact be animals that are 'left behind' if they have not accumulated enough fat reserves and will therefore not migrate. Non-pregnant females and juveniles specifically may benefit from staying in feeding areas for extended periods of time. They may possibly overwinter there in order to improve body condition in preparation for another reproductive cycle. In addition, there is no way to determine when the individuals first started foraging after the breeding season fast. For many of them, while their first sightings within the study area covered by the MICS have been recorded for each year, there is no way to know if, or for how long, they have been foraging in other areas of the Gulf. So, samples taken at the 'beginning' and the 'end' of the feeding season here, do not necessarily represent the 'beginning' or the 'end' of the season for all whales.

Using Aerial Photogrammetry and LSSAI

Morphometric data were obtained from images collected using a UAV. These were used to calculate a condition index that aimed to capture the variation in width across the body of the individuals. It was assumed that changes in width, and therefore girth of the animals, reflect changes in body fat reserves. The flat dorsal surface area of the most variable parts of the body was estimated for each individual, and used for the calculation of the Length Standardised Surface Area Index (LSSAI). The LSSAI was calculated for a total of 37 animals of all different sex, age and reproductive classes. Of these individuals, 21 were also biopsied and therefore had matched blubber cortisol data for comparison. This was a comparatively small dataset collected over the beginning of the feeding season, and no relationships were individually significant but some trends in the data were seen following model selection. The pregnant females were in the best body condition, with the largest LSSAIs, and lactating females in the poorest, with the smallest LSSAIs, as would be expected. There was considerable overlap suggesting that the variation in measurements within this small sample size was not enough to clearly distinguish between reproductive classes. Overall, males had a larger LSSAI than females which was unexpected as females are known to be larger in size than males (Clapham, 1996), but this suggests that males are able to deposit larger energy reserves.

There was no significant relationship between blubber cortisol concentrations and the LSSAI. Individuals with larger energy reserves were expected to have lower cortisol concentrations, as blubber cortisol concentrations were negatively correlated with morphometric indices of condition in balaenopterids in Chapter 3. The positive, but non-significant, correlation seen here could be a result of the small range in the LSSAI estimates between individuals compared to the morphometric measurements taken from the stranded individuals in Chapter 3. Future work should aim to also image and sample both males and females on the breeding grounds. The greatest range in body condition estimates could be obtained for comparison. Depending on the stage of gestation, the shape of females may also change. Changes in body shape throughout pregnancy should not be confounded with changes in energy reserves. Longitudinal images of pregnant females through the early, middle and late stages of gestation would help to better take potential changes in body shape into account.

To overcome the challenge of obtaining direct morphometric measurements from free-ranging cetaceans, the use of photogrammetry has become increasingly popular. Aerial photogrammetry appears to be the most promising method of image analysis for cetaceans, and has received the most attention with recent advances in UAV technology. Newly developed UAV methods are particularly popular as information about allometric relationships are not required. In addition, the full dorsal surface of the body can be imaged, and the high costs of taking aerial photographs from fixed-winged aircrafts and helicopters have been removed. The use of UAVs has thus made aerial photogrammetry techniques much more accessible. Different morphometric measurements from UAV images of cetaceans have been collected from a number of species (Christiansen et al., 2016; Christiansen et al., 2018; Durban et al., 2015; Durban et al., 2016), and these body dimension estimates have also been used to assess body condition (Christiansen et al., 2016; Christiansen et al., 2018; Fearnbach et al., 2018). It is expected that with a larger sample size of individuals, especially between feeding and breeding seasons, greater differences in body condition estimates would be observed with the LSSAI method used here. When coupled with endocrine profiling to determine reproductive status, this could be a valuable tool to measure individual and population health.

Conclusions and Future Directions

Our results demonstrate variation in hormone concentrations at a population level over time, highlighting the importance of longitudinal studies and linking endocrine measures with observational data. Pregnancy rates in this population appear to be very low, and targeted sampling of known, adult females would help to improve these estimates and assess reproductive success rates as well. There is evidence of inter-annual differences in measured cortisol concentrations that correlate with the estimated pregnancy rates. The variability in the cortisol concentrations suggests different physiological states in terms of animals either going through lipolysis or lipogenesis, and therefore provides a ‘snap shot’ of tissue metabolism at the time of sampling. Depending on how long the individuals have been on the feeding ground, and how successful their foraging has been, their physiological state will likely change. The addition of environmental data on primary productivity, prey abundance and sea ice extent, for example, could be added to these results to better investigate the drivers of inter-annual changes in cortisol concentrations and pregnancy rates. Taken together, the results of this study provide preliminary results to improve our understanding of the reproductive biology and physiology of humpback whales on their feeding grounds, which will assist monitoring, management and conservation efforts in the future.

Compared with conventional aircraft, UAVs are less expensive and safer to operate, and can be operated in more remote regions. This inexpensive and non-invasive approach provides a valuable tool to monitor the health of cetacean populations because it is applicable across different species and different habitats. Further work should prioritise how measurement errors can be quantified and incorporated into analyses of body condition estimates. Further work in this field should also prioritise the development of a standardised condition index for cetaceans from morphometric data collected by UAV image analyses to allow for comparison with other populations.

CHAPTER 6: INVESTIGATING THE PROTEOME OF CETACEAN BLUBBER TISSUE

Dr. Catherine Botting at the Biomedical Sciences Research Complex in the School of Chemistry at the University of St Andrews performed the protein identification part of this work using nanoflow Liquid Chromatography Electrospray Ionisation in tandem with Mass Spectrometry.

The harbour porpoise data analysis and discussion part of this chapter has been published in Conservation Physiology (2018).

Kershaw, J.L. Botting, C.H. Brownlow, A. Hall, A.J. 2018. Not just fat: investigating the proteome of cetacean blubber tissue. *Conservation Physiology*. 6;1.

1. ABSTRACT

Mammalian adipose tissue is increasingly being recognized as an endocrine organ involved in the regulation of a number of metabolic processes and pathways. It responds to signals from different hormone systems and the central nervous system, and expresses a variety of protein factors with important paracrine and endocrine functions. This study presents a first step towards the systematic analysis of the protein content of cetacean adipose tissue, the blubber, in order to investigate the kinds of proteins present and their relative abundance. Full depth blubber subsamples were collected from dead-stranded harbour porpoises (*Phocoena phocoena)* (n = 21), and remotely obtained, shallow, biopsy samples were collected from live minke whales (*Balaenoptera acutorostrata*) (n = 10) to assess the feasibility and reliability of applying proteomic methods to different sample types across species. Three total protein extraction methods were trialled. The highest total protein yields with the lowest extraction variability were achieved using a RIPA cell lysis and extraction buffer based protocol. Extracted proteins were separated using 1D Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), and identified using nanoflow Liquid Chromatography Electrospray Ionisation in tandem with Mass Spectrometry (nLC-ESI-MS/MS).

A wide range of proteins were identified across the two species (n = 409), and classed into ten functional groups, the most abundant of which were involved in cell function and metabolism, immune response and inflammation, and lipid metabolism. While a number of different individual proteins were identified in the extracts from the two species, the overall patterns in terms of the relative abundance of each functional group were very similar. These proteins likely originate both from the various cell types within the blubber tissue itself, and from the circulation. They therefore have the potential to capture information on the cellular and physiological stresses experienced by individuals at the time of sampling. The importance of this proteomic approach is two-fold: Firstly, it could help to assign novel functions to marine mammal blubber in keeping with current understanding of the multi-functional role of adipose tissue in other mammals. Secondly, it could lead to the development of a suite of biomarkers to better monitor the physiological state and health of live individuals though remote blubber biopsy sampling.

2. INTRODUCTION

White adipose tissue, the predominant type of adipose tissue in most mammals, is made up of adipocytes containing usually a single lipid droplet and a non-centrally located nucleus. Adipose tissue provides an excess fat storage site in the form of triglycerides that are metabolised depending on fluctuating energy requirements (Ahima and Flier, 2000). As well as its importance as a highly metabolically active energy storage organ, adipose tissue in mammals is increasingly being recognised as an endocrine organ in its own right through the secretion of proteins that are actively involved in energy homeostasis and the regulation of neuroendocrine, autonomic and immune functions (Ahima and Flier, 2000; Gimeno and Klaman, 2005; Kershaw and Flier, 2004). To date, however, the potential role of blubber, as the main white adipose tissue deposits in marine mammals, as an endocrine organ in terms of contributing to whole body metabolism has yet to be explored.

As well as the fully differentiated adipocytes themselves, mammalian adipose tissue is made up of numerous other cell types including fibroblasts, pre-adipocytes, macrophages and other immune cells, endothelial cells and smooth muscle cells, connective tissue matrix, nerve tissue and blood vessels (Frayn et al., 2003; Gimeno and Klaman, 2005). These components function as a structured whole which is known to express and secrete a variety of bioactive peptides which include proteins, metabolites and hormones, and are collectively known as adipokines (Trayhurn and Wood, 2004). These can act at both a local and systemic level (Kershaw and Flier, 2004; Trayhurn and Wood, 2004), and are derived from both adipocyte and non-adipocyte fractions of the tissue (Gimeno and Klaman, 2005). Considerable metabolic heterogeneity has been documented among various adipose deposits with the secretion of these bioactive peptides that include the sex steroids (eg. androgens and estrogens), glucocorticoids (e.g. cortisol and corticosterone), complement factors (e.g. adipsin and acylation-stimulating protein), proinflammatory cytokines (e.g. tumor necrosis factor α and interleukin 6), growth factors (e.g. transforming growth factor-β) and adipokine hormones (eg. leptin and adiponectin). While most of these products act through paracrine or autocrine mechanisms in the adipose tissue itself, others, such as leptin, are released into circulation and are capable of influencing other target organs (Ahima and Flier, 2000).

In addition to these secreted signals, adipose tissue expresses numerous receptors that allow it to respond to signals from a variety of hormone systems as well as the central nervous system. Its functions are therefore regulated by multiple external influences, such as the rate of blood flow into the tissue and thus the delivery of substrates and hormones in the plasma, autonomic nervous system activity (Frayn et al., 2003), and localised signalling and feedback loops within the tissue itself (Frühbeck et al., 2001). These networks of adipose tissue signalling pathways enable the organism to adapt to a wide range of metabolic challenges including starvation, stress, infection and periods of energy excess (Frühbeck et al., 2001). Through this network, adipose tissue is integrally involved in coordinating a variety of biological processes including the regulation of appetite and energy balance, immune system function, insulin sensitivity, angiogenesis, inflammation and the acute-phase response, blood pressure, nutrient transport and lipid metabolism, and haemostasis (Trayhurn and Wood, 2004).

It is therefore now clear that adipose tissue is a complex and highly active metabolic and endocrine organ with fat cells playing an active role in modulating their own metabolism (Frühbeck et al., 2001; Kershaw and Flier, 2004; Trayhurn and Wood, 2004). The presence and concentrations of certain proteins and their metabolites in the tissue as well as circulating concentrations in the blood stream can therefore provide information on various processes and metabolic challenges. For example, circulating concentrations of the adipokines leptin and adiponectin, are positively and negatively correlated with adiposity respectively (for review see (Gimeno and Klaman, 2005). More generally, protein screening and identification are commonly used as diagnostic markers in medicine to detect disease and perturbations to metabolic pathways (Steffen et al., 2016). Thus, both adipokines and other metabolic factors within the tissue are of interest to understand whole tissue function, the regulation of whole body metabolism and overall systemic health.

I aimed to investigate whether cetacean blubber could show equivalent pleiotropic functions to the adipose tissue in terrestrial animals by starting to identify some of the main protein components in the tissue. To achieve this, I had two main aims. Firstly, to reliably extract and separate the protein components in cetacean blubber. Secondly, to identify these components and link their presence to potential blubber functions. This process was split into three main stages. Firstly, method development for the extraction and separation of total protein from cetacean blubber tissue for the first time. Secondly, the identification of the extracted proteins by mass spectrometric analysis of protein digests and searching against a protein database for matches to homologous proteins from other vertebrate species. Thirdly, an investigation of which types of proteins were present and most represented in the extracts to make inferences about potential blubber functions and links to metabolic state.

Two cetacean species, the harbour porpoise (*Phocoena phocoena*) and the minke whale (*Balaenoptera acutorostrata*) were studied. Full depth blubber samples from stranded harbour porpoises were used for protein extraction method development, and to assess the range of proteins present through the full blubber depth. Minke whale biopsy samples collected from live animals were used to assess the applicability of this method to remotely obtained, shallow, biopsy samples collected from different species. The separation and identification of proteins in blubber tissue from different species are important steps in establishing a database of baseline, identifiable proteins, and the subsequent use of various proteins as biomarkers of health and condition in live animals with otherwise limited sampling opportunities.

3. METHODS

3.1. Method Development and Optimisation

3.1.1. Sample Collection and Preparation

Full-depth skin, blubber, and underlying muscle samples were collected from dead harbour porpoises by the Scottish Marine Animal Strandings Scheme (SMASS) between 2013 and 2015. Only freshly dead animals, specifically those that either stranded alive or had recently died, and thus showed no evidence of bloating and decomposition, were used in order to investigate blubber proteins that had not been subject to extensive degradation and metabolism after death. Samples showed no evidence of trauma or bruising as blood in the tissue would disproportionally represent proteins in circulation rather than proteins present in the blubber matrix itself. Samples were collected from the dorsal area immediately caudal to the dorsal fin. As previously described, this site was chosen as this area is typically sampled through remote biopsy of free-ranging individuals and therefore has the most relevance for investigating the potential use of these total protein extraction methods on samples collected from live animals. A total of 24 samples were used from 21 individuals that were a mixture of both males (n = 10) and females (n = 11), and adults (n = 12) and juveniles (n = 9). The cause of death was determined following post-mortem examination to classify individuals as either acute or chronic cases (as described in Chapter 1). For full details of the samples used for analysis see Table 6.1.

Samples were collected and frozen in individual plastic vials at -20^{0}C prior to analysis. For the total protein extraction, sub-samples were taken on ice while the tissue was still frozen, and care was taken to remove all skin and muscle from the blubber. Full depth subsamples were used for total protein extraction in order to investigate the proteins through all layers of the tissue.

3.1.2. Total Protein Extraction Methods

Protein extraction from a tissue typically involves three stages: 1) tissue and cell disruption through homogenisation 2) precipitation of the protein fraction of the homogenate into a pellet form 3) re-suspension of the protein pellet into solution for quantification and downstream applications.

In a comparative study of different detergent-free protein extraction protocols using these three stages, the most suitable method for the extraction of white adipose tissue proteins from a wide range of cellular and structural compartments was a de-lipidation protocol based on the Bligh and Dyer method (1959) (Sajic et al., 2011). The optimal tissue and cell disruption part of the protocol described by Sajic and colleagues was replicated here using blubber samples collected from harbour porpoises. Then, two different protein precipitation methods were trialled. A protein precipitation method using a methanol-chloroform solution adapted by Friedman and colleagues (Friedman et al., 2009) for the recovery of proteins in dilute solution in the presence of detergents and lipids (Wessel and Flügge, 1984), was trialled first (method 1). A second protein precipitation method using a trichloroacetic acid (TCA)-acetone solution was also trialled which aggressively removes non-protein compounds (Wu et al., 2014) (method 2).

TCA is often used for precipitation as it is effective at low concentrations (Wu et al., 2014). The sample volume therefore does not increase dramatically, and the protein concentration remains high which increases the efficiency of the precipitation (Wu et al., 2014).

Finally, a simpler extraction method using radio immunoprecipitation assay (RIPA) cell lysis and extraction buffer was trialled to assess if fewer sample processing stages, without the precipitation of protein into a pellet and resuspension into solution, results in a higher protein yield and less extraction variability (method 3). RIPA cell lysis buffer is highly effective for protein extraction from a variety of cell types because it contains three non-ionic and ionic detergents. One disadvantage, however, is that this detergent formulation is incompatible with certain downstream applications compared to other lysis reagents, and there is no possibility to suspend the extracted protein in a different buffer for further analysis. For a summary of the three sample processing methods (methods 1, 2 and 3) with their different stages see Fig 6.1.

3.1.2.1. Method 1: Methanol-Chloroform Precipitation with Methanol Pellet Wash

Full depth, duplicate blubber subsamples from eight individuals (n=16) were accurately weighed (0.4 - 0.6g) and homogenised with 0.5mL of isolation medium (50mM Tris, 150mM NaCl, 0.2mM EDTA and 10ug/mL protease inhibitors), and 1.875mL of 1:2 chloroform/methanol. Samples were then placed on ice for 15 minutes with thorough mixing every three minutes. 625ul of chloroform and 625ul of deionised water were added to the sample. The sample was vortexed and centrifuged at 800g for 5 minutes at 4^0C. This creates three layers, a lower lipid layer, an upper protein layer and a protein disc between the two. The upper protein phase and the protein disc were collected and transferred to a new tube.

Three volumes of water, four volumes of methanol and one volume of chloroform were added to the solution and vortexed vigorously for 5 min so that only one phase was visible in the tube. The mixture was centrifuged at 4700g at 4^0C for 30 min. This forms two immiscible layers with the protein precipitate at the interface between the two. The water /methanol top layer was removed, and care was taken not to disturb the interface as often precipitated proteins do not form a visibly white surface. Another four times the volume of methanol was added to wash the precipitate, and the mixture was again vortexed vigorously for 5 min. The mixture was centrifuged at 4700g at 4^0C for 45 min. This forms a protein pellet in the bottom of the tube. The supernatant was removed and the pellet was dried under nitrogen at room temperature. Care was taken not to over dry the pellet causing it to become flaky and stick to the centrifuge tube which reduces the resuspension efficiency. Finally, the pellet was resuspended in 500ul of SDS/Tris (0.1% SDS in 40mM Tris).

3.1.2.2. Method 2: TCA-Acetone Precipitation with Acetone Pellet Wash

Using another 16, different, full-depth blubber subsamples from eight individuals, the same protocol as previously described was followed up to the collection of the upper protein phase and the protein disc, and its transfer to a new tube. 10% TCA in acetone solution was added to the sample with a ratio of 1:3 of sample to TCA-acetone solution. The sample was frozen overnight at -20^0C. The sample was allowed to warm to room temperature and then centrifuged

at 4700g at 4^0C for 45 minutes the following day. Proteins form a pellet in the bottom of the tube. The pellet was washed twice by adding 1mL of ice cold acetone which was then discarded. Any remaining acetone following the final wash was evaporated under nitrogen at room temperature. Care was taken not to over-dry the protein pellet, and the pellet was resuspended in 500µl of SDS/Tris. Of the two protein precipitation methods, using a TCA-acetone solution showed least total protein inter-assay variability (Fig. 6.2). For this reason, alterations were made to improve this protocol in an attempt to firstly, further clean the extract, and secondly, to improve protein pellet resuspension by altering the pellet washing procedure.

Alteration 1: TCA-Acetone Precipitation with Butanol De-Lipidation and Acetone Pellet Wash

As cetacean blubber tissue has such a high lipid content, an extra butanol de-lipidation step was added before the precipitation of the protein pellet in an attempt to further 'clean' the extract (Zhao and Xu, 2010). Another 16 blubber subsamples were taken, as previously described, from the same individuals used for the TCA-acetone precipitation protocol. Following the collection of the upper protein phase and protein disc (after homogenisation and centrifugation), butanol was added in a ratio of 1:3 sample to butanol. This was then centrifuged at 1750g at 10^0C for 10 min and the upper phase containing any remaining lipids was removed and discarded. 10% TCA in acetone was added to the lower phase, and the precipitation and pellet wash continued as described above. The final protein pellet was resuspended in 500ul of SDS/Tris.

Alteration 2: TCA-Acetone Precipitation with Water Wash

Poor resuspension of the protein pellet in the SDS-Tris Buffer could result in under-estimates of total protein content if some pellets resuspend less efficiently than others. The effect of a different pellet wash was tested by using de-ionised water instead of acetone for the final wash stage. This was an attempt not to dehydrate the pellet, and therefore make its resuspension back into solution more efficient. As before, 16 blubber subsamples were taken from the same eight individuals used for the TCA-acetone precipitation protocol. Specifically, following the precipitation of the proteins in TCA-acetone, care was taken to discard all TCA-acetone solution, and the pellet was washed by adding 1mL of deionised water. It was vortexed briefly, and then centrifuged again for 5 min at 4700g at 4^0C. The supernatant was again discarded, and the pellet washed a second time with deionized water. Care was taken not to disturb the washed pellet that was then re-suspended in 500µl of SDS/Tris.

3.1.2.3. Method 3 - RIPA Lysis and Extraction Buffer

Another 16, full depth blubber samples were used for analysis from 8 individuals. The subsamples were accurately weighed (0.1 ± 0.01g). The frozen tissue was placed on ice in a 1.5mL low protein binding micro-centrifuge tube and 200µl RIPA lysis and extraction buffer (Thermo Fisher Scientific) with 2X concentration of protease inhibitors (Pierce Protease Inhibitor Mini Tablets) was added to the tissue. The samples were homogenised with a pestle designed for a 1.5mL tube for two minutes to give a cloudy white solution and small piece of connective tissue that could not be further homogenised. The samples were replaced on ice and

then centrifuged at 4700g at 4^0C for 15 minutes before being replaced on ice. The supernatant, the hardened lipid containing fraction, was pushed to one side, and the protein containing infranatant was removed and placed into a clean, low protein binding microcentrifuge tube. Care was taken not to disturb the insoluble/unhomogenised connective tissue pellet. The protein fraction was centrifuged, as before, as a second de-lipidation step, and replaced on ice. Again, the infranatant was removed and placed in a clean, low protein binding microcentrifuge tube taking care not to disturb any remaining lipid supernatant and insoluble pellet. This solution was used for further analysis.

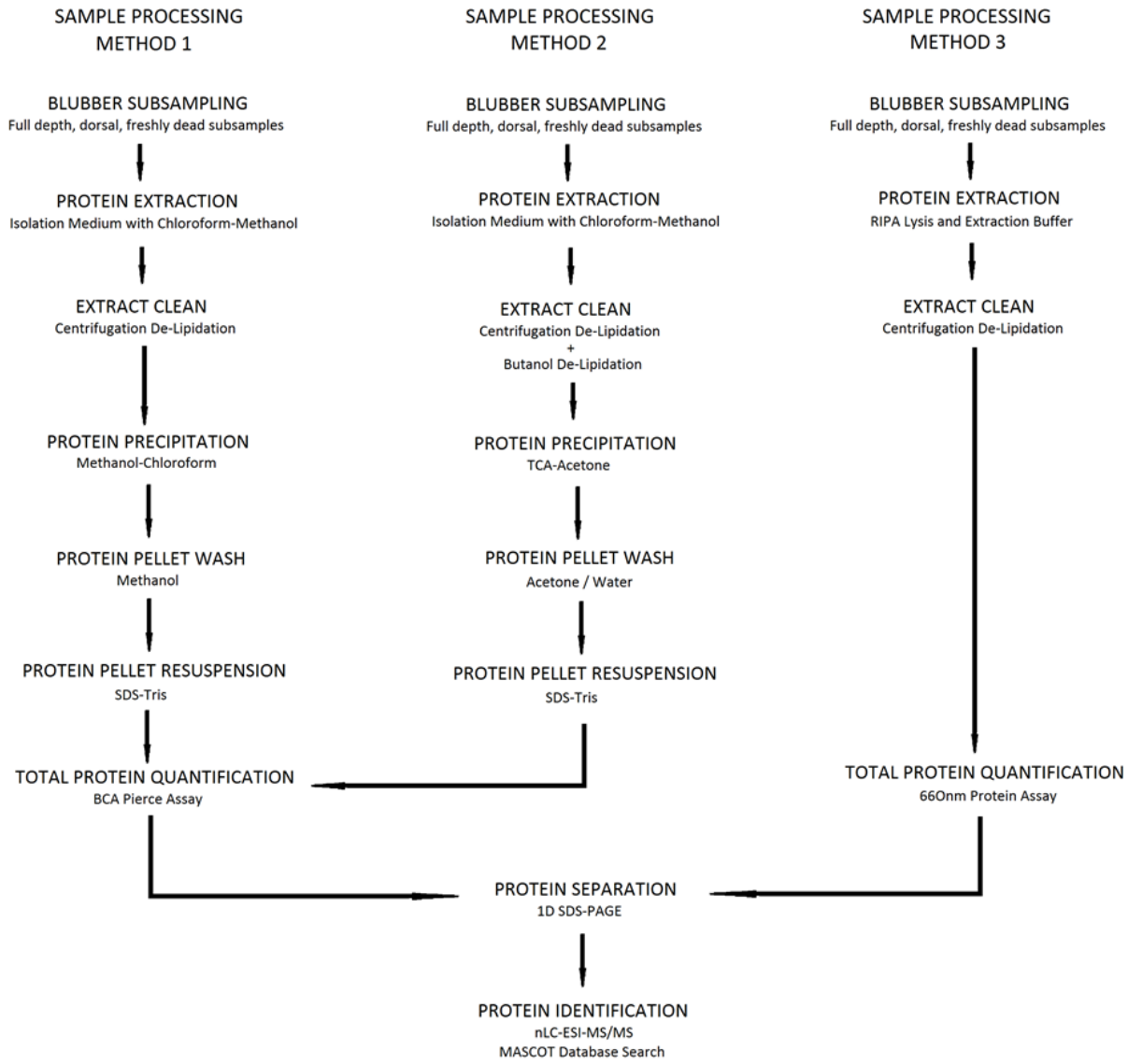

Fig. 6.1. Workflow showing the sample processing methods for protein extraction, quantification, separation and identification. Sample processing methods 1 and 2 involve various extract cleaning, protein precipitation and protein pellet wash stages. Sample processing method 3 involves fewer processing stages. The extracts using all three methods were used for downstream analyses; protein quantification, separation and identification.

3.1.3. Total Protein Quantification Assays

To assess the effectiveness of the different protein extraction methods, two different commercially available total protein quantification kits were used to measure the protein in the blubber extracts. Firstly, a Pierce™ BCA Protein Assay Kit (23225, Thermo Scientific, Rockford, USA) was used to quantify the total protein in the extracts resuspended in SDS-Tris using the chloroform-methanol and TCA-acetone precipitation methods. The assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colourimetric detection and quantitation of total protein. This assay has a broad working range of protein concentrations between 20-2000µg/mL. A series of dilutions of known concentrations of bovine serum albumin were prepared using the same SDS/Tris solution used to re-suspend the protein extracts, and assayed alongside the blubber extracts to determine their protein concentrations based on the standard curve produced using a four parameter log-logistic model. As blubber extract sample volumes are small, the microplate procedure was chosen for this analysis and assays were carried out following the kit instructions with the absorbance of the samples read at 560nm. Each extract was assayed in duplicate on every plate, a negative control of SDS/Tris was run on every plate, and total protein concentrations were reported as µg per wet weight of the sample. Each extract was assayed on three different plates in order to determine the inter-assay coefficient of variation (% CV), and the average concentration measured across the three plates was used as the final estimated total protein content of a sample.

Secondly, a Pierce™ 660nm Protein Assay (22662, Thermo Scientific, Rockford, USA) was used to quantify the total protein in the extracts processed in RIPA lysis and extraction buffer. This is a quick, ready-to-use colourimetric method for total protein quantitation, and is compatible with high concentrations of most detergents and reducing agents, and is therefore compatible with the RIPA buffer supplemented with 2X protease inhibitors used here. The blubber extracts were prepared following the assay instructions for cell lysates in RIPA buffer by adding Triton™ X-100 to a final concentration of 0.8% to the sample before performing the assay. The absorbance of the samples was read at 660nm. A series of dilutions of known concentrations of bovine serum albumin were used to generate a standard curve. A negative control of RIPA with 2X protease inhibitors was used as the zero standard on each plate. The absorbance measured for this negative control was used to correct the absorbance of the standard curve measurements which were then used to produce a 4 parameter log-logistic model and calculate the protein concentrations in the samples. The assay was performed following the microplate protocol and the absorbance read at 660nm. Each extract was assayed in duplicate and total protein concentrations are reported as µg per wet weight of the sample. Each extract was assayed on two different plates in order to determine the inter-assay CV (% CV), and the average concentration measured across the two plates was used as the final estimated total protein content of a sample.

3.1.4. 1D SDS-PAGE Protein Separation

The final stage of the method development was to optimise protein separation and visualisation using 1D SDS-PAGE (Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis) which separates denatured proteins based on their molecular weight. The XCell SureLock® Mini-Cell (Life Technologies, UK) gel electrophoresis unit was used to run 4-12% NuPAGE Bis-Tris mini gels (8.0cm x 8.0cm x 1.0mm) (NP0321BOX, Thermo Fisher Scientific, Paisley, UK) with a wide protein separation range of 3.5 kDa to 160 kDa. The recommended sample and running buffers for de-natured proteins as listed by the manufacturers were used to run the gels: 1x NuPAGE LDS Sample Buffer (NP0007, Thermo Fisher Scientific, Paisley, UK) and 1x NuPAGE MES SDS Running Buffer (NP000202, Thermo Fisher Scientific, Paisley, UK). Standard running conditions as recommended by the manufacturers were also chosen: a constant voltage of 200V and a current of 125mA for 35 minutes. A wide protein range ladder, Invitrogen™ Novex™ Mark 12™ Unstained Standard (LC5677, Fisher Scientific, USA) was run on each gel. The blubber extracts resuspended in SDS-Tris were run undiluted on the gels while the extracts in RIPA buffer were run at ½ and $^1/_3$ dilutions as the protein content of these extracts was much higher. NuPAGE LDS Sample Buffer (5µl of 4X) was added to 20µl of each of the blubber extracts (neat or diluted). 10µl of each of these were loaded onto the gel. Upon completion of the run, the gels were removed from the cassettes and rinsed in de-ionised water briefly to remove residual SDS that affects the staining procedure. The gels were stained in 500mL of Bio-Safe Coomassie Stain (1610786, Bio-Rad, UK) for 1.5 hours with gentle agitation on a platform shaker. This stain is fast, simple, sensitive and convenient in it's ready to use and non-hazardous solution. The gels were then removed from the stain, rinsed and de-stained in de-ionised water overnight. The de-stained gels were photographed on a white background using a BioDoc-It™ Imaging System (Ultra-Violet Products Ltd, Cambridge, UK).

3.1.5. Quality Assurance / Quality Control

Total protein extraction method comparison studies use various approaches to assess the protein extraction efficiencies for particular tissues. These approaches include assessing total protein yield, the distribution of molecular weights of extracted proteins separated using 1D SDS-PAGE, the reproducibility of protein bands with minimal streaking and background using 1D SDS-PAGE, the presence of specific protein markers from different cellular compartments using Western Blot techniques, the presence / absence of individual protein spots using 2D SDS-PAGE, and the reproducibility of protein spot patterns using 2D SDS-PAGE in both animal (Cilia et al., 2009; Jiang et al., 2007; Panchout et al., 2013; Sajic et al., 2011) and plant (Natarajan et al., 2005; Sheoran et al., 2009) studies. In keeping with such studies, here, the performance of the different extraction protocols in terms of their ability to efficiently and consistently extract protein from blubber tissue was assessed in four ways: by (i) measuring total protein yield; (ii) measuring extraction variability between duplicate extracts of the same sample extracted and assayed in tandem; (iii) measuring assay variability in terms of the protein measured in the same sample over multiple assays; (iv) and visually inspecting the molecular weight distribution, the number and reproducibility of protein bands separated by 1D SDS-PAGE.

All statistical analyses were performed using the statistical package, R, version 3.1.3 (R Core Development Team, 2015). A one-way analysis of variance (ANOVA) was used to compare between the mean protein yields of all extracts processed using the five different method variations (method 1, three alterations of method 2, and method 3) (Fig. 6.2). The extraction variability for each sample extracted in duplicate was calculated as a % coefficient of variation, and again, a one-way ANOVA was used to compare between the mean % extraction variability across the 5 method variations (Table 6.2). The inter-assay coefficients of variation for samples assayed multiple times across different plates were also assessed using a one-way ANOVA to determine differences in the repeatability of the measurements of the same extracts (Table 6.2). Finally, the 1D SDS-PAGE gels were assessed for the range of molecular weights of the bands that were separated, the number of protein bands separated and the consistency with which these bands appeared in multiple extracts and across multiple gels. For a comparative example of 1D gel images of extracts processed using the different methods, see Fig. 6.3.

3.2. Application to Minke Whale Biopsies

In order to investigate the use of the most appropriate total protein extraction method on biopsy samples from live animals of a different species, remotely obtained, shallow, biopsy samples collected from minke whales by the Mingan Island Cetacean Study in Quebec, Canada in 2013, were used for comparative analyses. Sampling occurred from June to September in the Jacques Cartier Passage of the Gulf of St. Lawrence (49° 36' N, 64° 20'W). Biopsies were collected from rigid-hulled, inflatable boats using a crossbow and hollow-tipped (40 mm in length and 8 mm in diameter) arrow system from the dorsal and flank areas of the individuals. All samples were stored in glass vials and on ice immediately after collection, and subsequently at −20°C until analysis. These animals were sexed genetically using the skin (Palsbøll et al., 1992), and were all females. Sub-samples of these biopsies were taken, and total protein was extracted using method 3 as described above. Total protein in the extracts was quantified using the Pierce™ 660nm Protein Assay, and then separated and visualised using the same 4-12% NuPAGE Bis-Tris mini gel running, staining, de-staining and imaging processes as described above.

3.3. Protein Identification

A total of 36 protein bands were excised from five harbour porpoise individuals run on 4 different 1D SDS-PAGE gels. A total of 11 protein bands were excised from four minke whale individuals run on the same 1D SDS-PAGE gel. All bands were stored in individual microcentrifuge tubes at 4 - 8^0C before subsequent protein identification. The bands were from blubber extracts processed using methods 2 (both alterations) and 3 (Table 6.1), and covered the full size range of separated proteins from the largest ones of more than 200kDa, down to the smallest bands visible at ~10kDa. The darkest stained bands (indicating highest protein concentration) were chosen, which allowed the clear visual separation of the band on the gels. In order to capture the full range of proteins that could be present in the tissue, bands from harbour porpoises were all of different molecular weights and were chosen from a mixture of

males and females, and from adults and juveniles with varying causes of death (Table 6.1). For the minke whale samples, in order to capture the full range in proteins present, bands from individuals sampled early in the feeding season (June) and late in the feeding season (September) were used for analysis. All bands were analysed using nanoflow Liquid Chromatography Electrospray Ionisation in tandem with Mass Spectrometry (nLC-ESI MS/MS) of in-gel trypsin digests.

The excised gel band was cut into 1mm cubes. These were then subjected to in-gel digestion, using a ProGest Investigator in-gel digestion robot (Genomic Solutions, Ann Arbor, MI) using standard protocols (Shevchenko et al., 1996). Briefly, the gel cubes were destained by washing with acetonitrile and subjected to reduction and alkylation before digestion with trypsin at 37°C. The peptides were extracted with 10% formic acid and concentrated down 20 times using a SpeedVac (ThermoSavant). The peptides were then injected on an Acclaim PepMap 100 C18 trap and an Acclaim PepMap RSLC C18 column (ThermoFisher Scientific) using a nanoLC Ultra 2D plus loading pump and nanoLC as-2 autosampler (Eksigent). The peptides were eluted with a gradient of increasing acetonitrile, containing 0.1 % formic acid (5-40% acetonitrile in 6 min, 40-95 % in a further 2.5 min, followed by 95% acetonitrile to clean the column, before re-equilibration to 5% acetonitrile). The eluate was sprayed into a TripleTOF 5600+ electrospray tandem mass spectrometer (Sciex, Foster City, CA) and analysed in Information Dependent Acquisition (IDA) mode, performing 250 msec of MS followed by 100 msec MSMS analyses on the 20 most intense peaks seen by MS. The MS/MS data file generated via the 'Create mgf file' script in PeakView (Sciex) was analysed using the Mascot search algorithm (Matrix Science), against the NCBInr database (Apr and Oct 2015 and Aug 2016) with no species restriction (65519838 to 93482448 sequences), trypsin as the cleavage enzyme, and carbamidomethyl as a fixed modification of cysteines and methionine oxidation as a variable modification. The peptide mass tolerance was set to 20 ppm and the MSMS mass tolerance to ± 0.05 Da.

A protein was accepted as identified if it had 2 or more peptides with Mascot Ion Scores above the Identity Threshold ($p<0.05$), and, for those proteins identified by only 2 peptides, the MSMS spectral assignments fulfil the criteria described in Jonscher, 2005 (Jonscher, 2005). The sequences matched homologous vertebrate proteins.

Table 6.1 - Blubber samples used for total protein extraction, separation and protein identification. 'Gel bands' indicate the number of individual protein bands excised from various gels that were analysed using nLC-ESI MS/MS. 2* - A mixture of gel bands were excised from samples processed using both alterations of method 2 were used for protein identification.

Species	Processing Method	Animal ID	Sex	Age Class	Cause of Death Class	Gel Bands
Harbour porpoises	1	M343/13	F	J	Acute Trauma	
		M396/13	F	A	Acute Trauma	
		M134/14	F	A	Acute Trauma	
		M055/14	M	A	Acute Trauma	
		M307/14	M	A	Acute Trauma	
		M355/14	M	J	Acute Trauma	
		M061/15	F	J	Chronic Debilitation	
		M020/15	F	A	Chronic Debilitation	
	2*	M072/13	M	J	Acute Trauma	2
		M018/13	M	A	Chronic Debilitation	
		M060/13	M	A	Acute Trauma	
		M040/14	M	J	Chronic Debilitation	
		M028/14	M	J	Acute Trauma	8
		M027/14	M	A	Acute Trauma	
		M068/14	F	J	Chronic Debilitation	8
		M147/14	F	A	Chronic Debilitation	
	3	M343/13	F	J	Acute Trauma	
		M315/13	F	A	Acute Trauma	
		M373/13	F	J	Acute Trauma	
		M265/13	F	A	Chronic Debilitation	
		M134/14	F	A	Acute Trauma	
		M307/14	M	A	Acute Trauma	14
		M131/15	M	A	Acute Trauma	4
		M144/15	F	J	Acute Trauma	
Minke whales	3	AC13001	F	A	NA	4
		AC13002	F	A		
		AC13005	F	A		
		AC13006	F	A		3
		AC13012	F	A		2
		AC13016	F	A		
		AC13017	F	A		2
		AC13025	F	A		
		AC13026	F	A		
		AC13027	F	A		

4. RESULTS

4.1. Method Development and Optimisation

There was considerable variation seen in both the total protein yields, and the extraction variability between duplicate subsamples (Fig. 6.2). Of the two precipitation methods, the TCA-acetone precipitation method was chosen for further development. An extra butanol de-lipidation step and pellet wash with water were trialled in an attempt to further optimise the method in order to improve consistency. However, even with these changes, the total protein yield was still poor, and there were no statistically significant differences between the overall protein yield between the four precipitation methods (method 1 and the 3 variations of method 2) (ANOVA; df = 3, F = 2.33, p = 0.08) (Fig. 6.2a). The highest total protein yield, by at least an order of magnitude, was obtained using the third extraction method with RIPA cell lysis buffer which was significantly higher than all other extracts (ANOVA; df=4, F = 280.1, p < 0.0001) (Fig 6.2b). The extraction variability was high and was not statistically different between methods (ANOVA; df = 4, F = 1.13, p = 0.36), but extracts processed using method 3 had a lower mean extraction variability and a smaller range across duplicate samples (Table 6.2).

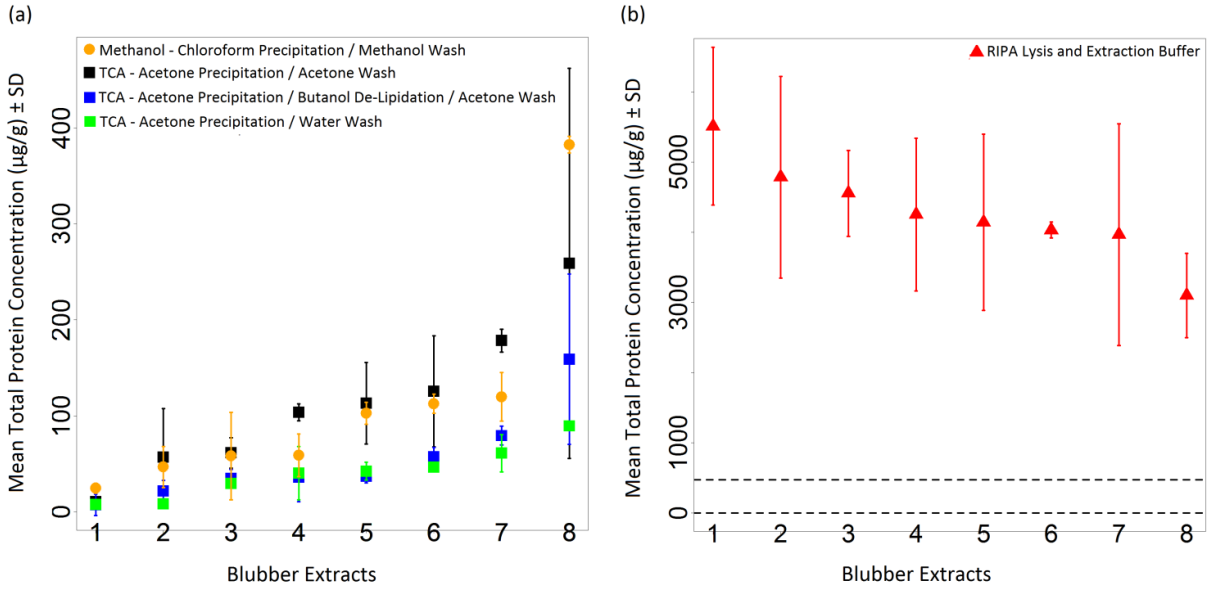

Fig. 6.2. Total protein yield in individual blubber extracts processed in duplicate using different sample processing methods 1, 2 and 3. a) Total protein concentrations measured in extracts processed using methods 1 and the three variations of method 2. b) Total protein concentrations measured in extracts processed using method 3. Overall, the total protein yield was an order of magnitude higher using this method although there was still high extraction variability. The horizontal dashed lines indicate the range of mean total protein concentrations measured using the other processing methods.

There was a wide range of inter-assay variability across all samples, particularly for methods 1 and 2, which were largely over the acceptable inter-assay % coefficient of variation threshold of 20%, based on general protocols for immunoassay validations (Grotjan and Keel, 1996) (Table 6.2). The extracts processed using method 1 had significantly higher inter-assay CVs than the other methods (ANOVA; $df = 4$, $F = 11.94$, $p < 0.001$), while the others were not significantly different to each other. There were also consistent trends of higher / lower protein concentrations between assays for method 1 (Supplementary Results, Fig. 1), suggesting that there is some change in protein resuspension or protein loss between repeated freeze-thaw cycles of the extracts. Three of the five extraction protocols generally gave low average intra-assay % CVs below the 10% threshold that was considered acceptable for three extracts assayed twice on each plate (Grotjan and Keel, 1996) (Table 6.2). As the total protein standard curves were all almost identical between different plates (Supplementary Results, Fig. 2), the high between and within assay variation measured here for the Pierce BCA assay is likely indicative that measurement error or artefacts due to the assay reagents and the assay process were not the cause of the variability. Instead, resuspension of the protein in solution was likely a problem for reliable and consistent measurement of the precipitated proteins.

Finally, visual inspection of the 1D SDS-PAGE gels showed that there was a wide range in molecular weights of the separated protein bands, with both more numerous and more consistent bands seen in the samples processed in RIPA cell lysis buffer (Fig. 6.3) For more example gel images, see Supplementary Results, Figures 3 and 4. This further supports the conclusion that method 3 is the most appropriate for replicable protein extraction from blubber tissue.

Table 6.2 – Summary of the total protein assay results for the three different extraction methods and alterations. (CV: coefficient of variation. SD: standard deviation)

	Extraction Methods 1 and 2				**Extraction Method 3**
	Methanol-Chloroform Protein Precipitation	**TCA-Acetone Protein Precipitation**			**RIPA Cell Lysis Buffer Extraction**
		Original: Acetone Wash	Alteration 1: Butanol De-lipidation	Alteration 2: Water Wash	
Total Protein Assay	Pierce BCA	Pierce BCA	Pierce BCA	Pierce BCA	Pierce 660nm
Minimum Protein Yield	14.4 ug/g	3.8ug/g	7.3 ug/g	7.7ug/g	3101.8 ug/g
Maximum Protein Yield	361.6ug/g	918.0ug/g	158.8 ug/g	89.3ug/g	5512.7 ug/g
Extraction CVs ($^{SD}/_{mean}$) x 100 duplicate protein extracts	28.4% (2.3 - 78.7%)	42.0% (6.7 - 90.0%)	50.6% (12.5 - 151.0%)	31.8% (2.1 - 68.8%)	21.9% (2.8 - 39.8%)
Inter-Assay CVs ($^{SD}/_{mean}$) x 100 different total protein plates	86.5% (47.8 - 133.1%)	42.5% (0.5 - 141.4%)	31.6% (4.1 - 103.9%)	23.4% (3.31 - 69.5%)	15.8% (1.5 - 64.9%)
Intra-Assay CVs ($^{SD}/_{mean}$) x 100 same total protein plate	0.7% (0.1 - 1.3%)	2.0% (1.2 - 3.3%)	28.7% (4.7 - 100.0%)	19.3% (2.5 - 105.6%)	4.5% (3.6 - 5.5%)

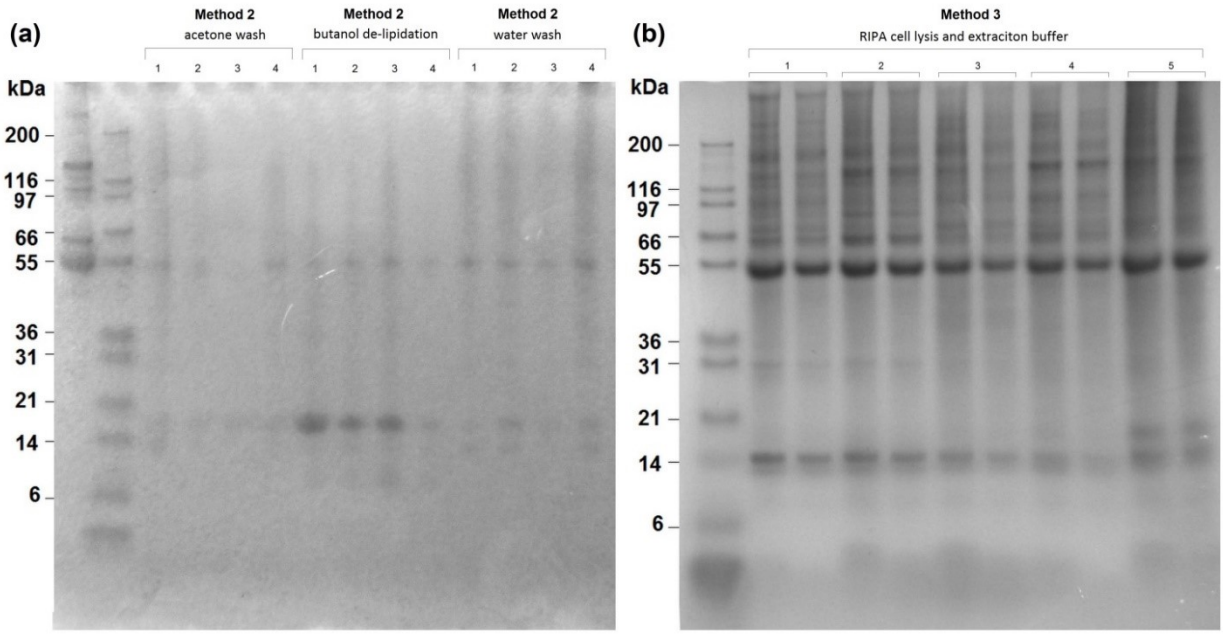

Fig. 6.3. 1D SDS-PAGE analysis of harbour porpoise blubber tissue extracts on 4-12% Bis-Tris gels stained with Bio-Safe Coomassie brilliant blue. (a) Protein extracts labelled 1 - 4 were extracted using TCA-Acetone precipitation with an acetone wash, TCA-Acetone precipitation with butanol de-lipidation, and TCA-Acetone precipitation with a water wash. (b) Protein extracts labelled 1-5, extracted in RIPA cell lysis buffer, and each diluted ½ and $^{1}/_{3}$.

4.2. Protein Identification

Harbour porpoises: In total, 295 proteins were identified across the 36 gel bands separated through 1D SDS-PAGE from 5 individuals. Many of the proteins, and protein fragments were identified across multiple bands from the same gel, and therefore did not show clear clustering around their expected molecular weight range. This was possibly due to protein degradation and / or some proteins being more abundant than others. The identified proteins were grouped into general subclasses firstly, based on their type, and secondly based broadly on their function using data from UniProt (http://www.uniprot.org) and a literature search. This resulted in the identification of proteins belonging to 5 main types: enzymes (proteins involved in the catalysis of various processes), immune proteins (proteins involved in the regulation of immune system function and activation as well as inflammation), carrier proteins (proteins that bind other factors for extracellular or intracellular transport), structural proteins (proteins responsible for the maintenance of cell shape and integrity as well as the extracellular matrix), and regulatory proteins (any other proteins involved in the regulation of other cellular processes) were grouped.

Proteins were then grouped into functional classes which resulted in the identification of 8 main groups: amino acid metabolism, lipid metabolism, tissue structure, cell structure, glucose homeostasis, biomolecule transport, immune response and inflammation and overall cell function and metabolism. As an indicator of relative abundance, the frequency with which each protein was identified across different gel bands and individuals was also recorded in order to

identify the kinds of proteins that appeared to be most abundant. Over half of all proteins identified were only seen once across all 36 gel samples analysed, while less than 5% of the proteins were seen more than 10 times. The top six most frequently identified proteins were haemoglobin, immunoglobins, serum albumin, fatty acid binding protein, myoglobin and annexins.

Proteins involved in amino acid metabolism, tissue structure, glucose homeostasis, biomolecule transport and cell structure were the least abundant of the protein functional groups, each making up less than 10% of all the identified proteins (Fig. 6.4). Proteins involved in lipid metabolism made up approximately 10% of the identified proteins and these included one of the adipokines, adiponectin, regulatory proteins including fatty acid binding proteins and perilipin-1, as well as enzymes including 3-hydroxyacyl-CoA dehydrogenase and acetyl CoA synthetase, for example (Fig. 6.4).

The second largest functional group was immune proteins. These were of varying sizes and functions, and made up approximately 15% of all identified proteins (Fig. 6.4). The immune proteins were dominated by two classes of immunoglobulin, IgA and IgG. Annexins and cyclophilins were also present, and are involved in the regulation of the inflammatory response (Bukrinsky, 2015; Sugimoto et al., 2016). Other proteins included transferrin, fibrinogen and fibronectin that are involved in the acute phase response (Kilicarslan et al., 2013). Dermicidin has antimicrobial properties (Schittek et al., 2001), and 4 complement proteins (C1, C3, C4 and C9) were identified that act as parts of the complement system which forms part of innate immunity (Nesargikar et al., 2012). B-cell antibody and galectins expressed by immune cells were also identified (Fig. 6.4).

Finally, the largest functional group was proteins involved in general cell function and metabolism, and made up approximately 45% of the proteins identified. These were proteins involved in a range of different processes including protein degradation and modification, biosynthesis, metabolic pathways (e.g. glycolysis), redox proteins, heat shock proteins, signal transduction, vesicle trafficking, cell cycle regulation and protein chaperones, to name just a few (Fig. 6.4).

Minke whales: Between the two species, a total of 409 different proteins were identified. From the four minke whales, 258 proteins were identified across the 11 gel bands separated through 1D SDS-PAGE. These were classified into the same five protein types as the harbour porpoises, and the same eight functional classes, with the addition of two extra classes. These were the peripheral nervous system (peripherin), and vasodilation and circulation (e.g. heparin and angiotensinogen). Across the two species groups, 55.8% of the proteins identified in the minke whale samples were also seen in harbour porpoise samples, so over 40% of the proteins were newly identified in the biopsy samples. Almost half (48.8%) of the proteins identified were only seen once, and just less than 20% were seen more than five times across these extracts. Similar to the harbour porpoise data, the top six most frequently identified proteins were haemoglobin, fatty acid binding protein, apolipoprotein, immunoglobulins, serum albumin and perilipins.

Proteins involved in the peripheral nervous system, amino acid metabolism, glucose metabolism and vasodilation and circulation were the least abundant of the protein functional groups, each making up less than 5% of all the identified proteins (Fig. 6.5). Proteins involved in tissue structure, biomolecule transport and lipid metabolism each made between 7 - 10% of the data. Cell structure proteins made up a larger proportion of the minke whale data than the harbour porpoise data, but immune response and inflammation proteins were again the second largest class for these data, making up approximately 14% of the identified proteins (Fig. 6.5). Only one class of immunoglobulin was identified in these samples compared to the harbour porpoises, but 3 extra complement proteins were identified (Fig. 6.5). Finally, the cell function and metabolism proteins were the largest protein functional group, making up over 40% of the data, and were involved in a range of different cellular and metabolic processes (Fig. 6.5). Thus, while a huge number of new proteins were identified, the overall patterns in terms of what proportion of the proteins were attributed to the different functional groups were generally the same between the two species.

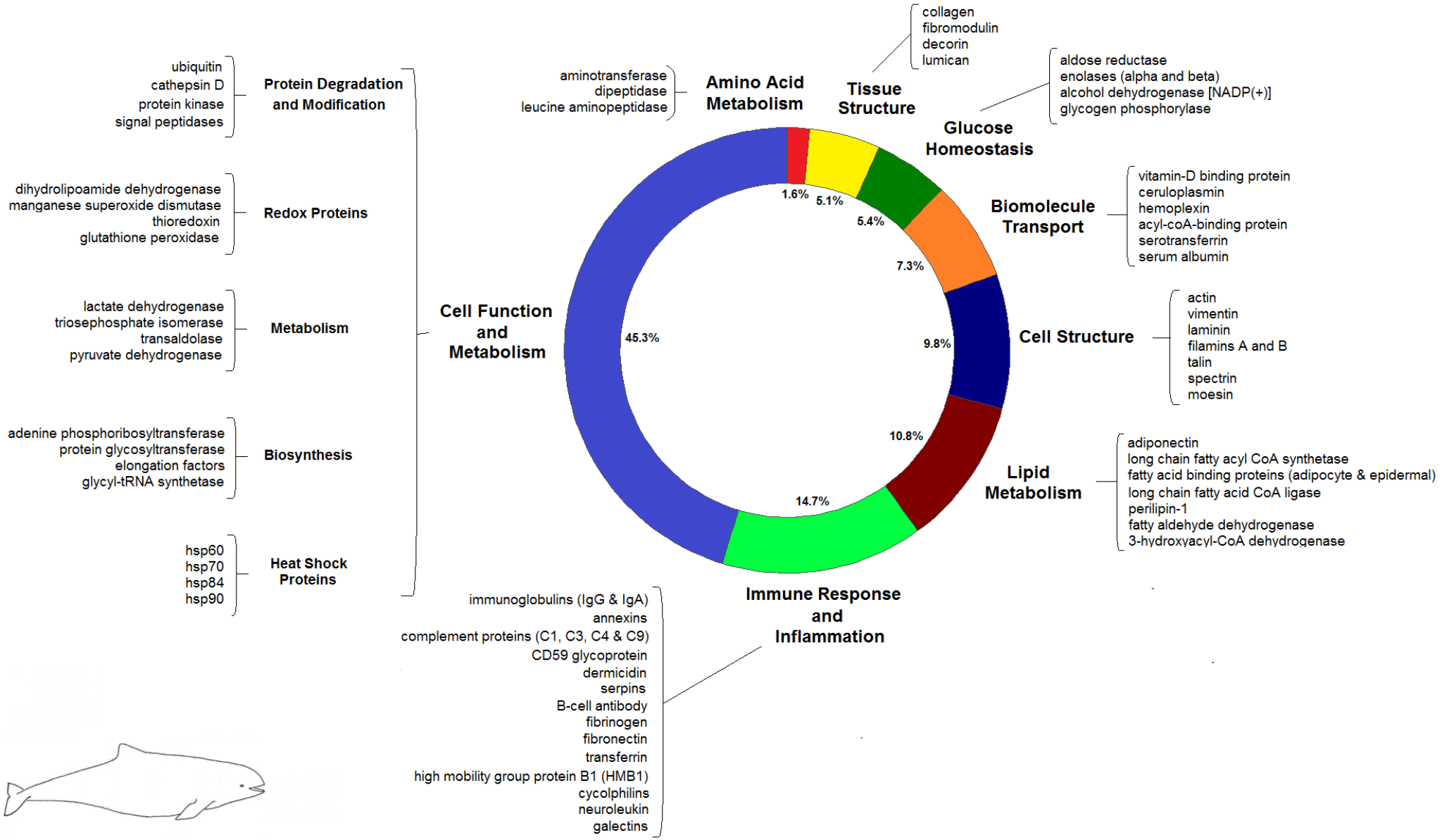

Fig. 6.4. Protein components identified from the harbour porpoise blubber extracts. The proportion of each functional group is indicated as well as examples of some of the most abundant and the most well-studied proteins that were identified. The most abundant functional group was proteins involved in cell function and metabolism followed by the immune response and inflammation.

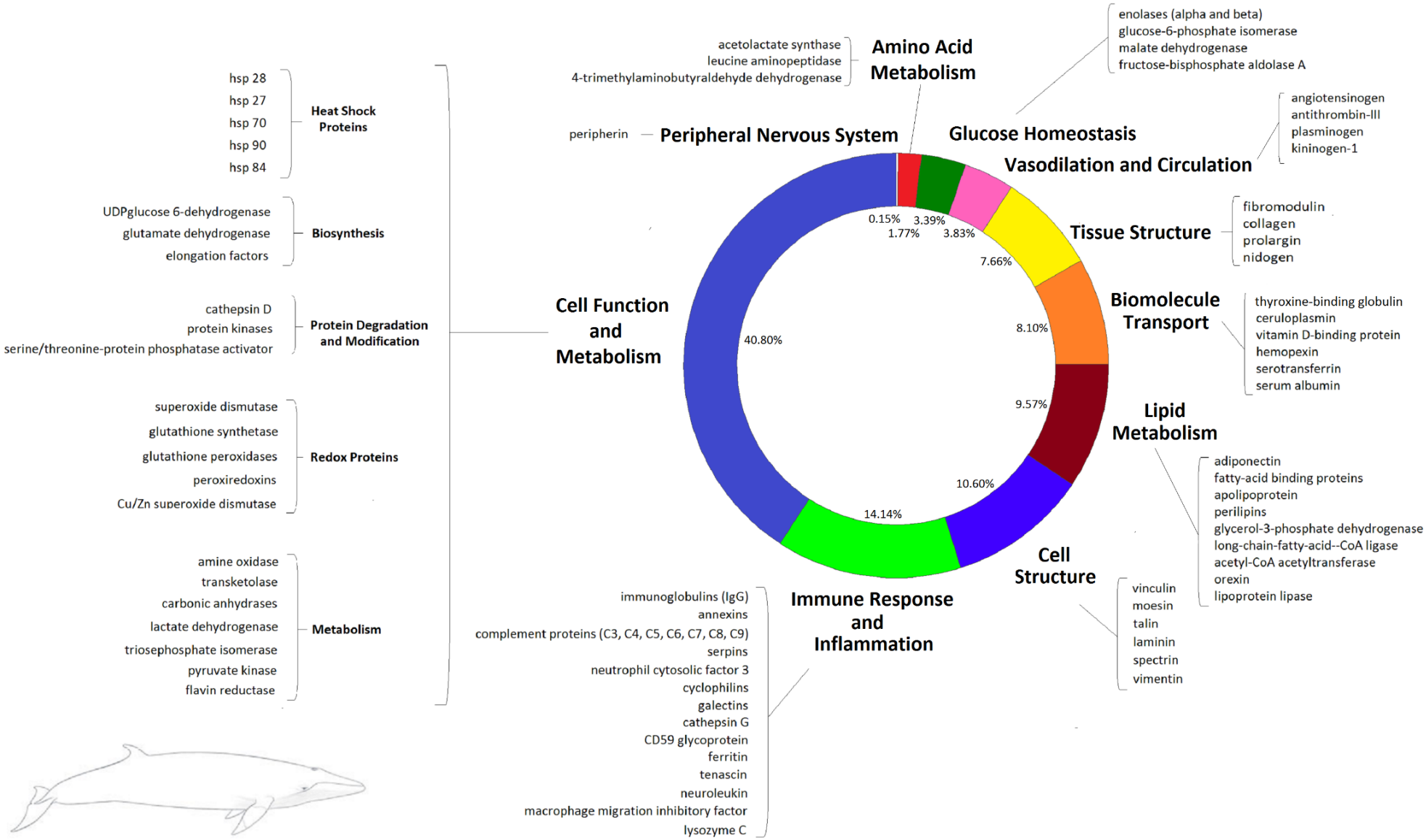

Fig. 6.5. Protein components identified from the minke whale blubber extracts. The proportion of each functional group is indicated as well as examples of some of the most abundant and the most well-studied proteins that were identified. The most abundant functional groups were the same as for the harbour porpoise data while two new groups were identified; the peripheral nervous system and vasodilation and circulation.

5. DISCUSSION

Method Development and Optimisation

Proteins in cetacean blubber tissue were extracted, quantified and identified. With all three extraction methods trialled here, there was variability in the total protein yields between individuals, between duplicate subsamples of the same individual and also between assays of the same extract. The variability may be the result of a combination of possible factors caused by the samples themselves including, for example, high levels of individual variation between porpoises, or fine-scale variation and patchiness of proteins within the tissue. The amount of total protein extracted could also vary depending on the physical properties of the tissue such that the effectiveness of the extraction may differ between samples with higher or lower lipid or collagen content, for example.

Other sources of variation are likely introduced during the different stages of the extraction protocol. Sources of variability in the extraction methods 1 and 2 include homogenisation, centrifugation, pipetting, evaporation and resuspension phases, which together lead to differences in the total amount of extracted or lost protein through the whole process. Specifically, it is likely that resuspension of the protein in solution was a problem for reliable and consistent measurement of the precipitated proteins using methods 1 and 2. These losses as a result of the multi-stage processing procedures, together with variable resuspension of the protein in solution which lead to the compounding errors associated with high inter- and intra-assay CVs, mean that these are not reliable methods for total protein extraction.

A protein precipitation method would be favoured in order to have the possibility of re-suspending the protein content of the blubber in a chosen buffer or solution that is compatible with subsequent analytical techniques (total protein quantification, SDS-PAGE, ELISAs, proteome profiler arrays, Western blots, for example). However, processing method 3, a simpler method with no precipitation and resuspension of the protein, and few processing steps, resulted in the most consistent results with the highest total protein yield. The final total protein yield was orders of magnitude higher than the previous two methods likely as a result of the streamlined extraction process that did not result in the same protein losses through the multiple-extraction steps used in the other two methods. This processing method results in an extract that is more limited in terms of its potential downstream applications as RIPA buffer is not compatible with a number of assays for protein quantification. Nevertheless, it is the preferred extraction method here for these preliminary proteomic investigations. Further work should prioritise the improvement of extraction methods that make the final product more compatible with downstream assays for individual protein identification and quantification.

The causes of the extraction variation using method 3 (approx. 20%) are still to be determined. It is likely that the protein content varies at a fine scale through the blubber tissue which gives rise to the extraction variability seen here. This natural variability is therefore a challenge to the usefulness of this technique in determining precise quantities as oppose to gross differences. The

applicability of the method will therefore be dictated by the acceptable level of precision for a particular study. Further work to investigate the sources of this variability should be prioritised. Similarly, further work should address the best means of standardising the amount of protein quantified from blubber extracts, instead of using wet weight of the tissue, as this may reduce some of the extraction variability. For example, when measuring biomarkers in urine, the osmolarity and concentration of creatinine are accepted methods of standardization (Reid et al., 2013). In general, variability in laboratory measurement procedures exist throughout laboratory medicine, and advances in analytical technologies and international agreements on improved approaches to test standardization have increased the accuracy of clinical laboratory tests over the last few decades (Bock and Eckfeldt, 2011). Advances in measurement science, called metrology, therefore depend on achieving international consensus on best practices (Bock and Eckfeldt, 2011), and in our field of marine mammal physiology, the way forward now is for inter-group collaborations to establish methods of best practice and standardisation to make these methods applicable and comparable across the widest range of study species possible.

1D SDS-PAGE was used here to separate and visualise, for the first time, the protein components of cetacean blubber tissue. Both the best band resolution and the highest number of bands were seen in the RIPA buffer extracts. This confirmed that this method increases both the total amount of protein extracted, as well as the range of proteins within the sample compared to methods 1 and 2. Linking a 'shotgun' proteomics approach to 1D SDS-PAGE was used here to identify proteins and, thus, investigate both the types of proteins and the most abundant proteins present in blubber. This work is based on the notion that known proteins from other organisms can help to identify cetacean proteins by homology. While there may be subtle differences in both protein structure and function, cetacean myoglobin compared to myoglobin from terrestrial species (Holm et al., 2016), or leptin in terrestrial animals compared to in phocid seals for example (Hammond et al., 2012), it was assumed that the proteins within the tissue could be positively identified based on regions of sequence identity with known proteins from vertebrate species within the NCBI database. However, due to the adaptation of marine mammals to an aquatic environment, the potential for protein structural modification could be high for some proteins. This could therefore lead to sources of error during protein identification, and should be considered when aiming to identify molecules that could serve as indicators of physiological condition.

Proteins Identified

As the protein gel bands extracted from the harbour porpoise samples were all of different molecular weights, from all sex and age classes that had suffered various causes of death, a wide variety of proteins present in the tissue were captured. Similarly, because the minke whale biopsy samples were taken over a 4-month period of the summer feeding season, animals were likely in varying states of lipolysis and lipogenesis so a wide variety of proteins were captured in these samples as well. The largest functional group for both species were proteins involved in general cell function and metabolism. Within this group, the proteins were further classed into more specific functional roles including proteins involved in biosynthesis, antioxidant proteins,

regulators of the cell cycle and signal transduction pathway proteins, for example. The variety of the proteins in this group with a range of different metabolic functions is in keeping with a recent transcriptomic study of Northern elephant seal (*Mirounga angustirostris*) blubber tissue which showed that the most significantly enriched pathway in the blubber transcriptome, compared to the human proteome, was metabolism (Khudyakov et al., 2017). The identification of important metabolic factors could therefore provide insight into localised tissue function. For example, heat shock proteins were identified here in the blubber extracts, and changes in gene expression for the heat shock response were detected in the transcriptome of the elephant seals (Khudyakov et al., 2017). Heat shock proteins are key cellular defences against stress and play crucial roles in the folding and unfolding of proteins, the transport and sorting of proteins, as well as cell-cycle control and signalling (Li and Srivastava, 2003). In phocid seals, greater requirements for heat shock proteins and other antioxidants have been hypothesised at certain times during the life cycle as a result of rapid protein synthesis and high metabolic fuel availability (Bennett et al., 2014). Six different heat shock proteins were identified between the two species groups, and the expression of these proteins could provide insight into cellular and physiological stresses of individuals.

The second largest functional group for both species was proteins involved in the immune response and inflammation. Some of the immune, and acute-phase response proteins that were identified included haptoglobulin, transferrin and eight members of the complement pathway. This is in keeping with the recognition of the extensive and direct involvement of white adipocytes in inflammation and the acute-phase response in other mammals (Frühbeck et al., 2001). It has been shown that adipocytes synthesise all of the proteins involved in the alternative complement pathway, specifically, factor C3. However, further research is required to determine the primary functions and regulation of this pathway in adipose tissue (Frühbeck et al., 2001). Other proteins involved in immune system function were also identified including immunoglobulins (IgA and IgG) which, together, were the second most abundant proteins in the extracts from both species. These were likely either in the circulation or were secreted directly from B-cell infiltrates within the blubber itself. Annexins, cyclophilins and dermicidin were among the other proteins identified that are involved in the regulation of inflammation and the immune response. Annexin A1, for example, is an endogenous glucocorticoid-regulated protein involved in the regulation of the inflammatory response by reducing leukocyte infiltration and activating neutrophil apoptosis to avoid tissue damage caused by excess neutrophils at a site of inflammation (Sugimoto et al., 2016). Both innate and the adaptive components of the immune system were therefore present in the tissue. Given the current understanding of the involvement of adipose tissue in immune system function, it is possible that cetacean blubber could show a similar role, and the presence of such proteins could provide information regarding immune system function and response in these animals.

As expected, there were also a range of factors identified that play key roles in lipid metabolism, and these made up the third largest functional group of identified proteins in the harbour porpoises and the fourth largest group in the minke whales. Fatty acid binding proteins are low molecular-

weight cytoplasmic proteins, and were particularly abundant here in both species. The adipose-specific fatty acid-binding protein has been shown to be involved in intracellular trafficking and targeting of fatty acids (Frühbeck et al., 2001), and may modulate lipolytic rate. Apolipoproteins were particularly abundant in the minke whale samples, and are known to play important roles in both the synthesis and catabolism of plasma lipoproteins, in lipid transport, and in the activation of various enzymes involved in lipid and lipoprotein metabolism (Donma and Donma, 1989).

Enzymes and regulatory proteins involved in lipolysis were identified, including the hormone adiponectin which was particularity abundant in the minke whale samples. Adiponectin is an adipokine produced by white adipose tissue and released into the circulation, and is important for whole body metabolic regulation by increasing adipogenesis and lipid storage in fat tissue, as well as increasing insulin sensitivity (Fu et al., 2005). Circulating adiponectin concentrations have been negatively correlated with total body fat stores in a number of terrestrial mammals (for a review see Fain et al., 2004), and, in marine mammals specifically, adiponectin is thought to be important in the development of blubber reserves in grey seal (*Halichoerus grypus*) pups (Bennett et al., 2015). Concentrations of this hormone in the blubber could therefore provide information on the physiological state of an individual in terms of current energy stores. Adiponectin signalling pathways were also identified in the transcriptomic study of Northern elephant seals when investigating the acute metabolic response to glucocorticoids (Khudyakov et al., 2017). Other differential gene expression was measured that promoted lipid catabolism and oxidation at the expense of lipid synthesis and storage (Khudyakov et al., 2017). The presence of factors involved in various stages of lipid metabolism could be used to assess whether the individual is undergoing a period of lipolysis or lipogenesis at the time of sampling. This is a good example of how the integration of proteomic and transcriptomic methods could result in a powerful assessment tool. Moving forward, the quantification of individual proteins could be coupled to the transcriptome so that transcription and translation can be linked.

Finally, for the harbour porpoises, although every attempt was made to obtain very fresh tissue samples, they were nevertheless collected from dead-stranded animals, so minor autolysis could have affected these findings. This would also complicate the functional interpretation of any proteins involved in *ante-* or *post-mortem* metabolic processes. Similarly, the remotely obtained biopsy samples from the minke whales were stored on ice immediately after collection, but a small amount of tissue degradation is possible as a result of the delay between sample collection and sample freezing. Ideally, samples should be collected and snap frozen immediately using dry ice or liquid nitrogen. However, the wide range of proteins and peptides identified here across a variety of metabolic pathways and processes suggests that proteomics is a robust tool to investigate tissue function using this biopsy sampling approach.

Tissue Specific and Circulatory Proteins

While a range of different molecular weight proteins were identified, there may be some size selective loss of protein species as well as some loss of the least abundant proteins through the

extraction procedure. There may also be some loss of more hydrophobic proteins that are more difficult to solubilise. Thus, if a protein was not identified following nLC-ESI MS/MS, this does not confirm its absence from the tissue, but this work does suggest that there are some proteins, and protein classes, that are more abundant than others. The relative abundance of some proteins compared to others is also likely the reason why over half of the proteins identified were only observed once. These proteins are probably the least abundant in the tissue, and were thus only identified once 'by chance' as a result of the combination of other proteins that were also within a particular gel band. There are therefore potentially a huge number of other proteins that were present in the samples, but were not identified. Conversely, serum albumin and haemoglobin, from the circulation, were two of the most abundant proteins identified in both species, and likely affected the detection of other proteins by swamping the samples. Further efforts to remove the albumin from the extracts would likely be required to detect less abundant proteins of potential interest. This could be achieved by fractionation of the extract for example or, the use of 'Cibacron Blue', a commercially available resin to remove albumins from solution. Alternatively, antibody columns could be used to target specific proteins. Similarly, targeted mass spectrometry could be used to detect particular peptides from a specific protein of interest to therefore detect the presence of proteins at lower concentrations within the samples.

Proteins identified here probably do not originate solely from the blubber tissue itself, but are a mixture of blubber proteins together with plasma proteins. Attempts were made to limit any external blood on the samples by using visibly 'cleaner' parts of the harbour porpoise tissue samples. The presence of plasma proteins in the extracts were therefore likely largely a result of their presence *within* the tissue vasculature rather than *on* each piece of tissue from contaminating sources during the necropsy sampling. The vascularisation of marine mammal blubber tissue is still not well understood, but one study comparing the microvasculature of deep diving and shallow diving odontocetes found that blubber tissue is more highly vascularised than adipose tissue in terrestrial mammals (McClelland et al., 2012). As such, blubber tissue sampling can provide information on both the proteins produced and metabolised *in situ* as well as those in circulation. Double sampling of freshly dead stranded animals before blood coagulation occurs would be useful for further comparisons of proteins present in both the blood and blubber of the same individuals. This would be an important next step to identify those proteins present in both matrices, and those that are found more exclusively in the blubber itself. For example, the absence of some proteins identified in the minke whale samples compared to the harbour porpoise samples could reflect differences in the vascularisation of the outermost part of the tissue compared to the full blubber depth.

The range of proteins identified here across both species, and the presence of a huge number of proteins identified exclusively in the full depth harbour porpoise samples or exclusively in the superficial minke whale samples, clearly demonstrate the heterogeneity of blubber tissue. A comparison of the proteins present through the different blubber layers from the same individuals would help to establish if this heterogeneity results from longitudinal differences in metabolic

activity of the tissue and / or different cell types through the blubber depth. While it has been well established that adipocytes express and secrete several endocrine hormones, many secreted proteins within the tissue are derived from the non-adipocyte fraction (Fain et al., 2004). For example, the innermost blubber layer has been shown to consist of a heterogeneous mix of white adipocytes, brown adipocytes and connective tissue as well as muscle and nerve fibres (Hashimoto et al., 2015). Therefore, as the full depth blubber layer was sampled in the harbour porpoises, proteins detected in these samples were most likely the result of differential gene expression in these different cell and tissue types which explains the presence of myoglobin, a muscle protein, across all samples. Thus, as well as the origin of certain proteins in terms of either the blubber or the circulatory system, an important next step would also be to establish the secreting cell types within the tissue. It would be possible to determine whether gene expression and protein secretion occurs within the mature adipocytes, or in the other cells that make up the tissue, either histologically (through *in situ* hybridisation) or by separation of the adipocytes from the stromal vascular fraction by collagenase digestion (Trayhurn and Wood, 2004).

Potential for Biomarker Development

While there were over 400 different proteins identified in the extracts from the two species, overall, the relative abundance of the proteins across the functional groups were very similar. Moving forward, the identification and quantification of different hormones and proteins involved in various metabolic pathways within blubber tissue could lead to the development of potential new protein markers of interest. Explaining and quantifying the natural variability in these protein markers in the context of different life histories or causes of death, for example, is the next step in developing this approach. Overall, proteomic studies also have the potential to identify key metabolic processes and pathways and therefore assign novel functional roles to marine mammal blubber tissue.

CHAPTER 7: THE CHALLENGES AND OPPORTUNITIES FOR QUANTITATIVE PROTEOMICS TO INVESTIGATE BLUBBER BIOMARKERS

1. ABSTRACT

Proteins extracted from blubber tissue likely originate both from the various cell types within the blubber itself, and from the circulation. They therefore have the potential to capture information on the cellular and physiological stresses experienced by individuals at the time of sampling. As a next step to further develop this analysis, more quantitative proteomic methods were trialled to assess how the variation in protein profiles between individuals could be explained by physiological states and other biological covariates of interest. Total protein was extracted and then separated using 1 dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D SDS PAGE) from four sample sets. These were samples from stranded harbour porpoises (*Phocoena phocoena*) (n = 32), stranded balaenopterids (n = 10), biopsy samples from minke whales (*Balaenoptera acutorostrata*) (n = 10) and biopsy samples from humpback whales (*Megaptera novaeangliae*) (n = 42).

Non-metric multidimensional scaling (NMDS) analysis was used to cluster individuals based on similar protein band profiles. Despite the range of proteins previously identified within blubber extracts, the NMDS results showed that separation and visualisation of different protein bands using 1D SDS PAGE was not at a fine enough resolution to be able to identify patterns that correlated with reproductive states, sex, age class, cause of death, season or area. It appears that the time in storage, the total protein yield extracted from the tissue and sources of error associated with running the 1D SDS PAGE affected the band profiles in some individuals. There are therefore some sample processing and measurement errors associated with these methods that require further investigation and quantification. However, for the largest sample set of humpback whale biopsies, linear regression models showed that the presence and absence of some protein band groups were associated with high or low blubber cortisol concentrations. In a semi-quantitative analysis, using the protein identification results from the minke whale nLC-ESI-MS/MS data in Chapter 5, the abundance of proteins involved in lipid metabolism and amino acid metabolism were higher in the band group associated with high cortisol concentrations compared to the full dataset. While this only provides a semi-quantitative investigation of the relationships between protein functional group abundance and the concentrations of other endocrine markers of interest, it does show that the application of multiple analytical methods together could lead to a better understanding of whole tissue function. Using this approach, biomarkers, like cortisol concentrations, can be linked to downstream changes in protein abundance.

2. INTRODUCTION

A huge number of proteins and peptides involved in a range of different metabolic processes and pathways were identified in cetacean blubber tissue extracts. Following this 'shotgun' approach as a preliminary investigation to assess what protein classes were present in the tissue and their relative abundance, the next step is to apply more quantitative proteomic methods to assess the variation in protein profiles between individuals, and how this variation can be explained by life history strategies and physiological states. This will allow the identification of potential blubber proteins and peptides of interest for further biomarker development. The use of proteomic biomarkers is a widely accepted approach in human medicine, and has been used to identify disease processes ranging from Alzheimer's to congestive heart failure (Zhang et al., 2004). These approaches have involved protein separation, characterization and identification techniques to create and compare protein profiles of normal and diseased body fluids including serum, cerebrospinal fluid and urine for example (for review see Zhang et al., 2004). The applicability of these methods for biomarker development using blubber tissue extracts here will depend on the magnitude of the explained, observed differences between individuals, and the consistency with which these differences can be identified. This will contribute to a better understanding of whole blubber tissue function as more than just an energy storage depot, in keeping with the current understanding of mammalian adipose tissue as an important endocrine organ (Kershaw and Flier, 2004).

Indicator proteins may be produced directly by the blubber itself, secreted either by the adipocytes or the stromovascular cells, or a combination of both. Equally, indicator proteins may have accumulated in the tissue from the circulation in a manner dependent on the individual's metabolic state. They therefore have the potential to capture information on a range of different metabolic processes and provide insight into the physiological stresses experienced by individuals. Of particular interest to assess energy stores and body condition would be the adipokines such as adiponectin, previously detected in both harbour porpoise (*Phocoena phocoena*) and minke whale (*Balaenoptera acutorostrata*) blubber extracts. Other markers of interest are enzymes and other regulatory proteins involved in lipolysis and lipogenesis. In addition, the range of immune proteins previously identified suggests that the blubber could be a valuable tissue for assessing immune system function and inflammatory responses.

For studies of complex protein mixtures, such as those extracted from blubber tissue, strategies for reproducible and accurate quantification are needed. 1 dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D SDS-PAGE) is a commonly used method for both fractionating complex samples by protein size, and visualising variations in individual protein content (Gautier et al., 2012). Using 1D SDS-PAGE to compare separated protein bands by their presence and their intensity has been applied to a wide range of proteomic studies in both human health sciences and other model species. For example, the biological pathways involved in human endothelial cell response to pro-inflammatory cytokines (Gautier et al., 2012), and the proteome of the human tear film (Green-Church et al., 2008) have been investigated using combinations of

1D SDS-PAGE and shotgun proteomics. In other species, 1D SDS-PAGE has been used to investigate changes in immune factors and other proteins in milk throughout lactation in seals (Lowe et al., 2017), and to provide diagnostic and prognostic information on urinary proteins in dogs with severe inflammatory response syndrome (Schaefer et al., 2011). These methods therefore represent a powerful analytical tool with applications to a range of different sample types of interest.

1D SDS-PAGE methods were applied here to separate the total protein content from blubber sample extracts collected from both dead, stranded individuals and biopsies from live animals. The blubber samples from dead stranded individuals consist of a small odontocete, the harbour porpoise, and balaenopterids of a mixture of species. The biopsies from live animals consist of samples from both minke whales and humpback whales (*Megaptera novaeangliae*) sampled on their feeding grounds in the Gulf of St Lawrence, Quebec, Canada by the Mingan Island Cetacean Study (MICS). Variations in protein band presence/absence, frequencies and relative concentration profiles generated though 1D SDS-PAGE were assessed in the context of both qualitative variables including species, sex, age class, reproductive status, cause of death, area and season. Quantitative variables were also assessed including morphometric body condition indices, blubber cortisol concentrations and total protein content extracted from the tissue. The aim was to interpret any variations in the protein band profiles between individuals with differences in physiological state, and thus identify particular protein bands or protein band patterns that can be further analysed for the development of health biomarkers in these species.

3. METHODS

3.1. Sample Collection

Stranded harbour porpoises: Full-depth skin, blubber, and underlying muscle samples were collected from 32 dead harbour porpoises by the SMASS between 2013 and 2015 as previously described in Chapter 5. Samples were taken from both males and females, and adults and juveniles. The cause of death was determined following post-mortem examination, and individuals were classes as acute or chronic cases as previously described in Chapter 1.

Stranded balaenopterids: In addition to the full depth samples from harbour porpoises, 10 full depth blubber samples were also collected from stranded balaenopterid individuals. These were a mixture of minke whales (sampled by the SMASS and MICS), humpback whales and a fin whale (*Balaenoptera physalus*). Again, these were a mixture of males and females, and adults and juveniles with varying causes of death.

Samples were collected and frozen in individual plastic vials at -20^{0}C prior to analysis. For the total protein extraction, sub-samples were taken on ice while the tissue was still frozen, and care was taken to remove all skin and muscle from the blubber. Full depth subsamples of each original sample were used for total protein extraction in order to investigate the proteins through all layers of the tissue. Of these 42 samples from stranded individuals, 34 were extracted in duplicate (Table 7.1) to assess the potential variability in total protein extracted from the tissue as a result of either protein heterogeneity within the tissue, or variability as a result of the tissue handling and processing.

Minke whales: Ten remotely obtained, shallow, biopsy samples collected from adult minke whales by the MICS in 2013 were used for total protein extraction (see Chapter 5 for details). These animals were sexed genetically using the skin as a source of DNA, and were all females.

Humpback whales: A total of 59 biopsies collected from humpback whales in Quebec, Canada, and in northern Norway between 2011 and 2017 were used for total protein extraction. These samples were all collected from simultaneously tagged and biopsied animals for a study to compare metrics of body condition estimated from tag data and from biomarkers in blubber biopsies. These samples were also used for cortisol and progesterone extraction (see Chapter 4 for details). A number of the biopsied animals from 2016 and 2017 also have accompanying photogrammetric information obtained from images captured from an unmanned aerial vehicle (UAV). These photogrammetry data consist of a Length Standardised Surface Area Index (LSSAI) used to capture variation in girth of individuals as a function of their total body fat stores, and therefore an index of body condition (see Chapter 4 for details). Overall, these were from a mixture of males and females in varying reproductive states, as well as some juvenile individuals.

All biopsy samples were stored on ice immediately after they were collected, and then stored frozen either in aluminium foil or in glass vials at -20^{0}C until processed. Sub-samples were taken

on ice while the tissue was still frozen, and care was taken to remove all skin from the biopsy and to cut the sample vertically so as to retain as much of the variation though blubber depth as possible.

Table 7.1 - Summary of the samples used for total protein extraction belonging to four separate groups. *SMASS: Scottish Marine Animal Stranding Scheme. *MICS: Mingan Island Cetacean Study.

Group	Species	Total	Duplicate Extracts	Source
Porpoises	Harbour porpoise	32	24	SMASS*
Balaenopterids	Minke whale	7	7	SMASS / MICS*
	Humpback whale	2	2	SMASS
	Fin whale	1	1	SMASS
Minke whale Biopsies	Minke whale	10	0	MICS
Humpback whale Biopsies	Humpback whale	59	0	Canada / Norway

3.2. Total Protein Extraction and Quantification

Total protein was extracted from 0.1g subsamples of either the full depth samples from the stranded animals, or the biopsy samples from live animals. The RIPA cell-lysis buffer protocol as described in Chapter 5 was used for total protein extraction of these samples, and extracts were assayed for total protein content using a Pierce™ 660nm Protein Assay (22662, Thermo Scientific, Rockford, USA) (see Chapter 5 for details). On each total protein plate, 4 samples were assayed in triplicate and an intra-assay CV of <10% was considered acceptable. Any plates with an intra-assay CV of > 10% were discarded and all extracts assayed again. Extracts were then separated on 1D SDS-PAGE gels the same day.

3.3. Total Protein Separation

The 1D-SDS PAGE total protein separation method, as described in Chapter 5, was used to separate and visualise proteins of different sizes within the extracts. Each extract was diluted by ½ to avoid protein overloading and smearing on the gel in order to produce good band visualisation. Four bovine serum albumin (BSA) standards of known concentrations were also loaded onto each gel for relative protein concentration determination. Gels were stained and destained as previously described in Chapter 5, and then photographed on a white background using a BioDoc-It™ Imaging System (Ultra-Violet Products Ltd, Cambridge, UK).

3.4. 1D SDS-PAGE Band Detection, Characterisation and Processing

Band Detection and Characterisation: Gel images were imported as jpeg files into freely available software for 1D gel electrophoresis image analysis software called 'GelAnalyzer 2010' (Fig. 7.1). The process of gel image analysis using the software is straightforward and covers automatic lane and band detection, background subtraction techniques, retention factor (Rf) calibration to correct for gel run distortions, and accurate quantity and molecular weight calculations with four different calibration curve types to choose from. Here, a combination of both automatic and manual band detection was used to assign band limits for the molecular weight markers, the BSA standards and the protein bands separated in each of the sample extracts. Log-linear models were fitted by the software to the molecular weight markers and the standards of known concentration in order to estimate, in an automated process, both the concentrations and the molecular weights of the bands identified in the sample extracts. Each band identified across all individuals and all gels was therefore assigned a molecular weight and a volume (concentration).

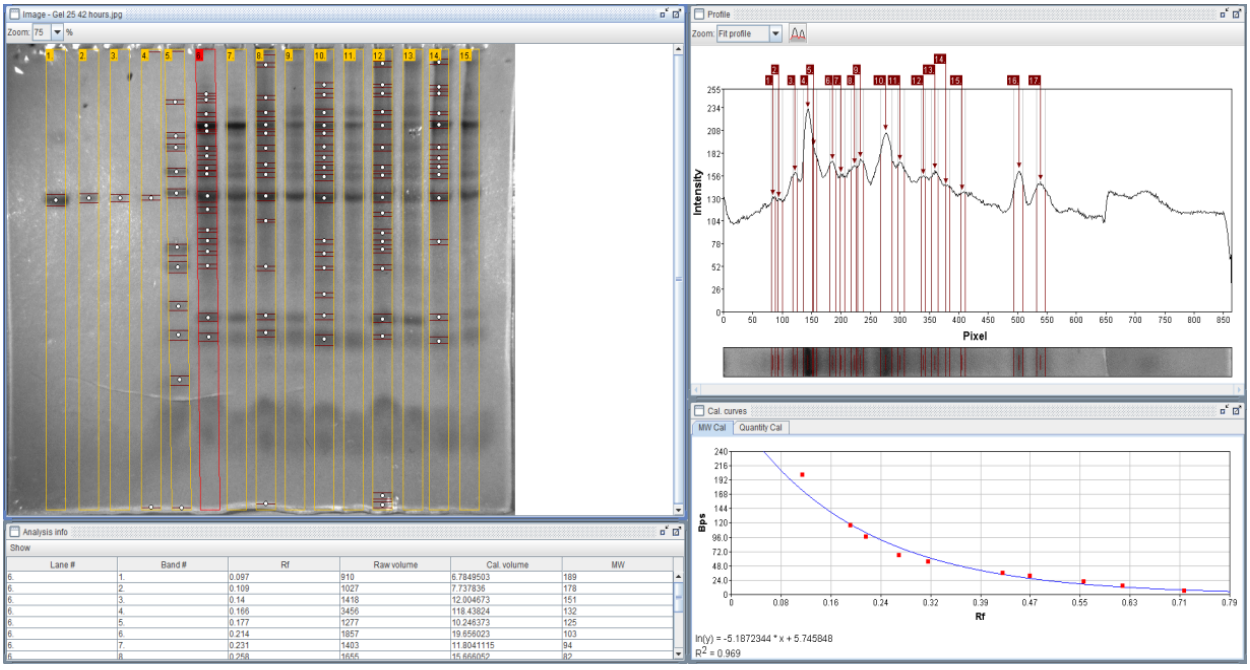

Fig. 7.1. 'GelAnalyzer' software interface for the quantitative assessment of the protein band patterns of blubber extracts. Top left panel: Gel image with identified bands marked with points and bordered by red horizontal lines. Top right panel: Intensity of the bands of an individual lane representing one extract. Intensity is used to calculate the volume (concentration of proteins) of a band. Bottom left panel: Calculated values for the bands in the lane highlighted in the trop right including Rf values (migration distance from the sample loading well compared to the migration distance of the dye front), calculated volume (concentration) and molecular weight (size of the proteins in that band). Bottom right panel: Visualisation of the model calibration curves fitted to the molecular weight markers.

Post Image Analysis Band Processing: As all gels ran slightly differently, the same band appeared as 17 kDa, 16 kDa or 15 kDa, on different gels for example, but this is the equivalent band separated across individuals. For this reason, there were a huge number of bands of different molecular weights detected in the harbour porpoise and the balaenopterid samples (Fig. 7.2a). Even when bands were grouped into 5 kDa bins (Fig. 7.2b), there was still a large number of bands that likely represented the same proteins but because of the slight differences in the running of different gels, the calculated molecular weights were marginally different. To overcome this problem, the bands were therefore separated into more manageable and meaningful units based on the maximum number of bands seen in an individual, and the distribution of separated bands down the gels (e.g. there are a greater number of different bands seen between 116 kDa and 55 kDa compared to between 200 kDa and 116 kDa), whilst taking into account the fact that there was greater separation of smaller proteins than larger ones (e.g. a greater migration distance between bands of 5 kDa and 15 kDa than bands of 90 kDa and 100kDa in size) (Fig. 7.3). This resulted in the separation of the bands data into 20 band groups shown in Fig. 7.3 named Band Groups 1 to 20. The smallest proteins were in Band Group 1 and the largest proteins in Band Group 20. While Band Groups 18, 19 and 20 consisted of proteins larger than the largest molecular weight marker at 200kDa, and the molecular weight of these bands has therefore been extrapolated and cannot be established with any certainty, the presence of these larger protein bands were retained for analysis as the presence and intensity of the bands was important for further interpretation rather than the accurate molecular weight.

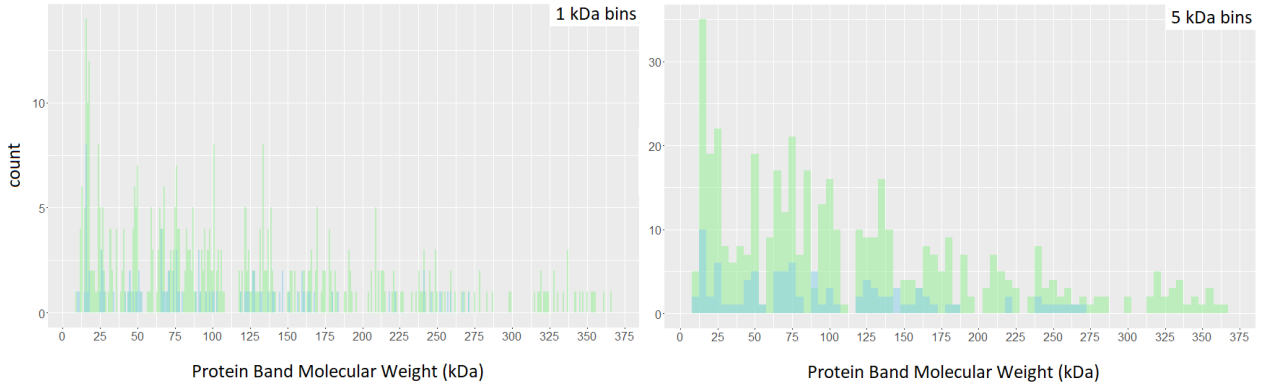

Fig. 7.2. Histograms of all protein bands identified across the harbour porpoise samples shown in green (n = 32) and the balaenopterid samples shown in blue (n = 10). These are broken down into 1 kDa bins (left), and 5 kDa bins (right). A total of 439 and 97 bands were identified across the harbour porpoise and balaenopterid individuals respectively. The largest bands were at 366 kDa and 271 kDa for the harbour porpoise and balaenopterid individuals respectively, while the smallest were at 9 kDa and 10 kDa for each species group.

In order to compare between individual extracts run on different gels, the relative band frequencies and band volumes (concentrations) were calculated for each individual:

Relative Frequency: The number of bands within a band group (e.g. Band Group 3; the number of bands between 16-20kDa in size detected for that individual), divided by the total number of bands detected for that individual (e.g. 17 bands were detected for the first individual shown in Fig. 7.1). This takes into account the fact that more bands were detected in some individuals than others. This relative frequency for each of the 20 Band Groups was calculated across all individuals and expressed as a proportion.

Relative Volume: The sum of the calculated volumes of the individual bands within a Band Group (e.g. Band Group 3; sum of the volumes calculated for any bands between 16-20kDa in size for an individual), divided by the total sum of the volumes calculated for all the bands detected for an individual. This is to overcome the confounding factor that the extracts from different individuals had varying total protein concentrations when loaded onto the gel.

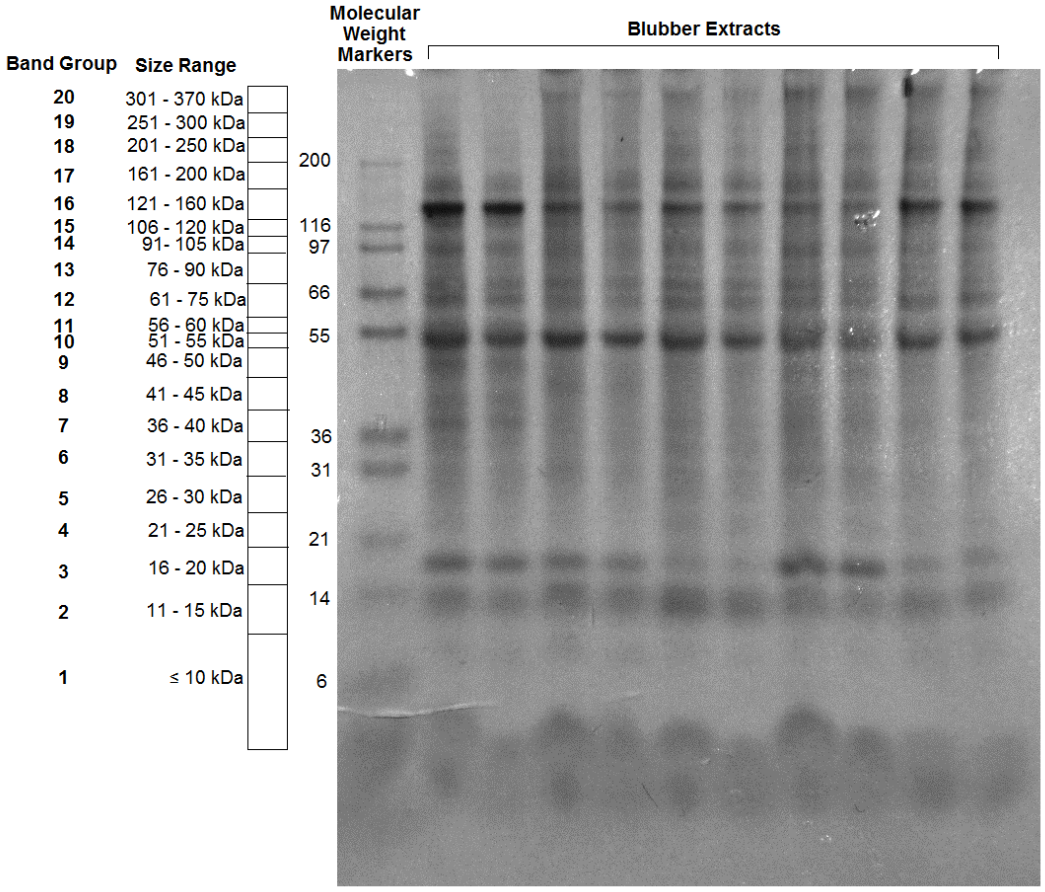

Fig. 7.3. Annotated image of a 1D SDS-PAGE gel. The molecular weight markers are shown in the lane on the left of the image, and the band groups of different molecular weights grouped together for analysis, named Band Groups 1-20, shown on the far left of the figure.

3.5. Statistical Analysis

Non-metric Multidimensional Scaling (NMDS): The objective of Non-Metric Multidimensional Scaling (NMDS) is to group data points into clusters of similar points based on a few explanatory variables so that they can be visualized and interpreted. Here, the aim was to investigate whether there were clusters of individuals that grouped together based on the frequency and volumes of different protein band groups measured in their SDS-PAGE profiles. Then, where clusters were observed, which of the qualitative variables listed in Table 2 could explain the observed groupings.

Similar to Principal Component Analysis (PCA), the aim of NMDS is to represent the original position of data in multidimensional space, as accurately as possible, using a reduced number of dimensions that can be easily plotted and visualized. PCA uses Euclidean distances which are the straight-line distances between two points in Euclidean space. However, these are sensitive to total abundances and absences. Unlike PCA, NMDS relies on rank orders for ordination. These rank orders are non-metric which omits some of the issues associated with using absolute distances, and allows for a much more flexible technique that can be used on a variety of different kinds of data, especially on data containing absences. The SDS-PAGE band groups data contained absences as not all protein band groups were present across the individuals sampled, which makes NMDS a suitable analytical approach as it is invariant to changes in units, it can recognize differences in total abundances when relative abundances are the same, and it is unaffected by zero-inflated data.

NMDS is an iterative procedure which takes place over a number of steps, the first of which is to define the original data point positions in multidimensional space. The number of reduced dimensions are then specified, typically two, and then an initial configuration of the data in a 2-dimentional (2D) space is constructed. The distances in this initial 2D configuration are regressed against the measured distances in multidimensional space. The disagreement or difference between the distances in the reduced, 2D space compared to the distances calculated in the complete multidimensional space is called the 'stress'. The aim is to optimise the stress, i.e. reduce the value as much as possible, by increasing the number of dimensions (e.g. from two to three) so that the configuration matches the data positions in the full multidimensional space as much as possible. To run the NMDS, the function '*metaMDS*' from the '*vegan*' package in R (version 3.1.3) was used to investigate the band group frequency data, and then to investigate the band group volume data for the four species groups separately; stranded harbour porpoises, stranded balaenopterids, minke whale biopsies and humpback whale biopsies.

Firstly, the points in 2-dimensions with a maximum of 30 iterations were assessed. The 'stress' value was assessed for the 2D model. Generally, a stress value of < 0.05 provides an excellent representation in reduced dimensions; between 0.05 - 0.1 is very good; 0.1 - 0.2 is good; > 0.3 provides a poor representation (Cox and Ferry, 1993). Secondly, this process was then repeated for the points in 3-dimensions to assess if the stress value could be further reduced and the representation of the points improved. If the stress values were reduced upon introducing a third dimension to the model, it was concluded that it was a better fit at capturing the variation in the data. Any clustering observed in the data by the qualitative variables listed in Table 7.2 was then

assessed visually to determine if any of the variation in the band frequency and band volume data can be explained using these variables of interest.

Linear Regression Models: For the harbour porpoise and humpback whale data, the two largest datasets for analysis, linear regression models were used to assess the relationships between the presence / absence of each band group with three dependent variables: 1) Morphometric condition index (mass / length^2 (Chapter 1) or Length Standardised Surface Area Index (LSSAI) (Chapter 4) for the harbour porpoises and the humpback whales, respectively). 2) Blubber cortisol concentration (Chapters 3 and 4). 3) Total protein yield. The presence / absence of the different band groups was chosen to include in linear regression models as the explanatory variables of interest because the relative frequency and volume data are unsuitable for this type of analysis. This is because proportion data like these are automatically negatively correlated with each other, they do not all have the same denominator and there are a lot of zeros in the data.

Here, a null model containing no explanatory variables for each of the three dependent variables for the harbour porpoise and the humpback whale dataset separately was generated. A full model was also generated that included each of the 20 band groups as a binary factor with 0 representing band absence and 1 representing band presence. Then, the *step* function in the *car* package was used as a forwards variable selection tool by specifying "*direction = forward*" whereby the variables listed in the full model were added to the null model one at a time. At each step, each variable that is not already in the model is tested for inclusion in the model. The most significant of these variables are added to the model based on a reduction in AIC compared to the null model and then subsequent models with important variables included. If the AIC is not reduced upon addition of a new variable, the variable is discarded as it does not contribute to explaining additional variation in the data. Forwards model selection was chosen here to better assess inclusion of such a large number of explanatory variables (20 band groups) with a relatively small dataset of individuals (32 harbour porpoises and 42 humpback whales). The band groups retained as being important were used for the final model interpretation for the effect of their presence / absence on each of the morphometric condition indices, blubber cortisol concentration and total protein yield for both species groups.

Table 7.2 - Variables used to explain variation in the band groups data for each species group. Qualitative variables used to investigate variation between individuals for band group relative frequency and relative volume data for all four species groups. Quantitative variables used to investigate variation in band group presence / absence for the harbour porpoise and humpback data only.

		Porpoises (n = 32)	Balaenopterids (n = 10)	Minke whales (n = 10)	Humpback whales (n = 42)
Qualitative Variables	Gel Number	✓			✓
	Sex	✓	✓		✓
	Age Class	✓	✓		✓
	Rep. Status[1*]				✓
	Species		✓		
	COD	✓	✓		
	Area				✓
	Season			✓	✓
Quantitative Variables	Mass/Length[2]	✓			
	Cortisol[2]*	✓			✓
	LSSAI[3]*				✓
	Total Protein	✓			✓

[1*] Rep. status refers to reproductive status as either a mature or immature male, or an immature, lactating, pregnant or resting female based on visual observations of females with calves and the concentrations of progesterone measured in the biopsies of humpback whales (Chapter 4).

[2*] Cortisol refers to blubber cortisol concentration measured in either the outer layer of blubber in harbour porpoise samples (Chapter 3) or the blubber biopsies collected from the humpback whales (Chapter 4).

[3*]LSSAI refers to the Length Standardised Surface Area Index estimated from photogrammetry measurements obtained from images captured by an unmanned aerial vehicle of humpback whales (Chapter 4).

4. RESULTS

4.1. Total Protein Yield and Extraction Variability

For the 42 samples from both the stranded harbour porpoises and balaenopterids, there was a range in total protein yield from 166.0 ng/g to 5426.4 ng/g (Fig. 7.4), with an average of 2735.8 ± 190.9ng/g. The extraction coefficient of variation (%CV) ranged from 2.2% to 66.6% with an average of 21.9 ± 2.7% for the 34 stranded animal samples extracted in duplicate. There was no difference between the duplicate % extraction CVs between the harbour porpoise and the balaenopterid samples (ANOVA; df = 1, F = 1.33, p = 0.72). A total of 69 biopsy samples were extracted in total from both the humpback whales and the minke whales. There was a range in the total protein yield from 64.9 ng/g to 9305.4 ng/g (Fig. 7.4) with an average of 2882.2 ± 249.9 ng/g, similar to the stranded animal samples.

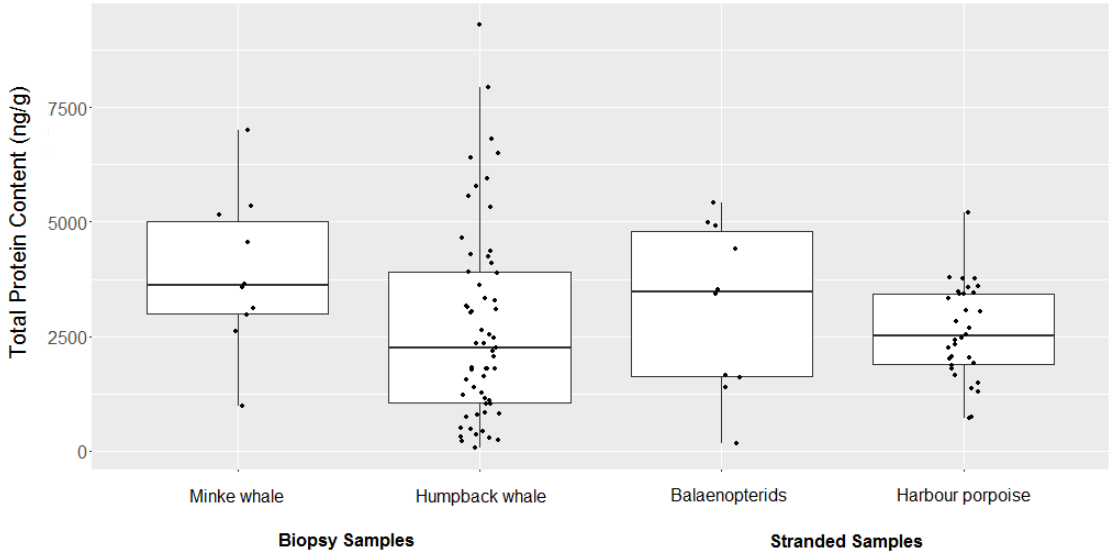

Fig. 7.4. Boxplots showing the raw data (points), the median and the inter-quartile range for the total protein content extracted from all four species groups. There were no differences between the average protein content extracted between the four groups (ANOVA; df = 3, F = 1.59, p = 0.21).

4.2. Sample Degradation through Storage

Of the 59 biopsy samples from humback whales that were used for total protein extraction and quantification, the results from 42 of them were used for protein band group analysis. While there were no statistical differences between the total protein yields of samples collected across the different years (ANOVA; df = 5, F = 1.085, p = 0.38), the older samples showed an overall lower yield (Fig. 7.5) as well as generally poorer band visualisation and separation (Fig. 7.6). The 17

samples that were excluded from the analysis were from 2011 (n = 3), 2012 (n = 8), 2013 (n = 2) and 2014 (n = 4) as they showed a combination of lower total protein content, and poor band separation. Thus, band group profiles in these samples likely represented the products of tissue degradation through storage rather than differences in the protein content of the tissue between individuals. There was no consistent pattern with all samples from one year showing poorer band separation compared to others however, which is why some of the oldest samples were still used for interpretation. Other factors, not accounted for here, therefore also likely affected the extent to which the samples were affected by time in storage.

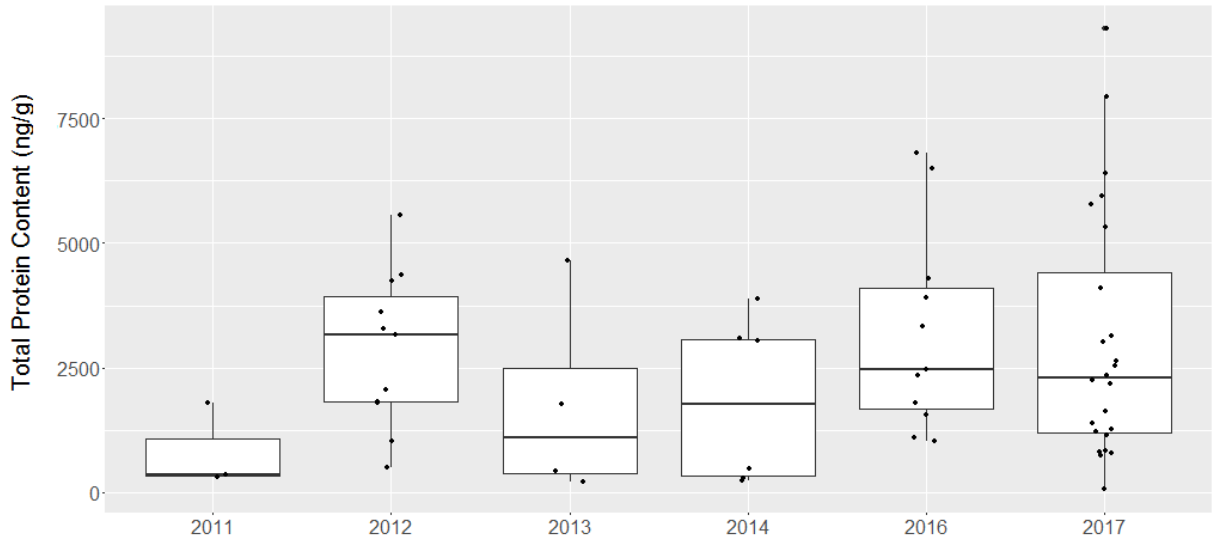

Fig. 7.5. Boxplot of total protein content measured in humpback whale biopsies collected between 2011 and 2017. The overall increase in total protein content from the 2011 – 2017 samples could indicate some protein degradation and loss with increased time in storage.

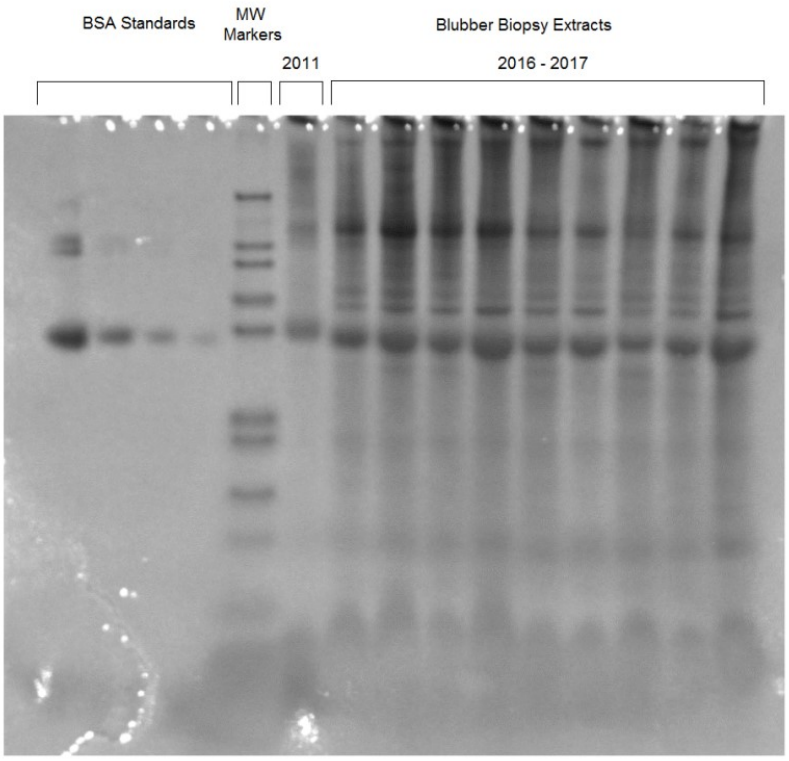

Fig. 7.6. 1D-SDS PAGE image of total protein extracts from humpback whale biopsies collected in 2011, 2016 and 2017. The 2011 extract shows lower protein content and poorer separation and visualisation of extracted proteins indicative of sample degradation. *BSA Standards: Bovine Serum Albumin standards used for protein band volume quantification. *MW Markers: Molecular Weight Markers used to determine the molecular size of protein bands.

4.3. Non-Metric Multidimensional Scaling (NMDS)

Stranded Animals Samples: Results of the NMDS analysis for the harbour porpoise and the balaenopterid band group datasets showed that using 3 dimensions produced better fitting (lower stress values) models compared to using just 2 dimensional scaling. Specifically, the harbour porpoise NMDS results data showed no clustering of individuals by sex, age class or cause of death, but did show some clustering of individuals based on the gel number on which they were run (Fig. 7.7). Overall, there were differences between gels 1 and 4 that showed similar band group patterns across individuals, and gels 2, 3, 5 and 6 that shared similar band group characteristics (Fig. 7.7). Again, there was no clustering by sex, age class or COD for the balaenopterid data. There was limited clustering by species for the band group frequency data, whereby the minke whales were more similar (more tightly clustered) than the fin whale and humpback whale samples, although this was a small sample size (Fig. 7.8). As all samples were run on the same gel, there were no differences between different gel trials to be investigated.

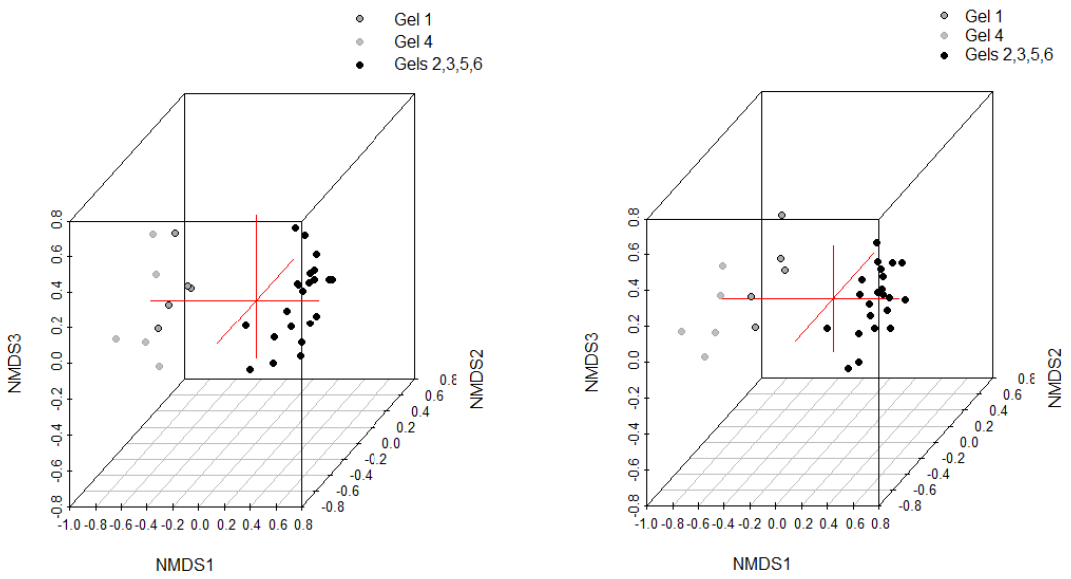

Fig. 7.7. 3D NMDS results for the harbour porpoise band group frequency (left) and volume data (right). There is some clustering by gel number. Band groups did not cluster by any other variable investigated.

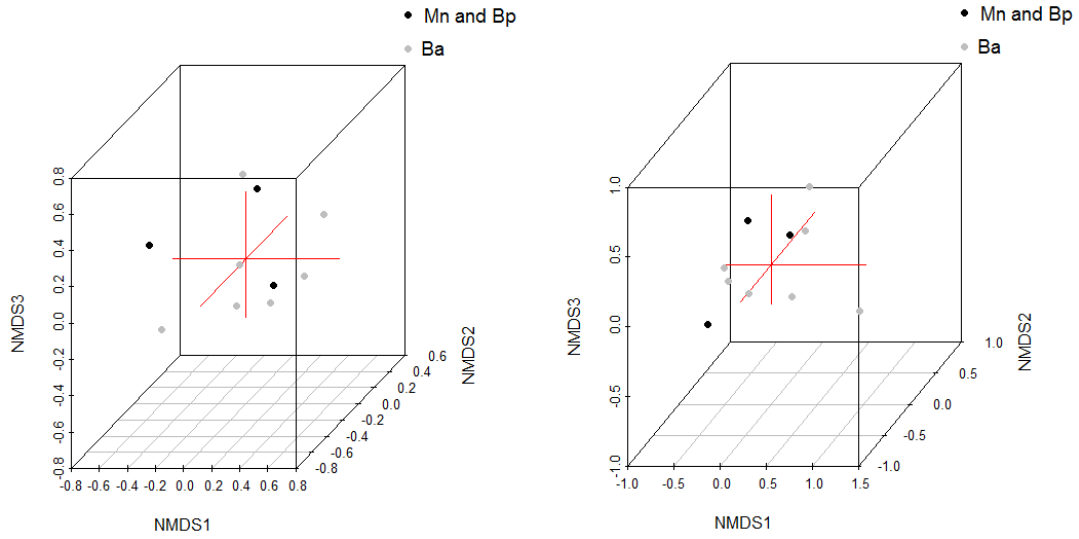

Fig. 7.8. 3D NMDS results for the balaenopterid band group frequency (left) and volume data (right). There is limited clustering by species. Band groups did not cluster by any other variable investigated.

Biopsy Samples: For the dataset of 10 minke whales, season was the only qualitative covariate to include in the analysis. For this dataset, using 3 dimensions did not reduce the NMDS stress values, so 2 dimensional scaling was used and results are shown in Fig. 7.9. There was no apparent clustering of these 10 individuals that consisted of 'early' (June and July) or 'late' (August and September) feeding season samples (Fig. 7.9). 3D NMDS results showed that, similar to the

harbour porpoise data, the clustering of band group frequencies and volumes in the humpback whale biopsy profiles was explained most strongly by the gel number on which the sample was separated (Fig. 7.10), and not by any of the other variables investigated. This clustering was most apparent for the band group volumes data (Fig. 7.10), but was not as strong as seen in the harbour porpoise data as there was still considerable overlap between extracts from individuals run on different gels (Fig. 7.10).

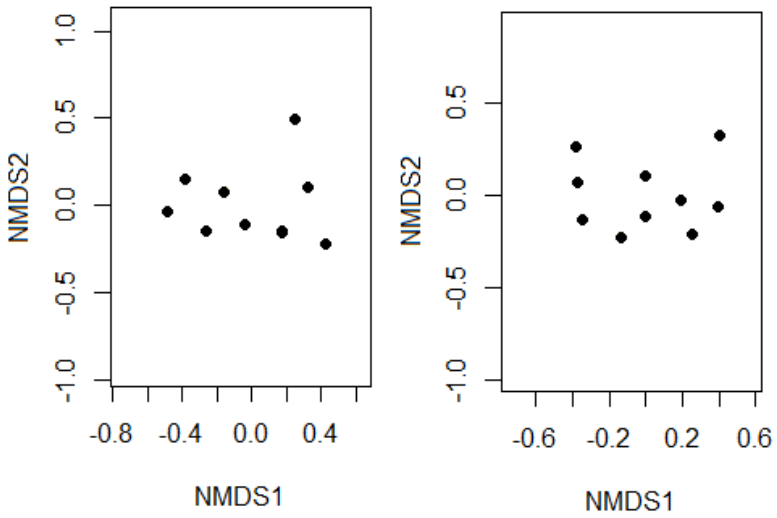

Fig. 7.9. 2D NMDS results for the minke whale biopsies band group frequency (left) and volume data (right). Neither show any clear clustering patterns.

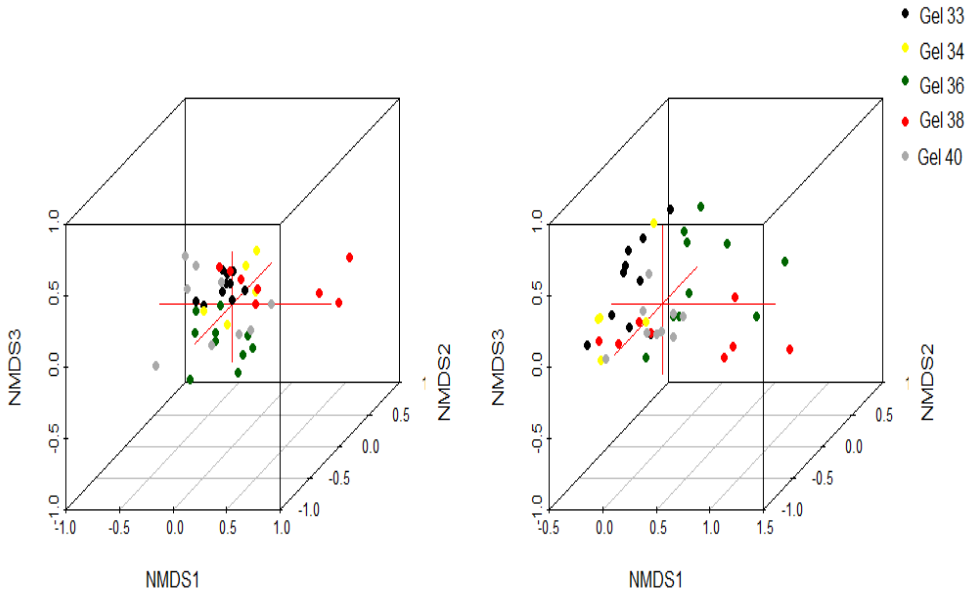

Fig. 7.10. 3D NMDS results for the humpback whale biopsies band frequency (left) and volume data (right). There was limited, clustering by gel number. Individuals did not cluster by any other variable investigated.

4.4. Linear Models

Plots of the band group frequencies and volumes with the morphometric condition indices estimated for the harbour porpoises and the humpback whales are shown in Fig. 7.11. While there was no obvious trend in the frequency or volume of certain band groups with increasing or decreasing condition, there appear to be differences in the two datasets in terms of the band groups that appeared more frequently and made up a larger proportion of the frequency and volume data than others (Fig. 7.11). For example, the 120-160kDa band group made up a large proportion of the data in the harbour porpoises, but not in the humpback whales (Fig. 7.11).

Harbour porpoise linear model: Forwards model selection showed that including the band group data did not improve model fit compared to the null model to explain variation in either mass/length^2 or blubber cortisol concentration. The inclusion of the presence/absence data for 10 of the 20 band groups improved model fit for total protein yield, six of which were individually statistically significant (p values all < 0.05), and explained almost 60% of the variation in the data (Table 7.3). Linear model checks including residual plots and checks for outliers showed that no model assumptions had been violated for this final model. This indicates that the total amount of protein extracted influenced the presence of certain band groups rather than individually relevant biological covariates.

Table 7.3 - Linear model selection results for the three dependent variables modelled against the band group presence / absence data.

Species	Dependent Variable	Sample Size	Model	R^2
Harbour porpoises	**Mass / Length^2**	32	Null	NA
	Cortisol Concentration (ng/g)	20	Null	NA
	Total Protein Yield	32	B1*; B2; B3*; B6*; B8*; B9*; B12*; B15; B18; B19	0.59
Humpback whales	**LSSAI**	24	B18*	0.16
	Cortisol Concentration (ng/g)	42	B4; B7*; B10*; B14*	0.62
	Total Protein Yield	42	Null	NA

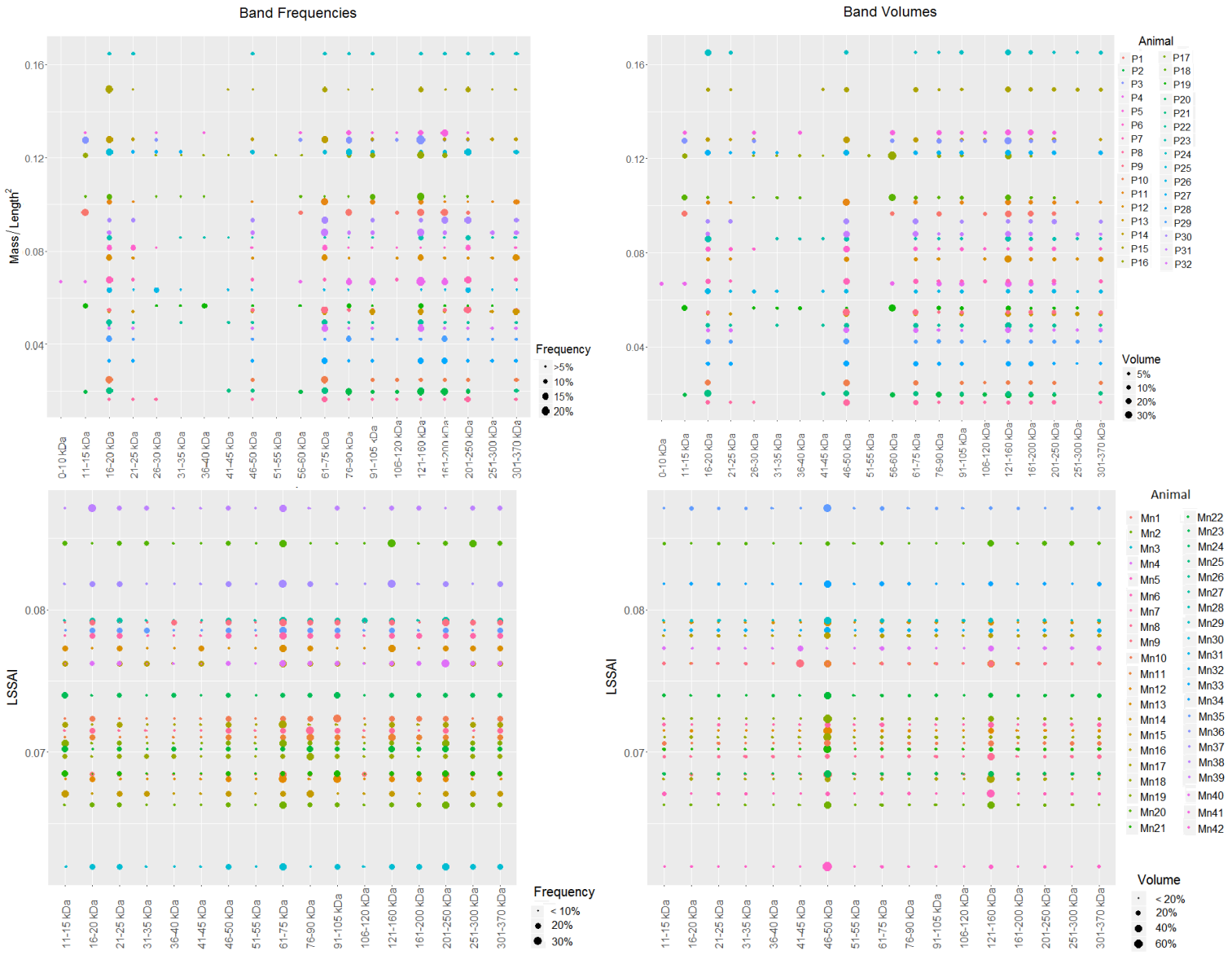

Fig. 7.11. Relative band frequencies and volumes plotted against the body condition index for the harbour porpoises (top panel) and humpback whales (bottom panel). Points are coloured by individual with the size of the points representing the relative frequency and volume for that individual. There was no clear clustering or patterns of band frequencies or volumes with animals of increasing or decreasing condition.

Humpback whale linear model: Unlike for the harbour porpoise dataset, forwards model selection showed that including the band group data did not improve model fit for the null model to explain variation in total protein yield (Table 7.3). The total protein extracted therefore had a lesser impact on the presence of the band groups in the extracts. Inclusion of the presence / absence data for band group 18 alone improved the fit of the LSSAI model, and while it was significant ($p = 0.04$), it was still a poor fit to the data with an R^2 of 0.16 (Table 7.3). Model checks showed strong clustering in the model residual plots, suggesting that other factors, not included in the model, were strongly affecting the LSSAI data and the model was thus a poor fit to these data. Finally, the inclusion of band groups 4, 7, 10 and 14 significantly improved the fit of the blubber cortisol concentration model, explaining 62% of the variation in the data (Table 7.3). These correspond to proteins of sizes between 21-25kDa, 36-40kDa, 51-55kDa and 91-105kDa for each band group, respectively. Specifically, the presence of band groups 4, 10 and 14 were seen in animals with lower blubber cortisol concentration, and band group 7 was seen in animals with higher concentrations (Fig. 7.12). Linear model checks including residual plots and checks for outliers showed that no model assumptions had been violated for this final model.

The next step was to assess what kinds of proteins were identified in these four band groups of interest from the nLC-ESI-MS/MS protein identification data (Chapter 5). The proteins identified in gel bands from the minke whale blubber extracts were used for comparative analyses. The proteins identified in the gel bands that fell within these four band groups were collated, and the functions of these identified proteins were used to generate functional profiles of each band group. Each functional protein class was assigned a proportion based on the total frequency of different proteins within that band group (e.g. 20% were tissue structure proteins, 5% were vasodilation and circulation proteins, 10% were immune function and inflammation proteins etc). The proportion of each of the ten previously described functional groups (Chapter 5) that made up the proteins identified in the four band groups of interest was then compared to the full identified protein dataset from the minke whale samples (Chapter 5). The relative proportion change in abundance of the proteins within each functional group between the full dataset and each of the four band groups was calculated (e.g. a change in the proportion of cell structure proteins from 10.6% in the full dataset to 12% in band group 4 is an overall 13.2% increase in band group 4).

As a pleiotropic hormone, cortisol is known to increase lipolysis, mobilise amino acids, and increase circulating concentrations of plasma proteins (Bergendahl et al., 1996; Exton et al., 1972). Changes in the relative abundance of proteins involved in lipid metabolism and amino acid metabolism are therefore of particular interest here in the context of differences in measured cortisol concentrations. As band groups 4, 10 and 14 are found in animals with lower cortisol concentrations (Fig. 7.12), we would expect either no change in the relative abundance of proteins involved in amino acid metabolism and lipid metabolism, or lower relative abundances in these groups compared to the full dataset. In contrast, as band group 7 is found in animals with higher cortisol concentrations (Fig. 7.12), an increase in lipid metabolism and amino acid metabolism related proteins would be expected.

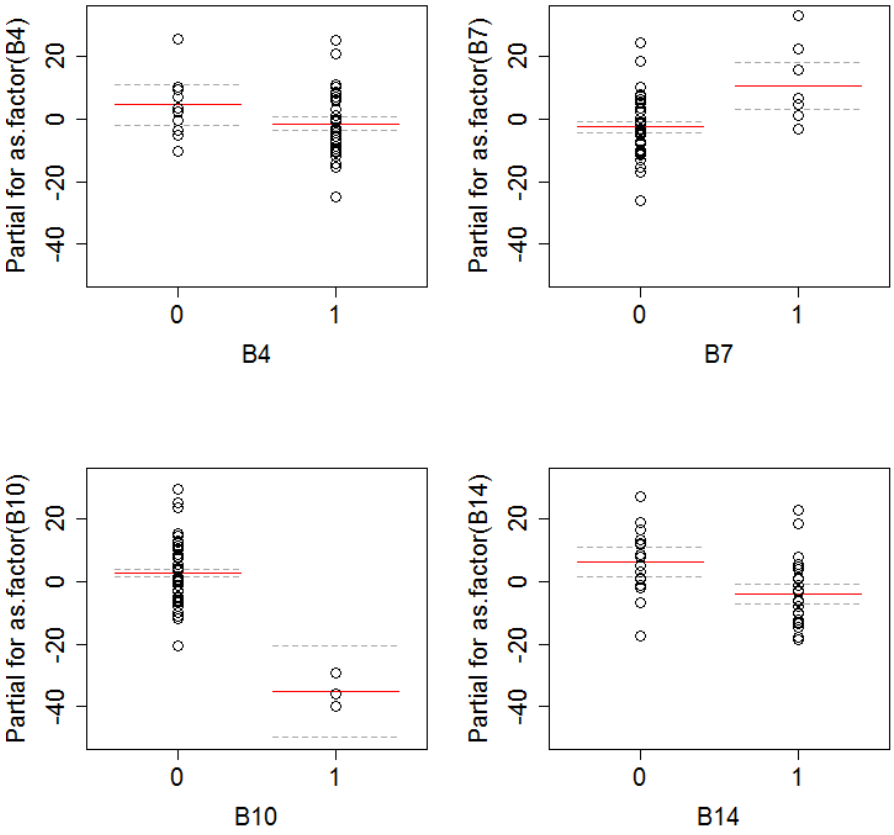

Fig. 7.12. Termplot for the final linear regression model for blubber cortisol concentration and protein band group presence / absence in humpback whale biopsies: lm(Cortisol ~ as.factor(B4) + as.factor(B7) + as.factor(B10) + as.factor(B14)). The presence of band groups 4, 10 and 14 are seen in individuals with lower cortisol concentrations, while band group 7 is seen in individuals with higher cortisol concentrations.

Changes in the relative abundance of proteins in these two functional protein groups were seen across all four band groups of interest, ranging from a ~40% decrease to a ~100% increase (Fig. 7.13). Of particular interest, as expected, was a 37.3% decrease in lipid metabolism related proteins in band group 4 with low cortisol concentrations (n = 50 proteins identified). A 34.7% and 99.9% increase in amino acid metabolism related proteins and lipid metabolism related proteins respectively in band group 7 with high cortisol concentrations (n = 42 proteins identified) (Fig. 7.13). In band groups 10 (n = 70 proteins identified) and 14 (n = 93 proteins identified) there were smaller increases and decreases in both functional protein groups of approximately 20% (Fig. 7.13). Variation in the relative abundance of proteins in other functional groups was also seen, but this is not explored here in relation to the cortisol data as there are likely many other factors that affect relative abundance of these other proteins.

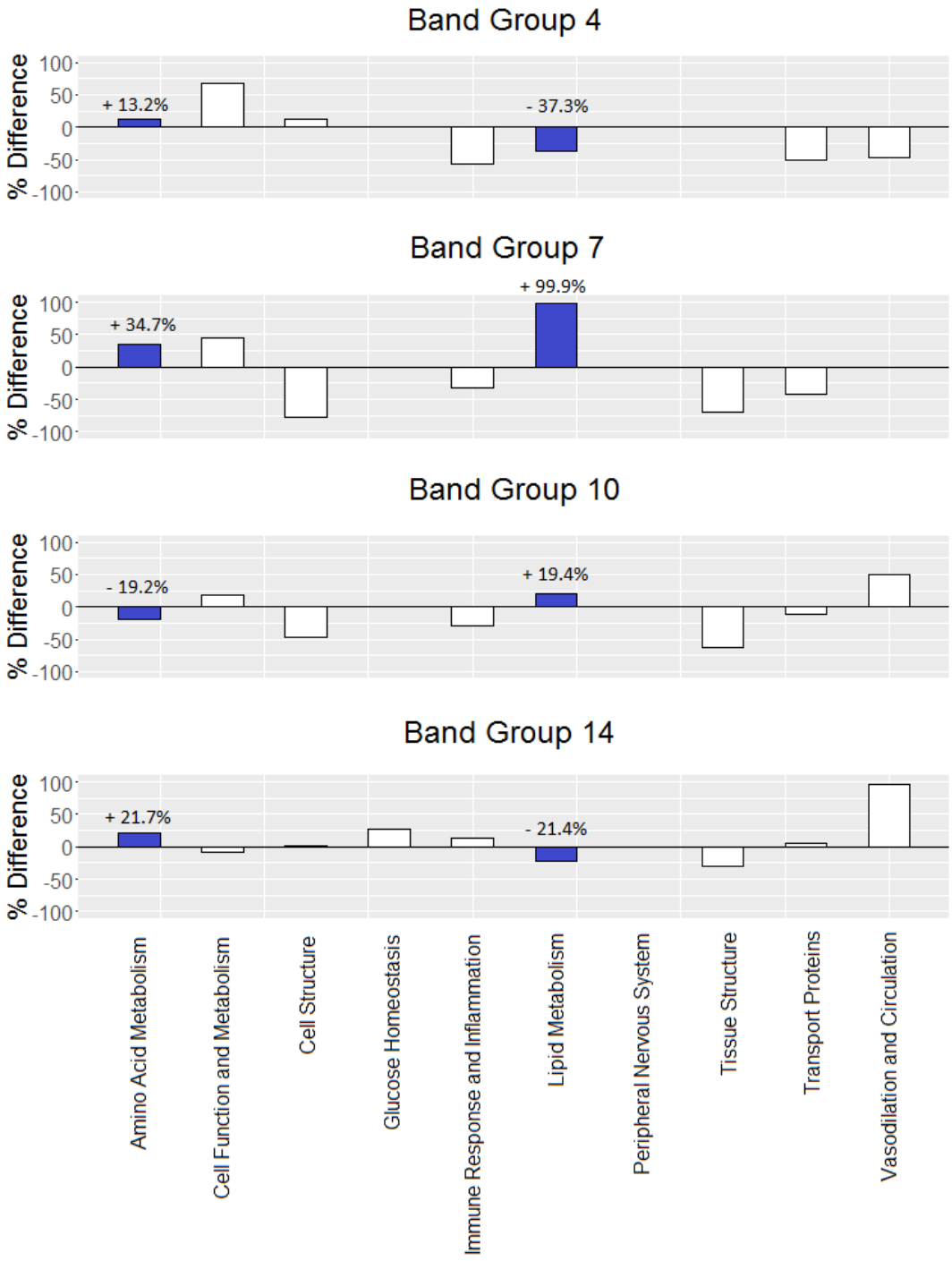

Fig. 7.13. Percentage change in relative abundance of proteins within each functional group compared to the full identified proteins dataset for band groups 4, 7, 10 and 14. Of particular interest were changes in the abundance of proteins involved in amino acid metabolism and lipid metabolism as these processes are known to be affected by circulating cortisol concentrations. The largest differences were seen in band group 7 with in an increase in the relative abundance of both of these functional group proteins.

5. DISCUSSION

This work represents a first step to explain and quantify the natural variability in the protein content of blubber tissue extracts. The variability is investigated in the context of different physiological states and life history parameters in attempts to develop this approach for the identification of useful protein biomarkers and to start to better understand whole tissue function and metabolism.

Sample Storage and Processing Confounding Factors

In terms of total protein extracted from the tissue samples, while the yield was typically high using the most efficient extraction method previously identified (Chapter 5), there was still high extraction variability measured between some of the duplicate samples. Additional sources of measurement error therefore need to be further evaluated for more robust quantitative inferences regarding total protein content of individual samples. Of particular interest would be attempts to better standardise the amount of protein extracted relative to some other marker instead of by weight of the tissue as this may reduce some of the variability. Further work to investigate the sources of this variability should be prioritised.

Three sample processing issues have also been identified that affect the protein band group profiles visualised on 1D SDS-PAGE gels. Firstly, time in storage seems to impact both the total amount of protein extracted from the samples, and also the quality of the protein band profile separation and visualisation on the gels. While the effects of freezing on blubber tissue have yet to be investigated fully, freezing meat tissue has been shown to cause lipid oxidation by lipid free radical generation which results in the production of toxic compounds (Morrissey et al., 1998). Oxidation through freezing has also been shown to change proteins through the modification of amino acid side chains, the formation of protein polymers, the reduction of solubility, an increase in carbonyl groups, a change in amino acid composition, an increase in proteolytic susceptibility and protein fragmentation or aggregation (Decker et al., 1993; Levine et al., 1990; Xiong, 2000). It is likely that some or all of these processes have occurred and altered the proteins in the older biopsy samples, which explains why the total protein yield and band profiles have been affected here. Additional factors unaccounted for throughout this analysis, such as time between sampling and sample freezing, any defrost-refreeze cycles and sample storage in plastic vials, glass vials or aluminium foil, for example, likely also affect tissue quality over time. In the future, in order to better account for these potential changes, and thus better interpret results from frozen samples, protein oxidation can be monitored as a means of assessing protein alterations by measuring the formation of carbonyl groups and the decrease in sulphydryl groups (Stadtman, 1990). It is therefore recommended that for protein content investigations, biopsies should be processed as soon as possible because prolonged storage at -20^0C appears to result in measurement artefacts associated with tissue degradation.

Secondly, the presence / absence of certain band groups in the harbour porpoise dataset helped to explain the variation in the total protein yield of different samples rather than other biologically relevant variables. The efficiency of the extraction in terms of how much protein is successfully

extracted from the tissue therefore appears to have strongly influenced the band group profiles for different individuals. This suggests that this method should be further developed to improve extraction consistency. While there were no clear trends with larger or smaller proteins being lost when less protein was extracted, some of the least abundant or least soluble proteins may not appear in these extracts.

There were no relationships between total protein yield and the presence / absence of different band groups in the humpback whale dataset, however. One possible explanation is that these were all outer blubber layer only samples, so the homogenisation and processing of the tissue was perhaps more uniform for these samples than for the full depth harbour porpoise samples. Additional sources of variation are potentially introduced depending on the relative proportions of the outer, middle and inner blubber layers that are included in the subsample. To evaluate this possibility, the difference in total protein content between blubber layers in full depth blubber samples should be investigated to assess this as a potential source of variation. The more vascularised inner blubber layer likely contributes more circulatory proteins to the blubber sample extracts, and is therefore a greater source of variability compared to the outer layer alone, for example. Another potential reason for the lack of relationship between total protein yield and the presence / absence of different band groups in the humpback whale biopsy samples, could be that the larger sample size of this dataset meant that the processing errors and sources of variation became more negligible compared to the smaller harbour porpoise dataset.

Thirdly, there appears to be some measurement error associated with running the 1D SDS-PAGE gels themselves. The harbour porpoise and the humpback whale samples were run on multiple gels, and the NMDS results showed that there was some clustering of individuals by gel number for both of these datasets. The humpback whale dataset showed this clustering to a far lesser extent. This suggests that while the running of the gels was standardised as much possible, it appears that there are sources of measurement variability inherent in this method which determine, to some extent, the band group profiles seen across individuals. For further development of this method, the same sample extracts should be assayed multiple times on different gels in order to identify and quantify the error associated with different 1D SDS-PAGE trials.

Band Group Profiles and Qualitative Variables

For all four datasets, there was no clustering of individuals based on the relative frequency or volume of their band group profiles by sex, season, cause of death, reproductive status, age class, area or species. As well as the sample processing sources of error and confounding factors mentioned above, another potential reason for the lack of any relationships in the band group profiles and the qualitative variables of interest is that these data are not of fine enough resolution to detect subtle changes in individual protein presence and abundance. The nLC-ESI MS/MS protein identification results from the harbour porpoise and the minke whale samples showed that each individual protein band is a mixture of proteins and peptides of the same size, not a single protein. This is because the extracts are a complex mixture of proteins of different sizes from different cell types. Then, for analysis purposes, the band groups are made up of a number of

individual protein bands. This means that a band group can potentially contain a huge variety of different proteins with different functions (Fig. 7.14). Thus, these band group pattern analyses are a summation of many different proteins, so subtle changes in certain proteins that are affected by the qualitative life history variables of interest are likely lost through this coarse analysis.

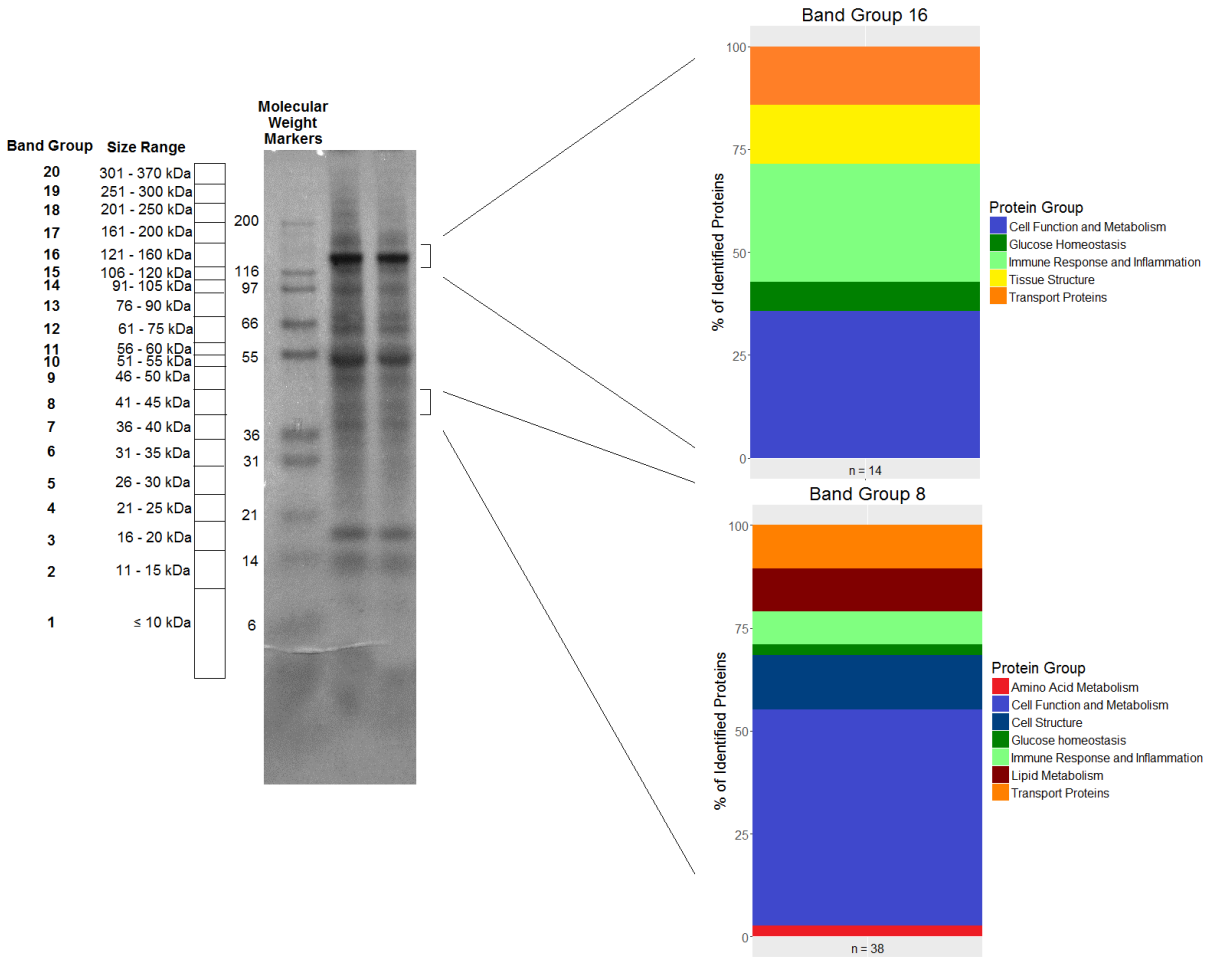

Fig. 7.14. 1D SDS-PAGE gel image to show the grouping of individual bands into band groups with associated identified proteins. The band groups numbered 1 to 20 used for NDMS analysis are shown on the left of the figure with protein identification results for band groups 8 and 16 from the harbour porpoise dataset of identified proteins shown to the right as examples. A total of 38 and 14 different proteins were identified in band groups 8 and 16 respectively. The contribution of different proteins that make up various functional groups are indicated.

Band Profiles and Quantitative Variables

The inclusion of the presence / absence data of the protein band groups did not improve model fit for the morphometric body condition (mass/length^2) or the blubber cortisol concentration models for the harbour porpoise dataset, and thus did not contribute to explaining any of the variation in these data. As discussed above, it appears that sources of variation in these data are largely driven by variation in tissue processing and analysis. Similarly, the morphometric body condition index (LSSAI) model for the humpback whales was not improved by the inclusion of band group presence/absence data. This could be because this was a small sample size of just 24 individuals with these matched data, so there was not a wide enough range in condition estimates between these individuals, and / or, as previously discussed, the protein bands data is not at a fine enough resolution to detect significant changes with overall physiological state. However, the presence / absence data for 4 band groups significantly improved the fit of the blubber cortisol concentration linear model. In a semi-quantitative assessment to explore which classes of proteins could potentially explain why the inclusion of these bands groups was important, changes in the relative abundance of different protein functional groups within these groups compared to the full dataset of identified proteins were assessed.

Overall, cortisol is a catabolic hormone which acts to increase the availability of all fuel substrates by mobilisation of glucose, free fatty-acids and amino acids from endogenous stores (Dinneen et al., 1993; Djurhuus et al., 2002). Therefore, high concentrations of cortisol seen in individuals with band group 7 in their profiles were expected to be correlated with increases in the proportion of proteins involved in lipid metabolism and amino acid metabolism. These expected increases were seen for the proteins identified within this band group, together with a decrease in lipid metabolism related proteins in band group 4 which was associated with individuals with low blubber cortisol concentrations. While this only provides a semi-quantitative investigation of the relationships between protein functional group abundance and the concentrations of other endocrine markers of interest, it does show that the application of multiple analytical methods together could lead to a better understanding of whole tissue function.

Future Directions

An important next step in the identification of different proteins and / or combinations of different proteins that can act as biomarkers of overall health and condition in cetacean blubber, is to employ a finer scale analysis. This can be achieved by using 2 dimensional PAGE to separate the proteins in blubber extracts. 2D-PAGE separates complex mixtures of proteins using two different properties of the proteins. In the first dimension, proteins are separated by their charge called the isoelectric point, and in the second dimension they are separated by their relative molecular weight (Saraswathy and Ramalingam, 2011). 2D PAGE can thus separate several thousand individual proteins in one gel. In addition, significant improvements have been made in 2D PAGE technology with the development of 2D fluorescence difference gel electrophoresis, which can be used to reduce gel-to-gel variations (Saraswathy and Ramalingam, 2011). Differentially expressed proteins can therefore be visualised by investigating different protein spot patterns and can then

be subsequently identified by mass spectrometric methods as with the 1D PAGE (Chapter 5). This would allow a higher resolution analysis of individual proteins or classes of related proteins e.g. heat shock proteins or adipokines that change in relative abundance with physiological state or life history stage.

As well as 2D-PAGE, trialling other extraction methods that are compatible with downstream protein quantification techniques should be prioritised. For example, the Minute^{TM} Total Protein Extraction Kit (Invent Biotechnologies Inc., AT-022, Plymouth, USA) for total protein extraction from animal cultured cells and tissues produces an extract that is directly compatible with EIA and ELISA quantification of specific proteins. The use of this kit was assessed here for overall total protein yield in comparison to the RIPA cell lysis and extraction buffer previously used, and was found to extract similar amounts of protein from blubber tissue (data not shown). However, when these extracts were used to quantify adiponectin concentrations, as one of the main adipokine hormones identified across multiple sample extracts by nLC-ESI MS/MS (Chapter 5), the ELISA trials did not pass quality assessment and control checks (data not shown). Further work is therefore needed to assess the cross-reactivity of cetacean adiponectin, and other adipokines of interest, with commercially available ELISAs, as well as the compatibility of the total protein extracts themselves with standard quantification assays.

Finally, another avenue of current research is to adopt a transcriptomic approach to examine changes in gene expression profiles in blubber tissue that can identify important markers and regulators of lipid metabolism and energy homeostasis (Khudyakov et al., 2017; Martinez et al., 2018). Specifically, using a non-targetted RNA sequencing approach to examine global gene expression profiles means that investigations into the molecular mechanisms involved in various physiological processes are not limited to a small set of target genes that are known to be important in terrestrial animal models (Martinez et al., 2018). The identification of upregulated and downregulated genes during different physiological states can then provide an additional resource for investigating and understanding the extreme metabolic adaptations of cetaceans, and other marine mammals, as well as markers of overall health and body condition.

CHAPTER 8: GENERAL DISCUSSION

1. Synopsis

1.1. Assessing Standard Indices of Condition

Despite its importance as a good predictor of fitness, there is currently no consensus on the 'best' way to estimate the overall body condition of cetaceans. Using data from stranded animals, I investigated morphometric indices of body condition across three cetacean families. These indices are commonly used in terrestrial ecology, and I identified the most appropriate indices as mass/length^2 and girth/length (where mass data were unavailable). Blubber thickness, the most commonly used index in cetaceans, was a consistently poor indicator of condition across all three families. Interestingly, beaked whales showed very little variability in their apparent energy stores. I used these morphometric indices to assess other potential markers of condition where a 'ground truthing' approach was required to evaluate novel biomarker methods.

Blubber lipid content was investigated as another commonly used condition index in cetaceans. Again, the variation in blubber lipid content was assessed across three cetacean families. Blubber thickness was not correlated with lipid content. This is likely because the blubber must maintain a certain thickness to fulfil its other roles, separate from energy storage, in thermoregulation, streamlining and buoyancy, for example. Therefore, the extent to which blubber can be reduced in thickness is limited. Together, the results from Chapters 1 and 2 demonstrate that using blubber thickness as a standard measure of condition should be avoided.

The lipid content analysis showed that blubber lipid content is not a suitable biomarker of body condition in these species for two reasons. Firstly, layering through the tissue means the outer layer accessible for biopsy sampling is not representative of the lipid available for mobilisation through the full blubber depth. Secondly, blubber lipid content was not correlated with morphometric indices of body condition. In keeping with the results of Chapter 1, there was little variation in lipid content both within and between the ziphiid samples, further suggesting little variation in energy stores. There may be trade-offs occurring between the different functions of blubber (energy storage, maintaining hydrodynamic shape, controlling buoyancy, contributing to water balance and preserving thermoregulatory properties) in these deep-diving species. The idea that the *main* role of the blubber is to act as an energy storage depot across all cetacean species is therefore challenged in Chapters 1 and 2. Its other roles may be of equal or even greater importance in some species depending on their reliance on endogenous energy stores during parts of their life cycle.

1.2. Investigating Novel Biomarkers of Condition

Cortisol is the main glucocorticoid hormone known to be important for the regulation of lipolysis and lipogenesis in mammals. As it has been previously suggested to be involved in the control of fasting metabolism in pinnipeds, it appeared to be a good candidate as a condition marker. Cortisol

was extracted and concentrations were measured in the blubber of stranded harbour porpoises and balaenopterids. Blubber cortisol concentrations showed a negative relationship with morphometric indices of body condition. It is suggested in Chapters 3 and 4, that concentrations of this hormone in the tissue are not a marker of an acute stress response, but are an integrated marker of physiological state. A long-term dataset of biopsy samples from female humpback whales on their feeding grounds in the North Atlantic showed significant inter-annual variation in cortisol concentrations.

Pregnancy rates were estimated for this population based on blubber progesterone concentrations. There was some evidence that inter-annual variation in pregnancy rates were negatively correlated with cortisol concentrations. Specifically, years with lower blubber cortisol concentrations corresponded to higher overall pregnancy rates. I hypothesise that this could be as a result of changing environmental conditions between years. The individual variability was probably a result of the multifunctional nature of cortisol that was not explained by differences in reproductive state, season, and a morphometric index of body condition obtained from aerial photogrammetry image analysis. Cortisol concentrations likely provide a 'snap shot' of the metabolic state of the tissue at the time of sampling.

The use of cortisol concentrations as a single marker to measure a 'snap-shot' in time highlights a general question of the applicability of using a single condition index to assess and compare animals across a range of life history stages or even different populations and ecotypes. Limiting the use of certain measures to compare within age classes, life-history stages or different environments may be more informative. In terms of life-history stage variability, using a single index with the same numerical value to compare the overall body condition of an individual at the end of a breeding season, to another individual the end of a feeding season, is meaningless unless the context in which the animal is sampled is considered. Specifically, animals should have accumulated significant energy reserves by the end of the feeding season, so two animals sampled at these different life-history stages with the same condition index value, does not mean that they are equally healthy. In terms of population-level variability, humpback whales tagged with suction cup tags in the Gulf of St Lawrence had higher overall body density values (indicative of lower total body fat stores), suggesting that they were in poorer body condition, compared to whales instrumented with the same tags in Northern Norway (Aoki, pers. comm.). However, differences in the water salinity, and therefore the density of these two aquatic environments, could have led the whales in these separate populations to adapt to an 'optimum' body density, close to neutral buoyancy, in order for locomotion, and therefore foraging, to be as efficient as possible in each environment. Thus, the use of a single body condition index, with a 'one size fits all' approach, does not apply in many cases as there are a number of other factors to consider when interpreting these estimates. For these reasons, there should be a movement towards multiple measures that, when integrated together, can provide composite indicators of body condition and overall health in order to add more detailed and robust information to condition index estimates.

Multiple other blubber biomarkers of interest that, together, reflect overall health and condition were investigated. The proteome of cetacean blubber tissue was explored for the first time. In Chapters 5 and 6, I developed a method for total protein extraction and applied it to samples from stranded animals and biopsies from live animals. Over 400 different proteins were identified and classed into ten functional groups based on their involvement in different physiological processes in terrestrial species. The most abundant proteins were involved in cell function and metabolism, immune response and inflammation, and lipid metabolism. The identified proteins are thought to have been secreted from the various cell types within the tissue, and to have accumulated from the circulation. These proteins capture the range of cellular and physiological processes experienced by individuals at the time of sampling. Protein band profiles generated through 1D SDS-PAGE across individuals of different ages, sexes, reproductive states, cause of death classes, morphometric body condition and species were compared. Analysis did not reveal any protein bands, or patterns of protein bands that explained the individual variation in protein profiles.

There was some evidence that the presence and absence of a number of protein bands were correlated with blubber cortisol concentrations in biopsy samples from humpback whales. These bands showed relative increases and decreases in lipid metabolism and amino acid metabolism compared to the full dataset of identified proteins. Using multiple analytical approaches, endocrine markers, like cortisol concentrations, can potentially be linked to downstream changes in protein abundance. A finer scale approach, using 2D PAGE for example, is necessary to identify individual proteins or classes of proteins that vary in relative abundance as a function of physiological state. The importance of furthering this proteomic approach is two-fold: Firstly, it could improve our understanding of tissue function, and its involvement in animal physiology by assigning novel roles to marine mammal blubber similar to those identified in the adipose tissue of other mammals. These include the regulation of appetite and energy balance, immune system and inflammatory responses and nutrient transport for example. Secondly, it could lead to the development of a suite of biomarkers that can be used as tools to better assess the health of live individuals though remote blubber biopsy sampling.

2. The Value of Strandings Scheme Data

One common theme through this work is that these method development and validation approaches would not have been possible without the samples and data provided by the Scottish Marine Animal Strandings Scheme. Trends in marine mammal stranding rates can provide useful information on life history parameters, seasonal and spatial species distribution, species abundance, human-induced mortality rates and disease (Gulland and Hall, 2007; Prado et al., 2016). Analysis of these spatial and temporal trends have been used with the aim of developing cost-effective ways of monitoring at-sea cetacean populations in order to provide indicators of conservation status, and thus focus conservation efforts to drive policy (McFee et al., 2006; Siebert et al., 2006).

As well as demographic data, necropsy data from stranded animals have been used to assess diet (Gannon et al., 1997; Santos et al., 2004), and contaminant concentrations in top marine predators

(Jepson et al., 2016; Law et al., 2012). The increased risk of mortality from infectious disease associated with high contaminant burdens has also been estimated from stranded animals (Hall et al., 2006). Strandings scheme data are vital for investigations of unusual mortality events and mass strandings. For example, examinations of carcasses and tissue analyses have linked unusual mortality events to harmful algal blooms (Fire et al., 2011; Lefebvre et al., 1999; Scholin et al., 2000), and the Deepwater Horizon oil spill (Venn-Watson et al., 2015; Williams et al., 2011). Studies of the gas bubble-associated lesions found in mass-stranded beaked whales following naval exercises have provided a possible explanation of the relationship between anthropogenic, acoustic (sonar) activities and the stranding of these deep diving animals (Fernández et al., 2005; Jepson et al., 2005). Finally, a collaborative effort and coordination of standings scheme resources across five countries allowed an international and comprehensive investigation into the largest sperm whale (*Physeter microcephalus*) mortality event ever recorded in the North Sea in 2016 (Ijsseldijk et al., 2018).

Strandings networks can therefore provide a unique opportunity for data collection on the biology of different species. Here, blubber tissue samples and accompanying metadata collected by the SMASS were invaluable for new biomarker method development and validation. The value of these data were three-fold. Firstly, using samples from stranded animals allowed the collection of tissue samples large enough for multiple and repeated processing and extraction methods. This overcomes the small sample mass constraints typical of biopsy samples from live animals. Importantly, different measures can then be compared for the same individuals. Secondly, samples from stranded animals allowed sampling of the full blubber depth in order to put the results of shallow depth, remotely obtained biopsy samples from live animals into context. Variation in biomarkers of interest through the full blubber depth also provides broader insights into blubber function and how this differs between species, as seen with variations in lipid content in Chapter 2. Thirdly, the metadata associated with each of the blubber samples are not available for free-ranging cetaceans. Information in terms of sex, age-class, pathological processes, cause of death and importantly, morphometric measurements allowed the variation in biomarker results to be put into life-history context.

These approaches highlight the importance of strandings scheme datasets and sample collection protocols in terms of the information that can be gathered from these efforts. Here, there were likely sources of variability in the way in which the morphometric measurements were taken. This could affect the accuracy of the long-term dataset. Personnel changes for example, or the difficulties of necropsies being performed under different conditions at the stranding site or in a laboratory, can introduce sources of measurement error. Sources of variation and measurement errors will also vary for animals of different sizes. Balaenopterids and beaked whales for example, are more logistically challenging to handle compared to the smaller porpoises and the delphinids. As a result, these errors were likely then propagated through downstream analyses. Even though the morphometric measurements and sampling protocols were standardised, it is not possible to retrospectively calculate the error associated with the morphometric measurements used here for

body condition indices calculations. These errors could have masked some relationships between the morphometric measures and other covariates of interest. Large sample sizes would help to ensure that while there will always be some measurement error, analyses are as robust as possible.

There may be some biases associated with sampling in terms of how representative dead stranded animals are of the population as a whole, and therefore how these results can be accurately extrapolated. In addition, using carcasses for sampling means that there is no control over which individuals are available for incorporation into any given study. For example, the samples from stranded balaenopterids in Chapter 2 and 3 were largely from juveniles which meant that robust analyses of differences between age classes was not possible. Of course, the issues associated with the representativeness of stranded animals, and the choice of which individuals to target for any particular study can be overcome by lethal sampling. While lethal sampling can provide huge amounts of physiological and ecological information about different species and different populations (Enoksen et al., 2016; Haug et al., 2017; Solvang et al., 2017), there are obviously ethical considerations and implications of these approaches that are not in keeping with the research objectives and ethos of many institutes and research programmes. So, even with these caveats, standings schemes can provide suiTablesamples and accompanying data for the investigation and validation of novel health markers. These investigations illustrate the need for their continuation into the future.

3. Improving our Understanding of Cetacean Physiology

The search for body condition biomarkers in cetacean blubber has led to an increased appreciation of the complexity and pleiotropic nature of blubber tissue, and by extension, further insight into the extreme physiological adaptations of these animals. Blubber is unique to marine mammals, and appreciating how its functions may contribute to physiological homeostasis can give us insights into the development and maintenance of the life-cycles and adaptations of marine mammals to their environment.

The notion that the blubber's primary function across all species is to act as an energy storage depot is too simplistic. Results from Chapters 1 and 2 demonstrate that the relationship between blubber thickness, blubber lipid content and overall body condition is not straightforward across different species. The importance of the blubber in terms of providing readily available energy reserves will likely be balanced with its roles in maintaining other optimal characteristics of the animals that allow them to occupy a particular ecological niche. The range of physiological demands on species, as a result of their ecology, will therefore affect the extent to which some functions are more important than others, and by extension, the extent to which the blubber is able to change in thickness and lipid content and still retain its structural integrity. A theoretical representation of this idea is shown in Fig. 8.1.

It appears that beaked whales are a great example of these trade-offs. The lack of variability in morphometric body condition indices or blubber lipid content shown in Chapters 1 and 2 suggests that these species may be adapted to live within very narrow physiological constraints, perhaps

driven by their extreme diving behaviour. Clues for why this may be the case come from previously published evidence of high wax ester content of the blubber. Other functional roles of the tissue in thermoregulation and buoyancy control that allow them to make continuous, prolonged, deep foraging dives (Tyack et al., 2006), unique to only a small number of cetacean species that share these blubber characteristics (Koopman, 2007), appear to be more important than energy storage. In contrast, baleen whales are able to take advantage of spatially and temporally separated breeding and feeding habitats through extreme fasting endurance. These species are therefore uniquely adapted to survive prolonged fasting periods where they rely heavily on energy reserves in the blubber.

Other, 'intermediate' species between these two extremes include some of the delphinidae family for example, and a number of pressures likely affect the blubber composition of the animals in this broad group. For example, hydrodynamic drivers that are responsible for high intensity or 'sprint' prey capture behaviours in some species (Aguilar Soto et al. 2008), will affect overall body shape through the deposition and mobilisation of the blubber to achieve optimum swimming efficiency (Koopman, 2007). Equally, some species will experience huge seasonal variation in ambient water temperatures which will dictate the extent to which the blubber layer is increased or reduced, and its conductance continually altered in line with the animals' thermoregulatory capacities and/or requirements (Fougeres et al., 2008; Noren et al., 2009; Koopman, 2007). There are likely very complex relationships between body size, thermal habitat, diving behaviour, lipid stratification, blubber thickness, reliance on endogenous energy reserves and overall metabolism that affect the structural and chemical composition of the tissue.

Marine mammal species that have highly energetically costly life history strategies, including long distance migrations (Burton and Koch, 1999; Lockyer, 1981), prolonged breeding seasons (Boness et al., 2002), and an annual moult in many pinniped species (McConkey et al., 2002; Thompson and Rothery, 1987; Worthy et al., 1992), undergo extreme physiological changes. However, an understanding of the regulatory pathways and how the blubber plays a role in allowing animals to readily meet these extreme, cyclical demands on their energy stores is limited. The functional adaptations governing these highly energetically costly life-history strategies warrant further investigation. With the results from Chapters 5 and 6 that identified, for the first time, a large number of proteins involved in various metabolic pathways in blubber tissue, I suggest that it may be involved in the regulation of a number of paracrine and endocrine metabolic processes, similar to the roles of white adipose tissue in terrestrial mammals (Ahima and Flier, 2000; Kershaw and Flier, 2004).

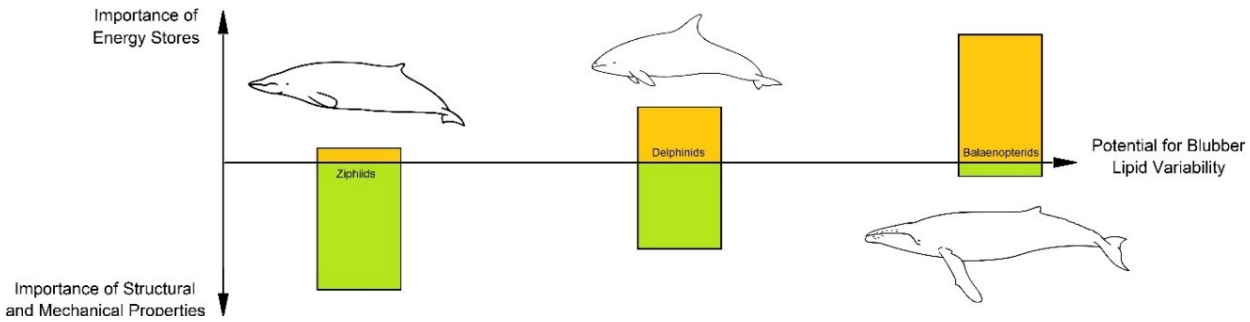

Fig. 8.1. Theoretical schematic demonstrating the relative importance of different blubber functions across three cetacean families with respect to the extent to which the tissue can vary in total lipid content. It is hypothesised that ziphiids prioritise the preservation of a constant blubber structure and lipid content to maintain the hydrodynamic, insulative and buoyant properties of the tissue. Balaenopterids however, show huge amounts of variability in blubber lipid content both within and between individuals, likely because they rely on blubber lipid stores during prolonged fasting periods of their life-cycles. Artwork by Chris Huh.

It is still not known whether marine mammal blubber produces and secretes hormones in the same way, and to the same extent as other mammalian adipose tissues. If it does, its importance in energy regulation is not only through the storage of dietary fats, but also through the secretion of hormones, such as cortisol and adiponectin, that are involved in the control of lipid metabolism, fasting energetics and the regulation of physiological processes that take place during periods of high energy demand. Recent evidence suggests that there is metabolism of corticosteroids in the blubber with the interconversion of cortisol and cortisone (Galligan et al., 2018). Cetacean blubber is therefore likely a site of active steroid metabolism. The localised production of hormones involved in lipid metabolism may help to explain how cetaceans have evolved an enhanced capacity for inhibiting unrestricted lipolysis and are able to finely control the lipid content of the tissue (Wang et al., 2015).

It is likely that there are a wide range of cellular and tissue-specific processes occurring constantly, and simultaneously in the blubber, and this work has only begun to scratch the surface as to the complexity of the tissue. As the blubber makes up such a large proportion of the total body mass in cetaceans, and other marine mammals, it is possible that the other roles of adipose tissue in the regulation of appetite and energy balance, the immune response, inflammation and the acute-phase response, blood pressure, nutrient transport and haemostasis (Trayhurn and Wood, 2004), contribute greatly to the overall metabolism of these animals. In fact, if blubber does act as an endocrine and paracrine organ, its ability to contribute to whole body homeostasis during different parts of the life cycle could help to explain some of the extreme life-history strategies characteristic of some species. For example, finely controlled metabolism of available lipid stores could contribute to the extreme, long-term fasting endurance of baleen whales (Chivers, 2009).

Endocrine signalling from the tissue could contribute to the mechanisms that signal the end of fasting and the initiation of feeding at the end of the extremely intense lactation periods of many female phocid seals (Schulz and Bowen, 2004). Finally, and very speculatively, combinations of the ability of the tissue to regulate vasoconstriction and vasodilation, together with the immune response and localised coordination of inflammatory and cellular defences, could help to explain how marine mammals are able to sustain severe injuries, often of anthropogenic origin, and survive (Barcenas-De la Cruz et al., 2017; Neilson et al., 2009).

These are speculative links between whole animal physiology and blubber tissue function, so further investigations of how the blubber contributes to different homeostatic processes are required. A major step forward in efforts to better understand blubber function is the recent use of blubber explants to investigate its metabolism in grey seals (*Halichoerus grypus*) (Bennett et al., 2017). Adipose explants are small pieces of tissue taken from live animals. Cells within the tissue are kept alive in a culture medium in order to maintain structure. Cells are therefore able to better retain their original metabolic characteristics compared to individual cells cultured in isolation. Experimental manipulations of these explants can be used to investigate adipose regulation in wildlife species when opportunities for whole organism experimental work are limited, as is the case for marine mammals (Bennett et al., 2017). Short term explant culture may therefore be a viable method to complement whole animal studies to improve our understanding of the molecular and cellular physiology of adipose tissue in general (Bennett et al., 2017). Future work should build on developing these novel experimental approaches to explore the pleiotropic nature of the blubber, and how it contributes to the extreme diving, fasting and breeding physiology of many marine mammal species.

4. Sentinels of Ecosystem Health

Widespread environmental changes have been documented as a result of climate change. Polar and sub-polar regions are particularly vulnerable environments, and the most accelerated changes have been documented in these areas (Moritz et al., 2002; Wassmann et al., 2011). Continued monitoring of environmental conditions in these environments is therefore of particular importance, but the remoteness of these habitats makes regular surveys and data collection logistically challenging. Thus, instead of attempting to estimate prey species biomass for example, or measure complex changes in environmental conditions through limited and costly surveys of these high latitude areas, a robust and cost-effective alternative is to focus on the integrated response that changes in prey availability elicit in dependent predator species, such as marine mammals. Estimating and monitoring the nutritive condition and reproductive success of these animals can therefore provide vital information on the ecosystem productivity and environmental quality (Bengtson Nash et al., 2018). In order for this to be effective, however, markers of individual health, reproductive status and body condition need to be identified and applied appropriately across populations. Continued efforts to develop heath and condition markers that can be used for large-scale analysis are therefore very important. Time-series data, like the

longitudinal sampling of humpback whales in the Gulf of St Lawrence presented in Chapter 4, will also be invaluable for identifying changes to, or disruption of marine ecosystem processes.

As well as using marine mammal health as an indicator of ecosystem productivity, an increasing number of studies have demonstrated the value of using marine mammal tissues to investigate the occurrence and concentrations of contaminants in the marine environment (Hoekstra et al., 2003). Despite regulations limiting the extent to which many harmful contaminants are released into freshwater and marine ecosystems, because of their longevity, many contaminants, including persistent organic pollutants for example, are still present at high concentrations in the marine environment (Tanabe et al., 1994). The impact of historic and current exposure on marine top predators is still largely unknown (Jepson et al., 2016). Measuring the contaminant burdens in the blubber of these species is therefore important in terms of assessing the potential risks to individual health associated with high contaminant burdens (Kannan et al., 2000) that could have population level impacts. It is also important in terms of long-term monitoring of the prevalence and persistence of contaminants in the ecosystem. The development of biomarkers that can be used to monitor the potential health effects of these contaminants will also be of particular interest as they have been linked to population declines in some species (Jepson et al., 2016).

5. Future Directions

Marine mammals face a wide range of potential anthropogenic stressors including, but not limited to, chemical and acoustic pollution, habitat loss, reduced prey availability and bycatch (Fair and Becker, 2000). There is therefore an urgent need to identify new approaches to improve our ability to estimate the potential cumulative effects of such anthropogenic stressors on marine mammal populations. Population Consequences of Multiple Stressors (PCoMS) models aim to link exposure to different stressors to behavioural changes (foraging, mating, vocalising etc.) which lead to physiological changes that can then affect overall health (energy stores, immune status, parasite load etc.) (Tyack et al., 2017). Changes in the health of individuals will ultimately lead to changes in vital rates of the population (survival, fecundity, age at first reproduction) (Tyack et al., 2017). These processes are all linked, and realistic predictions of the consequences of the cumulative effects of multiple stressors at a population level relies on our ability to accurately measure each of them. Currently, there is a gap in our knowledge of how to best measure health parameters in free-ranging marine mammals, particularly cetaceans. The development of biomarkers, not just of body condition, but also of contaminant exposure, immune response and disease processes would help to start to close this gap.

Recent advances in molecular sequencing as well as biochemical detection techniques offer the potential for the identification of suitable biomarkers of health and body condition using ‘omics’ approaches. Specifically, the detection of mRNA (transcriptomics) to investigate gene expression, proteins (proteomics) to investigate cellular and tissue processes, and metabolites (metabolomics) to investigate whole organism homeostasis (Horgan and Kenny, 2011), will likely prove to be particularly powerful tools. Ultimately, interdisciplinary approaches and collaborations that combine expertise across the fields of marine mammal science will provide the most innovative

opportunities for method development and optimisation. For example, the use of aerial photogrammetry techniques (Christiansen et al., 2018) combined with endocrine profiling (Pallin et al., 2018b), biomarker identification, and body composition analyses through tagging (Narazaki et al., 2018) could provide valuable physiological and ecological insight.

In keeping with the importance of inter-disciplinary approaches, an important next step to better predict the cumulative effects of multiple stressors, including the effects of climate change, is to combine long-term oceanographic, environmental data with markers of marine mammal health and condition. As top marine predators, and therefore sentinel species of ecosystem health, this work is important for the assessment of population resilience to environmental change. There is a timely and emerging need for such interdisciplinary studies to look at the 'bigger picture' as we aim to understand the cumulative effects of stressors on marine mammals, and what this could mean in terms of ecosystem health both now and in the future. From a conservation perspective, knowledge of wildlife population health and ecology is fundamental for formulating conservation policy, however, there is little information on how the health of populations of species living in particularly vulnerable environments. Research efforts should be focused in these areas.

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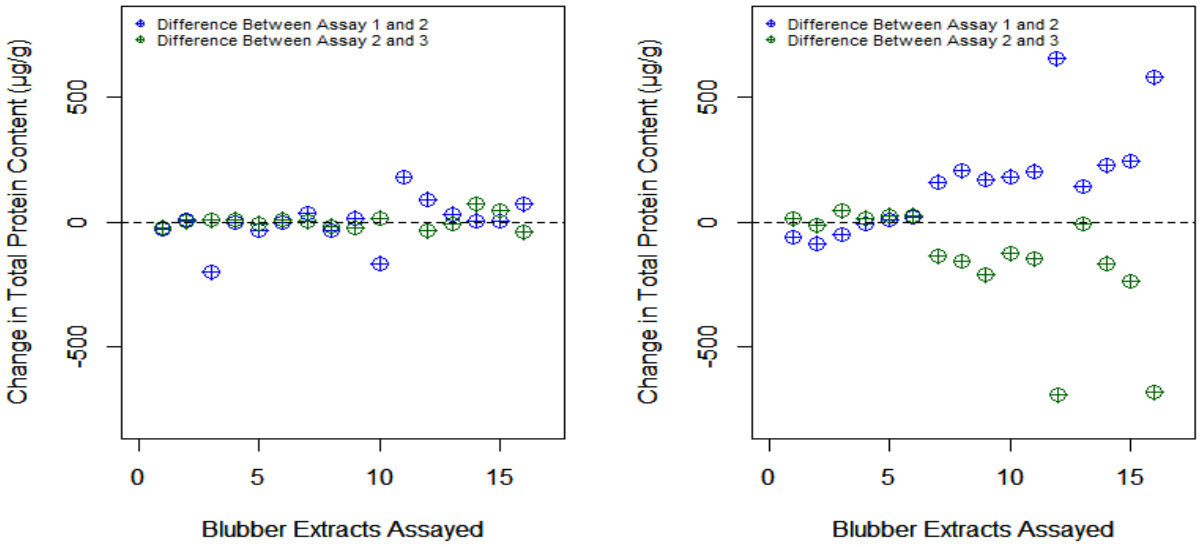
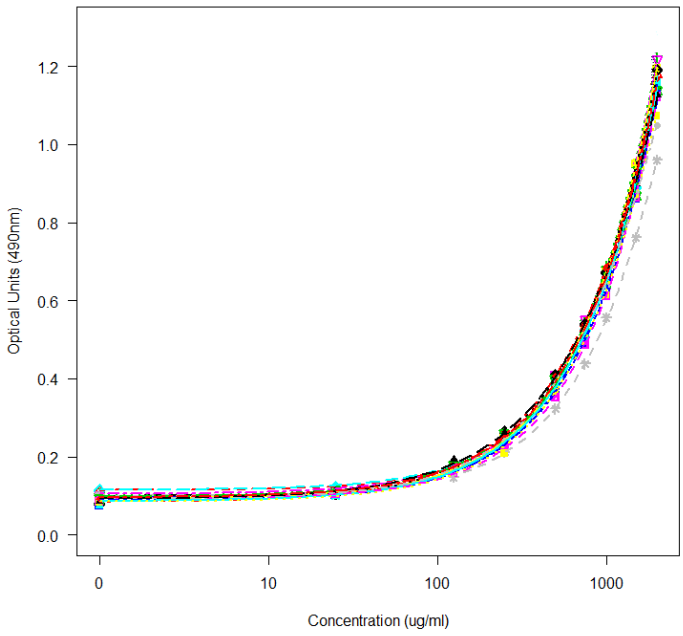
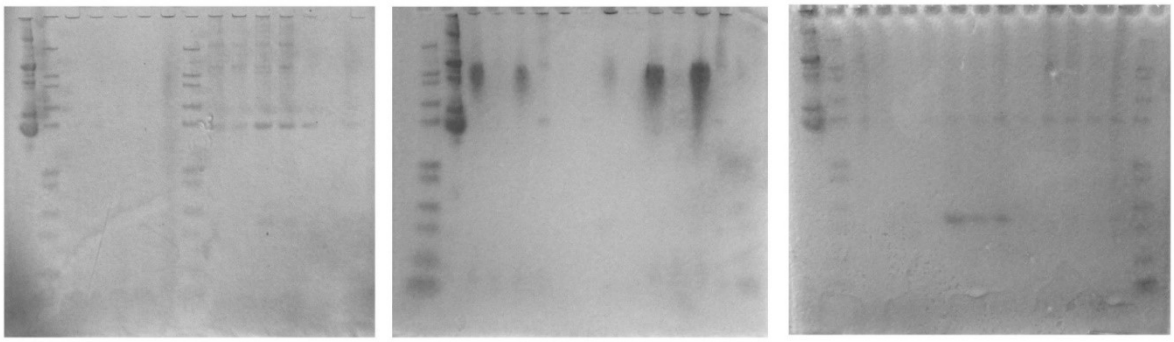
SUPPLEMENTARY MATERIAL



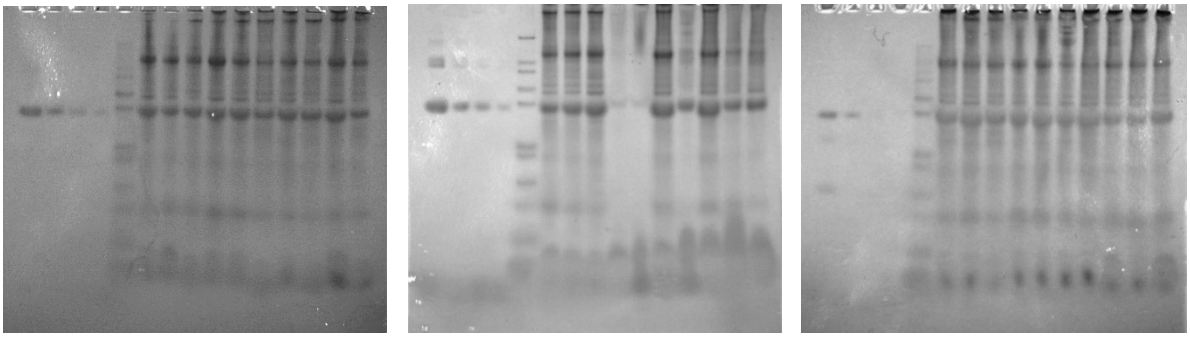
Supplementary Fig. 1. Variation in total protein concentration measurements in the TCA:Acetone (left) and the Methanol:Chloroform extracts (right) between consecutive Pierce™ BCA Protein Assays (23225, Thermo Scientific, Rockford, USA). There was no obvious trend in change in total protein content between consecutive assays for the TCA:Acetone extracts, but the Methanol:Chloroform extracts showed a consistent increase or decrease between assays suggesting that protein resuspension in solution was a problem for these extracts.



Supplementary Fig. 2. Total protein standard curves across the different Pierce™ BCA Protein Assays (23225, Thermo Scientific, Rockford, USA) used for total protein quantification in the extracts processed using methods 1 and 2. Standard curves were almost identical between assays, so measurement error as a result of the assay procedure or the reagents are unlikely to be the cause of the measurement variability in the sample extracts.



Supplementary Fig. 3. Example images of 1D SDS-PAGE analysis of harbour porpoise blubber tissue extracts processed using Methods 1 and 2 on 4-12% Bis-Tris gels stained with Bio-Safe Coomassie brilliant blue. Extracts show few protein bands only across the larger size range. There is high background and some protein smearing. In addition, there is poor reproducibility of bands across extracts and across gels.



Supplementary Fig. 4. Example images of 1D SDS-PAGE analysis of harbour porpoise blubber tissue extracts processed using Method 3 on 4-12% Bis-Tris gels stained with Bio-Safe Coomassie brilliant blue. Extracts show both more, and more consistent bands across the full size range down the gels.