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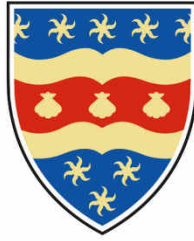
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Can we distinguish eustress from distress in marine mammals?

Trade-offs in expression of molecular stress markers and

consequences for cell damage and whole-animal fitness measures

in grey seals (*Halichoerus grypus*)

by

Holly Clare Armstrong

A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

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- Society for Experimental Biology Annual Main Meeting, Brighton, UK, July 2016. **Oral presentation.** 'Anti- and pro-oxidant gene expression and oxidative damage in the blubber tissue of grey seal (*Halichoerus grypus*) pups during suckling and the post weaning fast.'

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Abstract

CAN WE DISTINGUISH EUSTRESS FROM DISTRESS IN MARINE MAMMALS? TRADE-OFFS IN EXPRESSION OF MOLECULAR STRESS MARKERS AND CONSEQUENCES FOR CELL DAMAGE AND WHOLE-ANIMAL FITNESS MEASURES IN GREY SEALS (HALICHOERUS GRYPUS)

Holly Clare Armstrong

The ability to respond adequately to stress is crucial to an organism's survival and fitness. Stress influences reproductive success, development, energy balance and immune responses and thus shapes life histories, population dynamics and evolutionary trajectories. Little is known about the links between cellular and whole-animal physiological stress responses in marine mammals, and their impact on tissue damage and fitness. Although stress responses may be activated under specific conditions, we need to recognise that the induction alone does not always represent a state of distress. If we are to understand the role of stress as a driver of individual health, population dynamics and life history trajectories we need to be able to distinguish between eustress and distress at the whole-animal and cell levels. This thesis aimed to investigate the causes of cellular stress responses in tissues essential to fitness and survival of grey seal adult females and pups, which are routinely exposed to natural and anthropogenic stressors, and consequences for cell damage and trade-offs in energy investment at the whole-animal level.

Grey seals experience numerous physiologically stressful states throughout development and adulthood that induce cellular defences in other species. This includes fasting during the energetically demanding lactation and developmental periods, and large seasonal fluctuations in fat stores with consequently significant

variations in adipocyte size. Individual cellular stress responses to 'life-style' stressors may affect grey seals' ability to cope with additional stress, such as disease, disturbance and contaminants in prey.

The studies in this thesis investigated the nutritional and developmental changes in gene expression of key cellular defences, heat shock proteins (HSPs) and redox enzymes (REs), and to explore the role of intrinsic and extrinsic stressors in their induction at the gene expression level, in two vital tissues, blubber and leukocytes. Malondialdehyde (MDA) concentration (a marker of oxidative damage) was also used to measure tissue-level damage in blubber. Chapter 1 reviews current knowledge regarding eustress and distress in tissues and cells of marine mammals, with a focus on grey seals. Chapter 2 provides detailed methods that are utilised in this thesis to answer questions regarding causes of cellular stress and consequences for tissue function and whole animal health, filling knowledge gaps identified by Chapter 1. Chapter 3 and Chapter 4 consider relative gene expression of cellular stress markers (HSPs and REs) in blubber of breeding and pre-breeding females and pups during suckling and the post-weaning fast, respectively, because blubber is a key tissue in energy balance. Chapter 5 examines the same gene expression in leukocytes of both lactating-fasting females and pups, because animals may experience a trade-off between stress responses and immune function when resources are limited. These chapters identify nutritional and developmental causes of expression of cellular defences, and consider relationships with markers of tissue damage (MDA concentration) and whole-animal fitness. Chapter 6 used an experimental approach to examine causal relationships between GC and PCBs, and HSP gene expression in blubber. Identifying vulnerable life-history stages of grey seals, when animals experience specific additional stressors as 'distress', requires

correct interpretation of markers of cellular stress. This is essential to potentially

inform management of seal populations by predicting population level consequences

of exposure to specific stressors.

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Abbreviations

ABTS	2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AhR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
ANOVA	Analysis of variance
ATF	Activating transcription factor
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
BAT	Brown adipose tissue
BHT	Butylated hydroxytoluene
BiP	Immunoglobulin binding protein
BKI	*BestKeeper* index
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CAT	Catalase
CBG	Corticosteroid- binding globulin
CD	Cluster of differentiation antigen
cDNA	Complementary deoxyribonucleic acid
CGD	Chronic granulomatous disease
CHIP	Carboxy terminus of Hsc70 interacting protein
CoQ	Coenzyme Q

CSR	Cellular stress response
C_T	Cycle threshold value
CycA	Cyclin A
CYP	Cytochrome P450 enzyme
DAG	Diacylglycerol
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
ECM	Extracellular matrix
E. coli	*Escherichia coli* strain DH5α
EDTA	Ethylenediaminetetraacetic acid
E.L	Early lactation
EPWF	Early post-weaning fast
ER	Endoplasmic reticulum
EROD	Ethoxyresorufin-O-deethylase
E.S	Early suckling
ESR	Oestrogen receptor
ETC	Electron transport chain
Exo/Exo I	Exonuclease I
FA	Fatty acid
FBS	Fetal bovine serum
FFA	Free fatty acids
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoid

GC-MS	Gas chromatography-mass spectrometry
gDNA	Genomic deoxyribonucleic acid
GOI	Gene of interest
GPx	Glutathione peroxidase-3
GR	Glucocorticoid receptor
GRP	Glucose regulated protein
GSH	Glutathione
GSSG	Glutathione disulphide
GST	Glutathione-S-transferase alpha 4
HC	Hydrocortisone
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1α	Hypoxia-inducible transcription factor-1α
HNE	4-hydroxynonenal
H_2O_2	Hydrogen peroxide
HPA	Hypothalamic-pituitary-adrenal axis
HSC	Heat shock cognate
HSE	Heat shock element
HSF	Heat shock transcription factor
HSL	Hormone - sensitive lipase
HSP	Heat shock protein
HSR	Heat shock response
Ig	Immunoglobulin
IGF – 1	Insulin-like growth factor 1
IL	Interleukin
IoM	Isle of May

IP$_3$	Inositol 1, 4, 5-triphosphate
IRE1 - α	Inositol-requiring enzyme 1 alpha
kDa	Kilodalton
L$^\bullet$	Lipid radical
LB	Luria-Bertani
L.L	Late lactation
LME	Linear mixed-effect model
LOO$^\bullet$	Lipid peroxyl radical
LOOH	Lipid hydroperoxide
LP	Lipid peroxidation
LPWF	Late post-weaning fast
L.S	Late suckling
L8	Ribosomal protein L8
MDA	Malondialdehyde
MHC	Major histocompatibility complex
miRNA	Micro RNA
MPPM	Maternal postpartum mass
MR	Metabolic rate
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NO	Nitric oxide
Nox4	Nicotinamide adenine dinucleotide phosphate oxidase 4
O$_2^{\bullet-}$	Superoxide anion
OH$^\bullet$	Hydroxyl radical

ONOO⁻	Peroxynitrites
PAH	Polycyclic aromatic hydrocarbons
PBMC	Peripheral blood mononuclear cells
PCA	Perchloric acid
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PERK	Protein kinase R-like endoplasmic reticulum kinase
PKA; C	Protein kinase A; C
PMNC	Polymorphonuclear cells
PMNL	Polymorphonuclear leukocyte
Poldip2	DNA polymerase delta interacting protein 2
POP	Persistent organic pollutant
PPAR	Peroxisome proliferator - activated receptor
PR	Progesterone receptor
PTM	Post translational modification
RBC	Red blood cells
RE	Redox enzymes
RMR	Resting metabolic rate
RNA	Ribonucleic acid
ROS	Reactive oxygen species
mRNA	Messenger ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RS	Reactive species
SAP	Shrimp alkaline phosphatase

SCOS	Special Committee on Seals
SD	Standard deviation
SES	Southern elephant seal
sHSP	Small heat shock protein
SMRU	Sea Mammal Research Unit
SNS	Sympathetic nervous system
SOD	Superoxide dismutase-2 (MnSOD)
S9	Ribosomal protein S9
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
qRT- PCR	Real-Time quantitative reverse transcription polymerase chain reaction
UCP	Uncoupling protein
UPR	Unfolded protein response
UV	Ultraviolet
UXT	Ubiquitously expressed prefoldin-like chaperone
WAT	White adipose tissue
WBC	White blood cells
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

Chapter 1.
General Introduction

1. General Introduction

The ability to respond adequately to stress is crucial to an organism's survival and fitness (Müllner *et al.*, 2004; Blas *et al.*, 2007; Busch and Hayward, 2009; Goutte *et al.*, 2010). Stress influences reproductive success (Fossi *et al.*, 2003; Wingfield and Sapolsky, 2003; Jessop *et al.*, 2004), development (Lupien *et al.*, 2009), energy balance (Hayase and Takeuchi, 1986; Harris *et al.*, 1998; 2006; du Dot *et al.*, 2009) and immune responses (Monjan and Collector, 1977; Lahvis *et al.*, 1995; De Swart *et al.*, 1995; Padgett and Glaser, 2003) and thus shapes life histories, population dynamics and evolutionary trajectories.

Cannon (1932) and Seyle (1936) provided a foundation for the study of stress and the stress response, by developing the concept of homeostasis (Cannon, 1932) and the general adaptation syndrome (Seyle, 1936). Homeostasis was defined by McEwen and Wingfield (2003) as 'the stability of physiological systems that maintain life…maintained within [an optimal] range'. The definition implies consistency with a range of specific 'target values' for a number of physiological variables (e.g. glucose levels, body temperature, pH, oxygen tension). Cannon extended his concept of homeostasis by introducing the theory of the 'fight or flight response'. Also known as the 'acute stress response', it describes the reaction of an organism to a change in the external or internal environment that threatens homeostasis, by activating the sympathetic nervous system (SNS), which stimulates the adrenal gland to release epinephrine. This initially acts to recover homeostasis by accelerating heart rate, constricting blood vessels and raising blood pressure. Using this 'fight or flight response', Seyle went on to describe a general adaptation syndrome, as the non-

specific response of the body to any abnormal demand made upon it (Seyle, 1936; 1950), which recognised the fight or flight response as its first phase.

However, it is essential that the response to situations that threaten homeostasis, the stress response, is adaptive. Processes involved in the stress response need to enable an organism to cope with and adapt to changes in its environment that are perceived as stressful.

Allostasis is defined as the process that supports homeostasis and achieves stability through change (Sterling and Eyer, 1988; McEwen, 1998; McEwen and Wingfield, 2003). Allostasis allows the organism to maintain homeostasis by altering the 'target values' of key physiological variables; thereby adapting to an altered internal or external environment. 'Allostatic load' is the sum of all the adjustments an individual has to make to meet a number of challenges (e.g. the energetic requirements of reproduction added to the energetic requirements of a biological stress response). When the individual can meet the additional demands, it often becomes more resilient to current and subsequent stressors, and experiences eustress. If the energy requirements of all the adjustments exceed the capacity of the animal to replace that energy, then the individual experiences 'allostatic overload' and the deleterious consequences of distress, such as reduced health, fitness and survival chances (Romero, 2004). Using the above descriptions of homeostasis and allostasis as a foundation, this thesis will consider the definition of stress to be: a physiological response (at the whole-animal or cellular level) triggered by various stimuli that changes the 'normal' environment and disrupts homeostasis.

Mounting a stress response is energetically costly and costs occur at cell and whole-animal levels (Parsons, 1994; 2005; Segerstrom, 2007).

1.1 Energetic component of stress

Energy is allocated to essential life-history tasks such as growth, immunity, reproduction and survival (Gillooly *et al.*, 2001; Brown *et al.*, 2004; Pettersen *et al.*, 2018). Stress responses are energy demanding processes that alter metabolic demand and often divert energy away from other fundamental activities to deal with immediate requirements. Energy balance at the whole-animal and cellular level is therefore altered and organisms face trade-offs in allocation to energy-consuming biochemical and physiological cellular processes (Hardie and Hawley, 2001; Hardie, 2008; Romero *et al.*, 2009; Rabasa and Dickson, 2016). Energy is acquired and allocated to different tasks through metabolism, the network of biochemical reactions taking place in living cells which process energy and resources and convert them into life-sustaining products (Gillooly *et al.*, 2001; Brown *et al.*, 2004). Gillooly *et al.* (2001) defined metabolic rate (MR) as 'the rate at which organisms transform [these] energy and materials' and can be viewed as the capacity to perform life-history tasks. MR (e.g. basal/resting, standard) reflects an organism's capacity for growth, maintenance and reproduction (Pettersen *et al.*, 2018).

1.1.1 Allometric constraints to metabolism

As body mass increases, MR increases (i.e. there is a positive relationship between mammalian body mass and MR; Schmidt-Nielsen, 1984; White and Seymour, 2003), such that larger animals require more energy to sustain them. However, when considering metabolic rate per unit body size, this relationship changes; per unit body mass metabolic rate is higher in smaller animals, compared to that of larger animals (Schmidt-Nielsen, 1984). Thus smaller animals need more energy per unit mass.

1.1.2 Energetic costs of reproduction: maternal energy intake to support lactation

Reproduction is energetically costly and the reproductive output of wild animals is dependent upon the ability to acquire sufficient resources and allocate energy appropriately to support reproductive effort and reduce fitness trade-offs (Gittleman and Thompson, 1988; McNab, 2006; Speakman, 2008). This may affect their ability to withstand external and internal challenges, such as changes in their local environment and tolerating competition and predation, impacting fitness and survivorship (McNab, 2006; Speakman, 2008).

In mammals, reproductive costs are high due to pre-natal (gestational) and post-natal (lactation and maternal care) activities, both of which incur high metabolic costs (Gittleman and Thompson, 1988; McNab, 2006; Speakman, 2008). Energy has to be allocated effectively to meet both immediate costs (i.e. those associated with female maintenance and fetal growth) and those associated with energy storage (capital). Of all energetically demanding processes, lactation represents the most energetically demanding and expensive physiological state in a female mammal's life cycle and maternal investment in offspring during this time is higher than during gestation (Oftedal, 1984; Gittleman and Thompson, 1988; Prentice and Prentice, 1988; Clutton-Brock *et al.*, 1989; Butte and King, 2005). Lactation therefore represents a key period when female animals may face substantial trade-offs between allocation of energy to lactation and energy to stress responses, if required.

During lactation, energetic requirements can increase by two to five-fold (Oftedal, 1984; Gittleman and Thompson, 1988; Prentice and Prentice, 1988). Maintaining positive energy balance is essential for animals that can feed during lactation, because adequate energy reserves can determine reproductive fitness and

survival probability of the individual and its offspring (Calow, 1979). As income breeders, most lactating female mammals normally increase food intake to meet their increased metabolic demand (Oftedal, 1984; Gittleman and Thompson, 1988; Prentice and Prentice, 1988). This is true of rats (Cripps and Williams, 1975; Naismith *et al.*, 1982), guinea pigs (Künkele and Trillmich, 1997), ruminents (Arnold, 1985) including mountain goats (*Oreamnos americanus*; Hamel and Côté, 2009), koalas (*Phascolarctos cinereus*; Krockenberger, 2003), cheetahs (*Acinonyx jubatus*; Laurenson, 1995) and humans and other primates (Dufour and Suther, 2002) in which calorific intake can increase by up to 250% (Cripps and Williams, 1975; Zheng *et al.*, 2015) during lactation. In lactating humans' resting metabolic rate (RMR) and postprandial (i.e. after ingesting food) MR both increase, however with no major changes in metabolic efficiency compared to non-lactating females (Spaaij *et al.*, 1994), showing that the increased demand is directed to milk production and associated processes. Reproductive success is dependent upon the female's ability to bear energy costs of pregnancy and lactation, which in the case of pinnipeds, may be affected by body fat deposition and use of body fat when fasting.

Most phocid seals, including grey seals (*Halichoerus grypus*), are capital breeders meaning they support the energetic cost of reproduction by using body energy stores accumulated in advance of the breeding season (Jönsson, 1997; Boyd, 2000; Houston *et al.*, 2007). Smaller phocids, including the harbour seal, have adopted an income breeding strategy as their smaller body size means they cannot store enough energy to power them through the lactation period, if they are not supplementing energy intake. Most capital breeders, including grey seals (*Halichoerus grypus*), fast during lactation (Iverson *et al.*, 1993, 1995; Mellish *et al.*, 2000) without any apparent detrimental effects. This unusual strategy is undertaken

only by bears, phocid seals and baleen whales (Fowler *et al.*, 2014). Female phocid seals rely heavily on fat catabolism to meet metabolic demand (Fedak and Anderson 1982; Anderson and Fedak 1987; Reilly 1991; Iverson *et al.*, 1993, 1995; Mellish *et al.*, 2000; Bowen *et al.*, 2001). If trade-offs between energy allocation to stress response and reproduction are occurring, we might expect animals to be particularly vulnerable. Grey seals therefore represent an interesting and useful model in which to investigate the trade-off between allocation of limited resources to cellular defence and reproduction, and whether this is experienced as eustress or distress.

1.2 The physiology of pregnancy, lactation and weaning in pinnipeds

The annual reproductive cycle of pinnipeds can be divided into three basic phases: oestrus (when mating occurs), embryonic diapause (delayed implantation) and foetal growth and development (pregnancy after implantation; Figure 1.1).

1.2.1 Pregnancy

Significant physiological and anatomical changes occur during pregnancy to support the developing foetus and prepare the mother for labour, delivery and lactation (Lockitch, 1997; Soma-Pillay *et al.*, 2016). In mammals, gestation is generally associated with, amongst other physiological changes, higher blood volume and increased resting metabolic rate (RMR; Prince, 1982; Hytten, 1986; Lavigne *et al.*, 1986; Thompson and Nicholl, 1986). Mating systems (parturition and mating) are seasonal and highly synchronised in pinnipeds (Stirling, 1975; Cassini, 1999; Pomeroy, 2011). Pregnancies typically consist of five key stages: conception, delayed implantation, embryo reactivation and implantation, foetal development and parturition (Pomeroy, 2011; Figure 1.1).
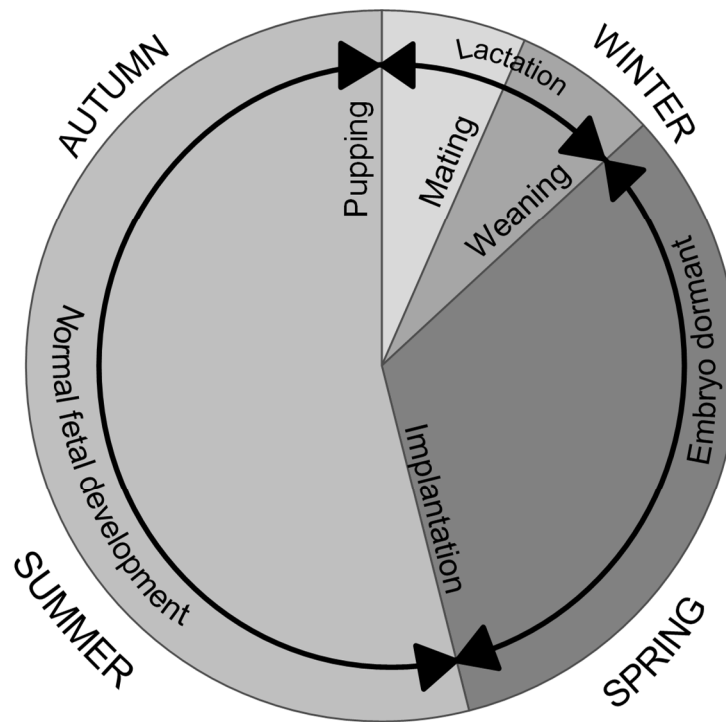
Figure 1.1 General annual reproductive cycle of pinnipeds, including lactation, delayed implantation and gestation. Adapted from Sumich (1996).

All species of pinnipeds are annual breeders, experience post-partum oestrus (except perhaps Weddell seals; Shero *et al.*, 2015) and delayed implantation (or embryonic diapause) and usually produce a single offspring at each reproductive attempt (despite massive variation in adult body size between pinniped species). Delayed implantation means time of birth can be determined by ecological/environmental factors that promote the survival of the offspring. This also enables females to give birth at the same time each year. Grey seals are the focal pinniped species in the studies that make up this thesis. Grey seal females have a gestation of approximately 9 months, exhibit site fidelity and give birth quickly (in comparison to other mammals; Pomeroy, 2011). Females enter oestrus near or after the end of lactation (Bowen, 1991). Males of the species play no part in pup rearing.

Grey seals are long-lived and males can live for over 20 years and though sexually mature at around age 6, do not begin to breed until around age 10, largely due to their inability to compete for occupancy of areas of the breeding colony close to fertile, receptive females and successfully weather aggressive encounters until this time (Hewer, 1964; Hammill and Gosselin, 1995; Tinker *et al.*, 1995; Lidgard *et al.*, 2005). Females commonly live beyond 30 years and reach sexual maturity around age five, with their first pup born between age five and six (Hewer, 1964; Hammill and Gosselin, 1995). The breeding season for grey seals in the UK is between August and December (SCOS, 2014). Grey seals, as for other pinnipeds, require a solid substrate for parturition (Bartholomew, 1970; Stirling, 1983; Tinker *et al.*, 1995). Breeding colonies encompass a wide variety of habitats including open sandy or pebbled beaches, rocky coastlines or grassy tops of remote, largely uninhabited islands (Antonelis, 2009).

In pinnipeds, little detail is known about specific anatomical and hormonal changes and energetic costs that occur during gestation, as females undergo pregnancy whilst diving and foraging at sea, which makes them difficult to study (Hückstädt *et al.*, 2018). Hückstädt *et al.* (2018) investigated diving behaviour of instrumented confirmed pregnant and non-pregnant northern elephant seals (*Mirounga angustirostris*) and found a potential cost of pregnancy, whereby diving capacity (i.e. time spent underwater) was reduced during the later stages of gestation, likely as a result of increased fetal oxygen demand. In contrast, previous studies of northern (Le Boeuf, 1994; Hassrick *et al.* 2010) and southern elephant seals (SES; *Mirounga leonina*; Campagna *et al.*, 1995) failed to find differences in diving patterns (e.g. dive duration; Hassrick *et al.*, 2010) between pregnant and non-pregnant individuals. Campagna *et al.* (1995) were limited by available technology

and were only able to transmit tag data for two and a half months, the first trimester of SES gestation, when Hückstädt *et al.* (2018) also found no difference between pregnant and non-pregnant individuals (Hückstädt *et al.*, 2018 classified individuals as pregnant if they successfully returned to land and gave birth to a pup or non-pregnant if they did not give birth).

The research that has been undertaken appears to show differences in energetic costs and diving behaviour of pregnant females. This could have implications for the management of different pinniped species (i.e. foraging areas that may be of importance to females during pregnancy) and help identify potentially vulnerable life-history states, when resource limitation may impact on energy allocation to key processes, including stress responses. This highlights the requirement for more in depth research into the physiology and energetics of pregnancy in pinnipeds.

1.2.2 Lactation

Milk is a complex biochemical secretion that provides the exclusive source of nutrients and bioactive molecules (including immunoglobulins, growth factors and metabolic hormones), essential for adequate growth, development and health of neonatal mammals (Capuco and Akers, 2009; Power and Schulkin, 2013). The process of milk production by female mammals is known as lactation and neonates are dependent upon maternal milk supply until they are weaned or begin feeding independently (whilst still receiving milk). Milk is synthesised in the mammary gland from dietary substrates or stored physiological reserves, such as adipose tissue (Boyd, 1998). Across the diverse range of mammalian species there are large differences in milk composition and output, length of lactation and frequency of

nursing (Appendix Table A4.1). These differences are likely to have evolved as a result of body size, nutrient requirements of the offspring, environmental pressures and the physiological constraints of the mother.

Pinnipeds are no exception and exhibit a diverse range of lactation strategies. Phocids, or true seals, have the shortest lactation periods of any of the placental mammals (Schultz and Bowen, 2004). As capital breeders, most phocids, including grey seals, transfer large amounts of energy via lipid-rich milk from the mother to the pup during an intense lactation period (lasting an average 4-50 days depending on the species; Oftedal *et al.*, 1987; Boness & Bowen, 1996; Costa,1991; Schulz & Bowen, 2004; Shero *et al.*, 2018). Phocid mothers fast on land or pack-ice and remain with their relatively inactive pups, increasing the opportunity to suckle. This allows pups to sequester large stores of subcutaneous blubber. Breeding grey seal females haul out onto land at ~33% body fat (Beck *et al.*, 2003) to give birth a single pup which is intensively suckled for 18-21 days (Fedak and Anderson 1982; Nordøy *et al.*, 1990; Pomeroy *et al.*, 1999) on milk which consists of 40-60% fat (Iverson *et al.*, 1993; Figure 1.3). During this period of simultaneous lactating and fasting, females lose approximately 40% of initial mass (Pomeroy *et al.*, 1999) and 61-84% of their fat reserves (Fedak and Anderson, 1982; Baker *et al.*, 1995; Figure 1.2). During suckling, pups gain mass at a rate of ~2.5 kg day^{-1} and triple in body mass, due primarily to the accumulation of subcutaneous blubber (Fedak and Anderson, 1982; Anderson and Fedak, 1987; Mellish *et al.*, 1999). Pups are also exposed to environmental toxins during suckling, as females transfer toxins to their pups via milk (Debier *et al.*, 2003).
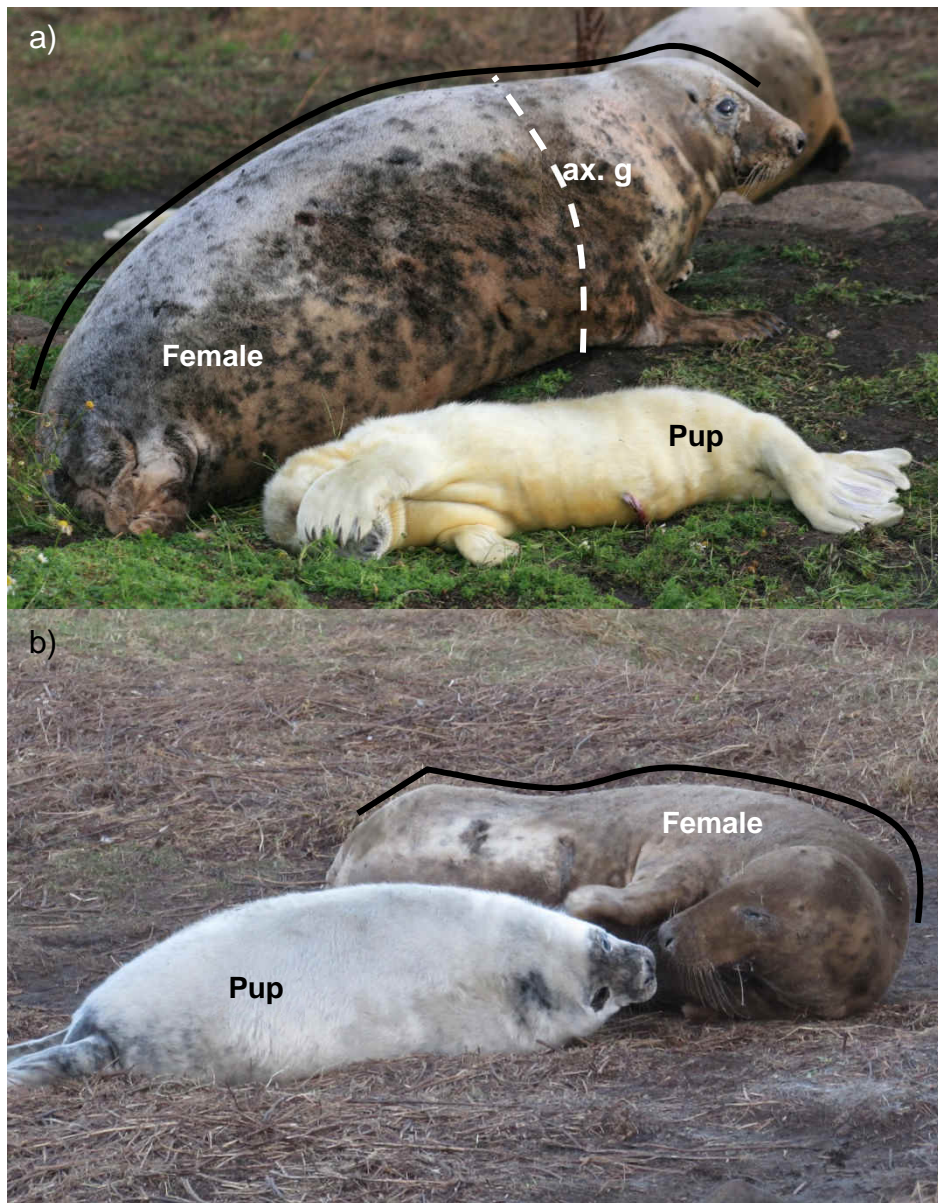
Figure 1.2. Grey seal female and pup, at a) early in lactation (the umbilicus of the pup is still visible suggesting the pup is between one and three days old) and b) late in lactation (pup is approximately 15 days old as image was taken at a LATE suckling/lactation capture) on the Isle of May breeding colony. Note the visibly increased size of the pup, largely due to the accumulation of subcutaneous blubber (but also increased general lean mass) over the suckling period. Mother's fat reserves are also visually decreased (see black lines, marking overall change in body outline). White dashed line on image a) indicates axial girth (ax. g) measurement. Images are not of the same mother-pup pair. Scale average lengths calculated using Isle of May (IoM) 2013 morphometric data. Image a) is courtesy of Kimberley Bennett.

Pups are weaned abruptly (Oftedal *et al.*, 1987; Reilly, 1991) when females return to sea to feed. Pups may not have entered the water at the point of weaning.

In contrast, otariids, which include fur seals and sea lions, and odobenids (the walruses), are income breeders and suckle pups over a much longer period of time (four to 12 months or more, depending on the species) with less energetically-rich milk. During lactation mothers regularly forage for extended periods, whilst pups are left to fast on land. Maternal care also involves extended swimming and diving with pups. Pups spend time in the water swimming and diving with their mothers during these extended/longer lactation periods.

Schultz and Bowen (2005) demonstrated in a phylogenetic analysis of pinnipeds and lactation strategies, that larger body mass was negatively related to lactation length (i.e. the larger the body mass, the shorter the lactation period).

Appendix 4, Table A4.1 shows how milk composition varies between pinnipeds and other mammals, depending upon life-history strategies.

1.2.3 Weaning

Weaning is a physiological event that terminates and/or reduces the input of maternal resources and is obligatory in the development of mammalian offspring (Mandalaywala *et al.*, 2014). Weaning is considered a stressful event for young mammals of various species (including pigs, rats and mice) and in cases of abrupt and/or early weaning events has been associated with intestinal and immune system dysfunctions (Campbell *et al.*, 2013).

Most phocid pups, including grey seals, are weaned abruptly and must learn to forage independently. Grey seal pups enter a post weaning fast of 10 days to > 4

weeks (Reilly 1991; Noren *et al.*, 2008; Bennett *et al.*, 2010). After weaning, the majority of phocids pups fast on land and rely upon endogenous energy reserves, mainly adipose tissue, accumulated whilst sucking (Reilly 1991; Noren *et al.*, 2008; Bennett *et al.*, 2010), before they are able to forage for themselves. During this time pups also experience physiological changes that allow them to develop diving capabilities and access marine prey resources; this includes increasing oxygen stores in both blood and muscle etc. (Bennett *et al.*, 2013).

The reduced volume of blubber fat that occurs in both fasting females and pups allows anthropogenic toxins to become concentrated (Debier *et al.*, 2003).

Grey seals naturally experience a physiologically stressful life history, which for adult female includes fasting during the energetically costly lactation period. Outside of the breeding season, grey seal females accumulate substantial fat reserves, which for other species is a stressful physiological state. Grey seal pups develop in the face of energy excess during suckling, then energy deficit when they are weaned and enter the post-weaning fast. Adipose tissue is the main source of metabolic fuel whilst fasting and is a major site of capital storage. Blubber therefore experiences dramatic seasonal changes in volume. Top predators are also regarded as good sentinels for ecosystem health (Sergio *et al.*, 2008; Estes *et al.*, 2011). Grey seals therefore represent an interesting and useful model in which to investigate the trade-off between the allocation of limited resources to stress responses and reproduction and development.

1.3 The multifunctional roles of blubber in energy provision, lactogenesis and thermoregulation

Adipose tissue is a remarkably complex secretory and metabolic organ (Trayhurn and Beattie, 2001; Kershaw and Flier, 2004; Trayhurn and Wood, 2005; Berry *et al.*, 2013). In mammals, there are two distinct types of adipose tissue that differ in histological appearance, anatomical distribution, function and molecular signature (Cousin *et al.*, 1992; May *et al.*, 2017). Brown adipose tissue (BAT) also generates heat, dissipates energy (Cannon and Nedergaard, 2003; Kajimura *et al.*, 2010) and is characterised by the presence of numerous mitochondria and high levels of expression of uncoupling protein 1 (UCP1; the protein that controls non-shivering thermogenesis; Lowell and Spiegelman, 2000; Ouellet *et al.*, 2012). Though most commonly associated with newborn infants (to help maintain normal body temperature), it has been established that BAT is also present in healthy adult humans (Virtanen *et al.*, 2009). White adipose tissue (WAT) is the predominant adipose tissue in mammals, and the tissue type considered in this study. It performs a number of vital functions that includes chemical energy storage in the form of triglycerides, which can be mobilised as an energy source (by hormone-sensitive lipase (HSL) action resulting in the release of fatty acids (FAs)) during resource limited periods, such as fasting (Sztalryd and Kraemer, 1994; Kraemer and Shen, 2002; Duncan *et al.*, 2007). In more recent times, adipose tissue has been identified as a key endocrine organ, involved in both the secretion and metabolism of hormones (adipokines, such as leptin and adiponectin; Zhang *et al.*, 1994; Scherer *et al.*, 1995; and steroid hormones such as cortisol; Stimson *et al.*, 2009; Hughes *et al.*, 2010) and cytokines (such as TNF-α and IL-6; Hotamisligil *et al.*, 1993), which control inflammatory responses, glucose homeostasis, insulin sensitivity, appetite

and energy balance (Kershaw and Flier, 2004; Kwok *et al.*, 2016; Wang and Scherer, 2016). In addition, WAT provides insulation against cold and protection against physical trauma (Berry *et al.*, 2013). Adipose tissue is important for fitness and survival in wildlife (Young, 1976; Barboza *et al.*, 2009). Since many wildlife species undergo profound changes in body condition as a result of life history cycles, such as moulting, migration, and reproduction, they are unlikely to have the same body fat regulation mechanisms as humans and other model species (Speakman *et al.*, 2008; Houser *et al.*, 2013; Fowler *et al.*, 2018; Halsey, 2018).

Adipose tissue is of great importance for marine mammals, providing a source of metabolic fuel, insulation, buoyancy and streamlining (Irving and Hart, 1957; Young, 1976; Koopman *et al.*, 2002; Strandberg *et al.*, 2008). Adipose tissue is essential for survival and fitness for different sexes and age classes (Pomeroy *et al.*, 1999; Hall *et al.*, 2002; Lidgard *et al.*, 2005). Blubber closer to the muscle, and a blood supply, is believed to be more metabolically active than blubber that is adjacent to the epidermis (Strandberg et al., 2008). Fasting seals largely use fatty acid oxidation to meet the energetic costs of fasting (Fedak and Anderson 1982; Anderson and Fedak 1987; Reilly 1991; Iverson et al., 1993, 1995; Mellish et al., 2000; Bowen et al., 2001) and blubber closest to the blood supply is more likely to be catabolised first. In addition blubber exhibits stratification in fatty acid composition (Koopman et al., 2007; Strandberg *et al.*, 2008) and contaminant load and type (Debier et al., 2003). Therefore ascertaining whether there was a difference in the expression of genes of interest in the inner and outer blubber core was important.

Size and distribution of adipocytes, which vary in harbour porpoise blubber cores (Koopman *et al.*, 2002), may also be a factor that has the potential to influence gene expression.

1.4 Endogenous sources of cellular stress: free radical production in tissues

In most metazoan cell types RS production is one of the major sources of cellular stress (Benhar *et al.*, 2002; Schieber and Chandel, 2014). RS with variable reactivities (Boveris, 1998), which include superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$), are generated as an unavoidable consequence of aerobic respiration and ATP production in the mitochondria. In the inner mitochondrial membrane the electron transport chain (ETC) is the primary source of RS. Briefly, the ETC is comprised of a series of electron carriers (enzymes and co-enzymes, known as 'complexes'), which accept and then donate electrons, resulting in energy production that stimulates ATP formation via oxidative phosphorylation (Lenaz *et al.*, 2006; Murphy, 2009). $O_2^{\bullet-}$ is produced when O_2 is reduced by one-electron donors (Murphy, 2009). In mammals, complex I catalyses the first step of the ETC: NADH (Nicotinamide adenine dinucleotide) is oxidised which transfers electrons to Coenzyme Q, also known as ubiquinone (CoQ; an electron carrier residing in the lipid bilayer of the inner mitochondrial membrane; Lenaz *et al.*, 2006). The resulting reduction of O_2 leads to the formation of $O_2^{\bullet-}$. CoQ transfers electrons to complex III via oxidation and reduction of quinone, via the Q cycle. Complex III also generates $O_2^{\bullet-}$ (Matsuzaki *et al.*, 2009). Cytochrome c proteins, which are part of complex III, accept electrons from CoQ and transfer them to complex IV, while pumping protons across the membrane. As the iron ion core of complex III is oxidised and reduced, electrons pass through. Complex IV, the final electron carrier in cellular aerobic respiration, is reduced by cytochrome c proteins and passes electrons to oxygen. Oxygen is held by cytochrome proteins c, a, and a3 until it is completely reduced. Oxygen then picks up two hydrogen ions and forms water. The resultant free energy from the electron transfer causes four protons to

move into the mitochondrial matrix. Oxygen is reduced and the proton gradient across the mitochondrial membrane allows ATP synthase to form ATP (Matsuzaki *et al.*, 2009; Murphy, 2009; Sun *et al.*, 2018). The highly reactive $O_2^{\bullet-}$ radicals that result from the ETC go on to form the less reactive H_2O_2 and $OH^{\bullet}$, which are directly linked to oxidative stress.

Oxidative stress occurs when there is an imbalance between RS generation and the capacity of the cells intracellular machinery to remove them (Betteridge, 2000; Valko *et al.*, 2007). RS play a key role in normal cell signalling pathways (Thannickal and Fanburg, 2000; Hancock *et al.*, 2001; Veal and Day, 2011; Delaunay-Moisan and Appenzeller-Herzog, 2015), however, when present at high concentrations, RS attack macromolecules and cause widespread damage to lipids, DNA and proteins (Halliwell and Gutteridge, 1999; Marnett, 1999; Valko *et al.*, 2007; Birben *et al.*, 2012). The unpaired electron in RS attacks macromolecular double bonds, and can decrease the functional ability and subsequent survival of cells and tissues (Hancock, 1997; Forman and Torres, 2001). RS can also be generated by phagocytic cells (Babior *et al.*, 2002), in response to high glucose levels and free fatty acids (FFAs; Inoguchi *et al.*, 2000) and the induction of 'pro-oxidants' such as the nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidases (Noxs; Brandes *et al.*, 2014). High glucose levels and FFAs contribute to an elevated push force through the ETC that can exceed the capacity of electron removal by oxygen and redox enzymes (Naudi *et al.*, 2012).

The process of uncoupling or proton leak in mitochondria can be mediated by uncoupling proteins (UCPs; Azzu and Brand, 2009). UCPs are a sub-family of mitochondrial solute carrier proteins that are believed to have a variety of functions, including adaptive thermogenesis, metabolic and energy balance and regulation,

adaptation to fasting, glucose sensing and decreasing RS production (Brand and

Esteves, 2005). UCP-1 is responsible for catalysing adaptive thermogenesis in

mammalian BAT, by increasing proton conductance of the inner mitochondrial

membrane (Cannon and Nedergaard, 2004). The leak of protons through UCP-1

uncouples the substrate oxidation of ADP to ATP, leading to rapid oxygen

consumption and subsequent heat production (Brand and Esteves, 2005). UCP-2

and UCP-3 are paralogues of UCP-1 and, rather than being involved in

thermogenesis, have been implicated in lowering RS production by 'mild' uncoupling

(Brand *et al.*, 2004). During 'mild' uncoupling there is: a limited increase in proton

conductance, protonmotive force is slightly lowered, respiration rate slightly

increased and ATP can still be synthesised (as opposed to full coupling where

proton motive force and ATP synthesis do not occur and respiration is maximal;

Brand and Esteves, 2005). This attenuates mitochondrial RS production and protects

against RS- related damage to cells (Rolfe and Brand, 1997; Vidal-Puig *et al.*, 2000;

Brand and Esteves, 2005).

Other RS include nitric oxide (NO) and peroxynitrites ($ONOO^-$). NO is

synthesised from the amino acid l-arginine by NO synthases. NO can diffuse rapidly

across membranes, exerting wide ranging effects (Wilson and Garthwaite, 2009).

NO has an unusual signalling mechanism, whereby signalling depends on the

chemical reaction of NO with protein targets (Lane and Gross, 1999; Thomas *et al.*,

2015). The covalent modifications of target proteins can result in a stable altered

protein structure, meaning NO can be involved in both normal and pathophysiology

(for example, ischemia-reperfusion injury; Lane and Gross, 1999). $ONOO^-$ is a

powerful oxidant and is formed as a result the reaction between NO and $O_2^{\bullet-}$

(Beckman *et al.*, 1994; Trujillo *et al.*, 2010). $ONOO^-$ is capable of modifying protein

function by oxidation and/or nitration of amino acids, such as cysteine, tyrosine and methionine, changing their physical and chemical properties (Gabrusiewicz *et al.*, 2017). Reactions of ONOO⁻ with proteins promote aggregation and turnover and disrupt signalling (Trujillo *et al.*, 2010). Therefore, NO and ONOO⁻ represent additional stressors that, at high concentrations, have the potential to impact cell function by altering proteostasis.

1.5 Environmental sources of cellular stress: POP and PCB accumulation in lipids

Persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs), cause chemical stress in cells. Due to their lipophilic properties POPs are found at high concentrations in lipid-rich tissues of contaminated organisms (Kawai *et al.*, 1988; Müllerova and Kopecký, 2007). Unsurprisingly, the accumulation of POPs in adipose tissue represents a significant internal reservoir of such contaminants (Yu *et al.*, 2011; Bourez *et al.*, 2012). In marine food chains, this phenomenon can lead to biomagnification with increasing trophic level (Muir *et al.*, 1988; Ross, 2000; Ross *et al.*, 2000; Burreau *et al.*, 2006), which is particularly problematic for top predators, such as marine mammals, as they accumulate substantial depots of adipose tissue and reside at the top of the food chain (Ross, 2000). Though it is apparent that PCBs are sequestered in the lipid droplet (Bourez *et al.*, 2012), little is known regarding the specific mechanisms that regulate the storage and release of lipophilic contaminants in/from adipocytes (Bourez *et al.*, 2012; 2013). Bourez *et al.* (2012) suggested a role of caveolae, which are small (50-100nm) invaginations of the plasma membrane, particularly numerous in adipocytes (Thorn *et al.*, 2003), in PCB uptake by adipose tissue. Such structures modulate

transmembrane FA movement and triglyceride storage (Pilch *et al.*, 2011) in adipocytes. Importantly, caveolae formation has been linked to PCB exposure in these cells (Lim *et al.*, 2007; 2008). However, Bourez *et al.* (2012) found that PCBs were not associated with the cell membrane and accumulated in lipid droplets even in primary adipocytes of mice deficient in the membrane protein caveolin-1 (cav-1), which is essential for caveolae formation (Fra *et al.*, 1995; Fujimoto *et al.*, 2000). To further test accumulation dynamics and PCB storage, Bourez *et al.* (2013) exposed two different models of cultured adipocytes (mouse embryonic fibroblasts and differentiated 3T3-L1 cells) to three PCB congeners (PCB-28, 118 and 153) and found that different cell types accumulated different amounts of the same congeners. This appeared to be related to the levels of stored triglycerides. PCB-28 accumulated in cells more rapidly than PCB-118 and 153, which suggests lipophilicity of the molecules is a key parameter governing uptake (Bourez *et al.*, 2013). Additionally, the presence of POPs in adipocytes has been linked to alterations in the regulation of lipid metabolism, implicating these contaminants in the development of type-2 diabetes and metabolic syndrome (Dirinck *et al.*, 2011; Lee *et al.*, 2011).

Environmental POPs, including PCBs, are widely distributed in terrestrial and marine ecosystems and detectable far from their anthropogenic sources of origin (Nisbet and Sarofim, 1972; Wolska *et al.*, 2012). Due to the physicochemical properties of PCBs (i.e. the number and position of chlorine atoms in biphenyl rings), they do not degrade easily by physical, chemical or biological means and remain in the environment indefinitely, resulting in chronic toxicity to wildlife (Loganathan and Kannan, 1994). PCBs affect wildlife in a variety of different ways: they disrupt endocrine and immune function (Hammond *et al.*, 2005; Hall *et al.*, 2009); behave as

carcinogens and induce DNA damage (Safe, 1994; Oakley *et al.*, 1996); impair

reproduction (Haave *et al.*, 2003; Pavlova *et al.*, 2016) and development (Bergman

et al., 1986); are associated with neurotoxicity (Tilson and Kodavanti, 1998); whilst

altering adipocyte function and energy homeostasis (Regnier and Sargis, 2014;

Routti *et al.*, 2016; Robinson *et al.*, 2018). The toxic effects of POPs are mediated by

the aryl hydrocarbon receptor (AhR).

1.5.1 PCB genomic signalling mechanism: aryl hydrocarbon receptor

The aryl hydrocarbon receptor (AhR) is a ligand activated transcription factor

that mediates the toxic effects of POPs, including dioxin-like PCBs, PAHs and

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; Hankinson, 1995; Gonzalez

and Fernandez-Salguero, 1998; Beischlag *et al.*, 2008). AhR is a member of the Per-

ARNT-Sim homology domain superfamily of regulatory proteins that also includes

the AhR's dimerization partner, Ah receptor nuclear translocator (ARNT; Hankinson,

1995; Gonzalez and Fernandez-Salguero, 1998). This superfamily also includes

hypoxia-inducible transcription factor-1α (HIF-1α), which mediates gene activation by

hypoxia and also requires ARNT as a dimerization partner to activate gene

transcription (Wang *et al.*, 1995). The AhR is ubiquitously expressed in almost all

organs and cells and it has been established that AhR has a required physiological

function (Birnbaum and Tuomisto, 2000; Beischlag *et al.*, 2008), playing an essential

role in development and cell signalling. Both Gonzalez and Fernandez-Salguero

(1998) and Lahvis and Bradfield (1998) reported growth abnormalities during the

post-natal period, including impaired liver development, as a result of targeted

deletion of AhR in mice (Fernandez-Salguero *et al.*, 1995). In the absence of a

ligand, AhR is present in the cytoplasm as a complex bound to two molecules of

HSP90 (Dolwick *et al.*, 1993; Carver and Bradfield, 1997). This interaction means

HSP90 has been identified as an important regulator of receptor activity (Whitelaw *et al.*, 1995; Tsuji *et al.*, 2014). Upon binding of receptor ligands, such as PCBs, AhR requires ARNT in order to translocate to the nucleus, bind to upstream dioxin-responsive DNA elements and activate transcription of target genes, including the xenobiotic metabolising enzymes cytochromes P450 CYP1A1, CYP1A2, and CYP1B1 (Hankinson, 1995; Rowlands and Gustafsson, 1997; Bemis *et al.*, 2005; Beischlag *et al.*, 2008). This induces biological and toxicologic responses (Kafafi *et al.*, 1993). Different congeners and isomers of POP compounds exhibit a variety of adverse biological effects (Müllerova and Kopecký, 2007), discussed above.

1.5.2 POPs inducing cellular stress

POPs, such as PCBs, also induce proteotoxic and oxidative stress in cells (Lee and Opanashuk *et al.*, 2004). PCBs and their metabolites can induce oxidative stress by increasing RS levels (Hennig *et al.*, 2002; Zhu *et al.*, 2009), leading to cell injury, mutagenesis and cell death (Zhu *et al.*, 2009). Therefore, metabolic oxidative stress caused by PCBs and their metabolites may play an important role in cytotoxicity in mammalian cells. Zhu et al. (2009) confirmed that PCBs (PCB-153, Aroclor 1254 mix and the 2-(4-chlorophenyl)-1,4-benzoquinone metabolite of PCB3 (4ClBQ)) increased levels of intracellular $O_2^{\bullet}$ and H_2O_2 through mitochondrial metabolism and induced superoxide dismutase (SOD) activity in human breast and prostate epithelial cells. Increased levels of RS could cause oxidative damage to proteins, highlighting the need for molecular chaperones to protect proteostasis.

Fossi *et al.* (2010) found exposure to high concentrations of PCBs induced heat shock protein 70 (*HSP70*) mRNA expression in the skin of fin whales (*Balaenoptera physalus*). In rats both HSP70 and HSP90 were induced in liver tissue by a highly toxic PCB congener (3,3',4,4',5-pentachlorobiphenyl; PCB 126) at a dose

of 25 mg kg^{-1} via intraperitoneal injection (Fukuda et $al.$, 1999). The same study also found induction of the same HSPs at concentrations as low as 0.5 mg kg^{-1}. For comparison, lactating grey seal female blubber has been found to contain an average 1.25 – 1.96 mg kg^{-1} lipid (inner vs outer blubber biopsy section) early in lactation, which increased to 4.06 – 2.99 mg kg^{-1} lipid late in lactation (inner vs outer blubber biopsy section; Vanden Berghe et $al.$, 2010). Conversely, no relationship between PCB concentrations and $Hsp70$ mRNA expression were determined in the blubber of free ranging killer whales ($Orcinus$ $orca$; Buckman et $al.$, 2011), which had previously been shown to contain high concentrations of PCBs relative to other cetaceans (Ross et $al.$, 2000a). This was also true for blood of free ranging adult and yearling harbour seals ($Phoca$ $vitulina$; Lehnert et $al.$, 2016). This disparity suggests that there is no clear link between PCB concentration and HSP expression or that the induction of HSPs by PCBs depends on specific conditions (for example, a particular PCB concentration or congener) that have not yet been identified.

However, POPs are sequestered in the fat droplet, which may protect cellular machinery from their disruptive and oxidative effects and avoid impact on cell function and distress. Furthermore, due to the mobilisation of PCBs from blubber that are eventually transferred to offspring during gestation and via milk during lactation, reproductive marine mammal females generally have reduced levels of PCBs, compared to males of the same species (Tanabe et $al.$, 1994; Westgate et $al.$, 1997; Debier et $al.$, 2003; Jepson et $al.$, 2016).

1.6 Defence against cell stress

The cellular stress response (CSR) is a universal feature of all cells. The proteins involved in key aspects of CSRs are conserved in all organisms, though are species and cell-type dependent, due, in part, to the proteome expressed in a cell at a particular time (Kültz, 2003; 2005). Kültz (2003) compared annotated proteomes of multiple organisms from a diversity of taxa, including humans (*Homo sapiens*), eubacteria (*Escherichia coli*), yeast (*Saccharomyces cerevisiae*) and archaea (*Halobacterium spp.*) to identify evolutionarily conserved stress proteins. The author identified approximately 300 proteins involved in cellular redox regulation, DNA damage sensing/repair, lipid and energy metabolism, protein degradation and molecular chaperoning, which were highly conserved across all taxa. This included: the molecular chaperones DnaJ/HSP40, DnaK/HSP70, HSP60 chaperonin and GrpE (cofactor of HSP70) and the redox regulating enzymes superoxide dismutase (SOD), glutathione reductase, thio- and peroxiredoxin, aldehyde dehydrogenase and aldehyde reductase. There are other, diverse molecular mechanisms involved in the CSR, but molecular chaperones and redox enzymes (REs) are the evolutionary conserved mechanisms that will be considered by this thesis. Focusing on mechanisms which are conserved across taxa may be key to understanding cellular adaptation to stress (Kültz, 2005).

The interaction with and intensity of stressors acting on essential macromolecules, such as membrane lipids, proteins and DNA, is an important initiator of the CSR (Kültz, 2003). Stressors that overwhelm the ability of the cell to respond effectively can lead to deformation of or damage to these essential macromolecules (Kültz, 2003; 2005), disrupting cell function and potentially damaging cell integrity, which is considered distress. Cellular stress may be

proteotoxic and/ or oxidative in nature. The cellular stress response consists of a number of intracellular pathways that can be initiated depending on the nature and severity of the stressor. Stress responses provide an elevated level of protection for the cell as result of heightened availability of cellular defences, which temporarily makes cells more resistant to subsequent insults (Samali and Cotter, 1996; Samali and Orrenius, 1998; Samali *et al.*, 1999), including oxidative and proteotoxic stress, which is eustress.

1.6.1 The heat shock response (HSR) and heat shock proteins (HSPs)

Protein quality control is an essential process that is closely linked to cell health and long-term survival (Cotto and Morimoto, 1999; Balch *et al.*, 2008; Morimoto, 2008; Roth and Balch, 2011). Under stable cell conditions this is governed by protein homeostasis, or 'proteostasis', which encompasses a series of complex molecular interactions that control the balance between concentration, conformation, binding interactions, cellular location and clearance of proteins that make up the cell proteome (Balch *et al.*, 2008; Morimoto, 2008; Hartl *et al.*, 2011; Hetz and Glimcher, 2011). To maintain protein homeostasis, cells use stress sensors and inducible pathways which includes the heat shock response (HSR; Westerheide and Morimoto, 2005; Balch *et al.*, 2008).

The HSR was first discovered in 1962 by Ferruccio Ritossa, who observed expanded puffs on salivary gland chromosomes after exposing the fruitfly *Drosophila busckii* to elevated temperatures and chemical treatments, including 2,4-dinitrophenol and sodium salicylate (Ritossa, 1962; Lindquist, 1986; Schlesinger, 1990). The appearance of this phenomenon and the concomitant reduction of pre-existing chromosomal puffs (also as a result of heat shock and chemical treatments),

indicated the HSR resulted in global changes in gene transcription patterns (Ritossa, 1962; Jamrich *et al.*, 1977; Richter *et al.*, 2010). Subsequent stress research aimed to identify the induced RNAs and corresponding proteins at a molecular level (Ashburner and Bonner, 1979; Peterson *et al.*, 1979; Lindquist, 1986). In 1974 Tissières and colleagues ultimately isolated and characterised *Hsp* genes (Tissières *et al.*, 1974). Since this time, the HSR has since been identified as a universal and ancient mechanism, highly conserved across taxa, in both prokaryotic and eukaryotic cells (Kelley and Schlesinger, 1978; Lemaux *et al.*, 1978; Lindquist, 1986; Richter *et al.*, 2010). Importantly, though the HSR was first observed to be triggered by elevated temperature (hence the 'heat-shock' nomenclature), it has since been established that the HSR is induced by a number of different stressors (Figure1.3), including changes in cellular redox potential and levels of RS (Snoeckx *et al.*, 2001; Wyttenbach *et al.*, 2002; Kültz, 2005; Guo *et al.*, 2007), elevated heavy metal concentrations (Tamás *et al.*, 2014), hypoxia and reoxygenation (Nakano *et al.*, 1997; Lin *et al.*, 2001; Gupta and Knowlton, 2002; Baird *et al.*, 2006), fluctuations in glucose availability (including glucose deprivation; Pelham, 1986; Valentim *et al.*, 2003; Wang *et al.*, 2012) and the presence of steroid hormones (including cortisol; Udelsman *et al.*, 1993; Murphy *et al.*, 1996; Pratt and Toft, 1997; LeBlanc *et al.*, 2012) and chemical stressors (including phenolic compounds, n-alcohols, 2,4-dinitrophenol and diethylstilbestrol; Neuhaus-Steinmetz and Rensing, 1997).

1.6.1.1 Heat shock factors (HSFs)

The HSR is primarily controlled by heat shock transcription factors (HSFs) and HSF1, 2, 3 and 4 are present in mammalian cells (Fujimoto *et al.*, 2010; Åkerfelt *et al.*, 2010; Mahat *et al.*, 2016). In their inactive state HSFs exist as monomers

within multi-subunit complexes, which include HSPs (Lee *et al.,* 2013). Upon activation HSFs are trimerised which allows them to enter the nucleus and bind to heat shock elements (HSEs; Perisic *et al.*, 1989; Westwood *et al.*, 1991), which in turn induces the expression of HSPs. HSEs are promoter elements, characterised by inverted repeats of the pentanucleotide 5'-nGAAn-3' (Fernandes *et al.*, 1994). In most HSEs the proximal promoter regions of HSP genes are made up of three or more adjoining inverted 5'-nTTCnnGAAnnTTCn-3' repeats, where n is any nucleotide (Amin *et al.*, 1988; Perisic *et al.*, 1989). Promoters of HSF target genes can contain more than one HSE, allowing binding of multiple HSFs. HSF-HSE binding occurs in cooperatively, where the binding of one HSF facilitates the binding of the next HSF trimer (Åkerfelt *et al.*, 2010). HSF1 is the major stress-induced transcription factor and plays an important role in the molecular response to newly synthesised non-native proteins (Goodson and Sarge, 1995; McMillan *et al.*, 1998; Zhong *et al.*, 1998). HSF2 also takes part in the HSR to stress as it can also bind to HSEs, however this can only be achieved in the presence of HSF1 (Östling *et al.*, 2010). There is evidence to suggest that HSFs do not only act in the HSR, but are essential transcription factors that regulate other physiological functions, such as the development of oocytes pre- and post-fertilisation (Christians *et al.*, 2003; Metchat *et al.*, 2009; Åkerfelt *et al.*, 2010).

1.6.1.2 Heat shock proteins (HSPs)

HSPs are major components of the CSR, highly conserved (Ali and Banu, 1991; Feder and Hoffman, 1999) and vital for cell survival as molecular chaperones (Garrido et al., 2006) in all animals. In the normal cell state HSPs facilitate the proper folding of newly synthesised native proteins and assist in their translocation around

the cell (Feder and Hoffman, 1999; Hartl and Hartl-Meyer, 2002). During the stress response, HSPs aid misfolded and degraded proteins, either helping them retain their correct formation or regulating their removal from the cell to avoid the formation of cytotoxic aggregates (Parsell and Lindquist, 1993; Hartl, 1996; Fink, 1999). HSPs are classified by molecular weight (in kilodaltons, kDa), which allows them to be grouped into five major families: HSP100s, HSP90s, HSP70s, HSP60s and small HSPs (sHSPs). A brief overview of different HSP families and their cellular locations is displayed in Table 1.2.

Table 1.2. The main families of HSPs. Adapted from Srivastava, 2002a. This list is not all-inclusive. Highlighted in bold are those that are included as biomarkers of cellular stress in this thesis.

HSP family	Members	Intracellular location	Is function ATP dependent?
Small HSPs	**HSP27**	Cytosol	No
HSP40	**HSP40**, DNAJ	Cytosol	No
HSP70	**HSP72**, **HSC70** (HSP73) SSC1, SSQ1 GRP78 (BiP)	Cytosol Mitochondria Endoplasmic reticulum	Yes
HSP90	**HSP90**, HSC84, HSP86 gp96	Cytosol Endoplasmic reticulum	Yes
HSP100	HSP104, HSP110 HSP78	Cytosol Mitochondria	Yes

1.6.1.2.1 HSP70 and HSC70

The 70kDa HSP family includes HSP70, the stress inducible isoform, and the constitutively expressed heat shock cognate 70 (HSC70) which is present in the cell cytoplasm under basal conditions (Flaherty *et al.*, 1990; Bozaykut *et al.*, 2014). The distinction between HSC70 and HSP70 lies, not only in their expression, but in the structure and function of their ATPase and carboxyl-terminal domains, which confer different affinities for substrates and provides specificity to their chaperone roles (Flaherty *et al.*, 1990; Tutar *et al.*, 2006; Liu *et al.*, 2012). HSP70 has a diverse range of roles influencing many aspects of protein quality control and turnover (Nollen *et al.*, 1999; Garrido *et al.*, 2006). In its inactive state HSP70 is complexed with other proteins including HSF1, ESR (oestrogen receptor) and GR (glucocorticoid receptor; Echeverria and Picard, 2010). In eukaryotes, such chaperone-substrate complexes are known to influence the biological activity of these regulatory proteins. For example, GR only binds hormone effectively as part of a chaperone-substrate complex (this includes HSP90 and HSP40; Dittmar *et al.*, 1998; Mayer and Bakau, 2005). Through these interactions the HSP70 and other chaperone systems have involvement in major cellular functions (i.e. signal transduction and cell cycle regulation) and whole-animal physiology (Mayer and Bakau, 2005; Echeverria and Picard, 2010). This highlights the importance of HSP70 and other molecular chaperones outside of the CSR.

HSP70 functions with its co-chaperone, HSP40. Via its J-domain, HSP40 recognises and binds to unfolded proteins and delivers them to HSP70, whilst also stimulating HSP70 ATPase activity (Minami *et al.*, 1996; Mayer and Bakau, 2005; Li *et al.*, 2009). This highlights that key elements of the HSR are energetically costly.

1.6.1.2.2 HSP40

HSP40 is characterised by its J-domain and can bind to non-native polypeptides (Gething and Sambrook, 1992; Li *et al.*, 2009). HSP40 family members are either Type I, II or III; Type I and II HSP40 family members have the J-domain at the N-terminus, whereas Type III HSP40s can have the J-domain located at any position within the protein sequence (Li *et al.*, 2009). Type I and II HSP40 proteins can function as ATP-independent chaperones for non-native polypeptides (Langer *et al.*, 1992; Hartl and Hartl-Meyer, 2002). Type I HSP40s have been reported to bind polypeptides and prevent protein aggregation, whereas the Type II family require HSP70 to be able to play such a role (Muchowski *et al.*, 2000). HSP40 also plays an important role in the HSC70 ATPase cycle which is essential for polypeptide binding (Minami *et al.*, 1996; Liu *et al.*, 2012).

1.6.1.2.3 HSP90

HSP90 is highly abundant and can make up 1-2% of total protein in the cell (Csermely *et al.*, 1998; Chen *et al.*, 2005; Li *et al.*, 2012). HSP90 is also involved in binding to unfolded proteins and inducing correct folding, but it predominantly mediates the function of its bound proteins to ensure they stay in a functional folded state (Marcu *et al.*, 2000; Bozaykut *et al.*, 2014).

1.6.1.2.4 HSP27

HSP27 is a small HSP that functions independently of ATP (Ehrnsperger *et al.*, 1997; Rogalla *et al.*, 1999; Shashidharamurthy *et al.*, 2005; Garrido *et al.*, 2006). The main function of HSP27 is to prevent protein aggregation (Ehrnsperger *et al.*, 1997). Although HSP27 is a small HSP, it can form large oligomers of 1000 kDa

(Rogalla *et al.*, 1999; Garrido *et al.*, 2006). Oligomerisation appears to regulate the chaperone activity of HSP27 by altering its affinity for proteins to be chaperoned (Rogalla *et al.*, 1999; Shashidharamurthy *et al.*, 2005).
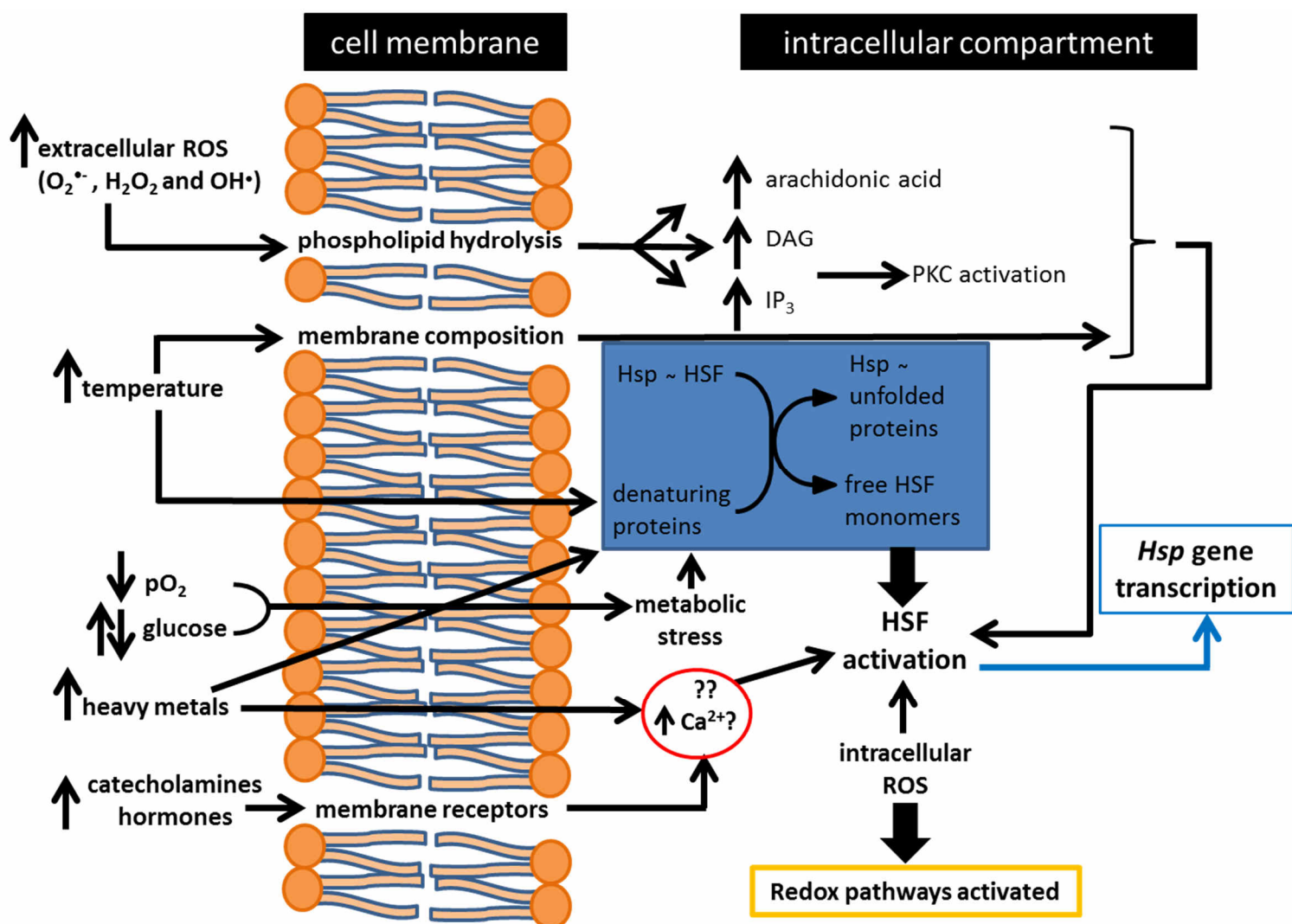
Figure 1.3 Extracellular and intracellular stressors that can activate heat shock factor (HSF) and subsequent transcription of heat shock protein (*Hsp*) genes. ROS, reactive oxygen species; DAG, diacylglycerol; IP$_3$, inositol 1, 4, 5-triphosphate; PKC, protein kinase C; Hsp˷HSF, binding between Hsp molecules and HSF; Hsp˷unfolded proteins, binding between Hsp molecules and unfolded proteins. Where increasing Ca^{2+} and ?? are circled in red this indicates that, although calcium has been found to increase in response to those stressors, it is not certain that this is the significant activator of HSF and the complete transduction pathways have not been fully elucidated i.e. increasing concentrations of heavy metals have been shown to directly interfere with protein folding (for example, as a result of binding to functional side groups; Tamás *et al.*, 2014), however there is evidence that increasing calcium ions linked to heavy metal toxicity may also be involved in HSF activation. Adapted from Snoeckx *et al.* (2001).

1.6.2 Redox enzymes (REs)

Reactive species (RS) formation and changes in cellular redox state are closely linked to the CSR (Kültz, 2005). Almost every gene implicated in the response to stress is also affected by changes in cellular redox potential and levels of RS (Sen and Packer, 1996; Monteiro and Stern, 1996; Piette *et al.*, 1997; Adler *et al.*, 1999).

Anti- and pro-oxidant enzymes regulate the redox state of the cell, are sensitive to changes in cellular redox state and can be involved in reactions that result in oxidation or reduction. Therefore, the oxidative stress markers considered by this thesis will hereafter be referred to as redox enzymes (REs), rather than being defined purely as either anti- or pro-oxidant. Figure 1.4 below highlights major pathways of reactive oxygen species generation and metabolism and introduces the REs that will be discussed and used a markers of oxidative stress in this thesis.
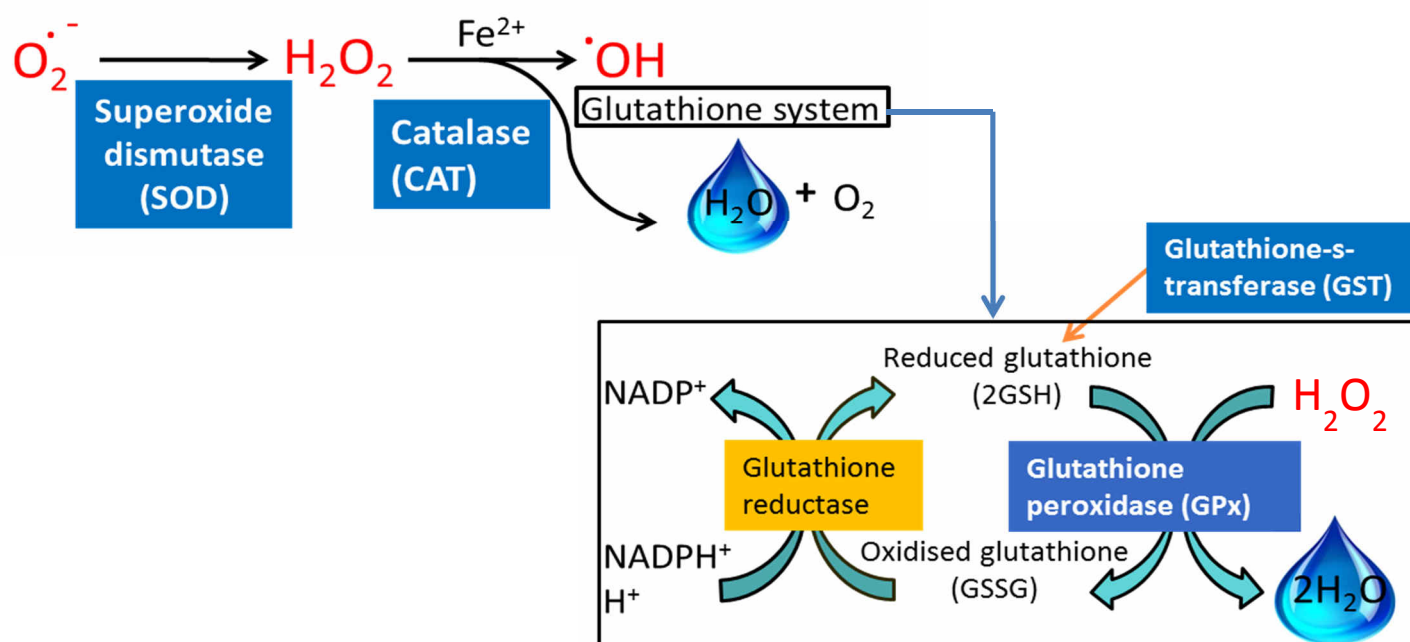
Figure 1.4. Major pathways of reactive oxygen species generation and metabolism. REs can control the production of free radicals and limit the impact of oxidative stress by detoxifying RS in cells of aerobic organisms. Superoxide dismutase (SOD) catalyses the reaction that converts superoxide anion to H_2O_2. Catalase (CAT) prevents the formation of the highly reactive hydroxyl radical (OH$^\bullet$) by breaking down H_2O_2 to water and oxygen. The glutathione system is also involved in scavenging free radicals. Glutathione peroxidase (GPx) decomposes H_2O_2 and lipid peroxides, whilst glutathione-*s*-transferase (GST) catalyses glutathione conjugation reactions. Highlighted by blue boxes and white font are the REs that will be discussed below and used a markers of oxidative stress in this thesis.

1.6.2.1 Glutathione peroxidase (GPx)

GPxs have both selenium dependent and independent forms that oxidise their essential cofactor, glutathione (GSH), an important scavenger of RS, to glutathione disulphide (GSSG), which eliminates H_2O_2. As GPxs rely on the presence of GSH, the role of catalase (CAT) may become much more important when GSH is depleted by intense periods of oxidative stress (Zubkova and Robaire, 2004).

1.6.2.2 Catalase (CAT)

CAT is a ubiquitous enzyme, found mainly in peroxisomes, which catalyses the breakdown of hydrogen peroxide to water and oxygen (Zámocký and Koller, 1999; Chelikani *et al.*, 2005; Alfonso- Prieto *et al.*, 2009). Each active site of this enzyme contains a heme group which is responsible for its catalytic activity and allows CAT to interact with H_2O_2 (Groves *et al.*, 1981). CAT can act both catalytically and peroxidically depending on the concentrations of H_2O_2 (Chance *et al.*, 1979; Ścibior and Czeczot, 2006) in the cell.

1.6.2.3 Glutathione-S-transferase (GST)

Glutathione transferases (GSTs), which also require GSH as a co-factor, catalyse reactions which conjugate electrophilic substrates to reduced GSH (Hayes *et al.*, 2005). GSTs also have non-catalytic roles, including synthesis of and binding to biomolecules such as thyroid and steroid hormones (Ishigaki *et al.*, 1989; Tew *et al.*, 1993; Hayes *et al.*, 2005).

1.6.2.4 Superoxide dismutase (SOD)

The family of SOD enzymes are classified by their different forms, which utilise different catalytic metal ions at the molecules core. These metal ions function as

electron donors/receivers and also provide structural support for the molecule (Perry *et al.*, 2010). The different forms of SOD are found in different cellular compartments; CuZnSOD is found in the cytosol and the intermembrane mitochondrial compartment, whereas MnSOD is found in the mitochrondrial matrix (Perry *et al.*, 2010).

1.6.2.5 Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (Nox4)

Nox4 is a 'pro-oxidant' enzyme, requiring the p22^{phox} subunit and the interacting protein Poldip2 to generate RS (usually in the form of $O_2^{\bullet-}$and H_2O_2) via the reduction of O_2 (Ushio-Fakai *et al.*, 1996; Lyle *et al.*, 2009; Figure 1.5). Nox4 is one of the most abundant and widely expressed Nox isoforms (Ushio-Fakai *et al.*, 1996; Lyle *et al.*, 2009) and generates H_2O_2 constitutively, which suggests a role in resting cellular homeostasis (Miller Jr., 2009).
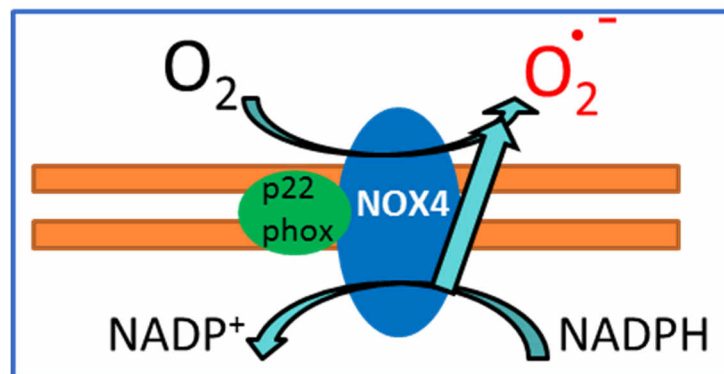
Figure 1.5. Schematic representation of NOX4 oxidase in the cell membrane. NOX4 binds to a smaller p22phox subunit, which is essential for enzyme activity. NOX4 utilises NADPH as an electron donor and catalyses the transfer of electrons to molecular oxygen to generate superoxide ($O_2^{\bullet-}$; shown above) and hydrogen peroxide (H_2O_2).

1.6.3 Linking the HSR with redox pathways and vice versa

As discussed in section 1.2.3, RS and changes in cellular redox state are key triggers of the CSR (Kültz, 2005) and almost all CSR related genes are sensitive to such fluctuations (Sen and Packer, 1996; Monteiro and Stern, 1996; Piette *et al.*, 1997; Adler *et al.*, 1999). Therefore, it is unsurprising that there is a body of evidence which suggests that the HSR plays an important role in protecting proteins from oxidative stress and it has been demonstrated that HSPs are induced in response to RS and redox changes (Figure 1.3; Schoeniger *et al.*, 1994; Wheeler *et al.*, 1995; Ehrenfried *et al.*, 1996; Gasbarrini *et al.*, 1998; Zhong *et al.*, 1998; Nishizawa *et al.*, 1999; Madamanchi *et al.*, 2001; Mustafi *et al.*, 2009). Much of the literature considering the interactions between heat shock and redox systems comes from the biomedical sciences; for example, redox changes and the activation of the HSR or the regulation of the HSR by RS are generally considered in the context of disease pathologies (Snoeckx *et al.*, 2001; Wyttenbach *et al.*, 2002; Vila *et al.*, 2008).

HSP70 and HSP90 are known to bind to electrophiles, including 4-hydroxynonenal (HNE; Vila *et al.*, 2008). HNE is a major end product of lipid peroxidation (Uchida *et al.*, 1999; Negre-Salvayre *et al.*, 2008; Ayala *et al.*, 2014) and is associated with the development of diseases with oxidative stress pathology. HNE is accumulated in numerous oxidative stress-related diseases (including metabolic syndrome, cancer and cardiovascular diseases) and also induces cell death (Vila *et al.*, 2008; Dalleau *et al.*, 2013; Ayala *et al.*, 2014).

HSP27 has been shown to reduce levels of ROS in neuronal and kidney cells transfected with vectors expressing mutant huntingtin (httEx1; Wyttenbach *et al.*, 2002). Huntingtin is a protein which is a key factor in the induction of

neurodegeneration associated with Huntington's disease, in part by increasing ROS production, leading to oxidative stress and cell death (HDCRG, 1993; Sari, 2011).

Ahn and Thiele (2003) showed that heat and the RS H_2O_2 induce mammalian HSF1 in a manner that is both reversible and redox regulated. Furthermore, activity of stress-stimulated HSF1 is reduced when antioxidants including catalase and ascorbic acid are observed to suppress intracellular RS levels (Nishizawa *et al.*, 1999; Ozaki *et al.*, 2000; Kim *et al.*, 2001). Paroo *et al.* (2002) also found that there was increased incidence of HSF1 binding to HSEs as a result of a depletion of the intracellular pool of reduced glutathione (GSH).

These examples demonstrate the complimentary links between these two cellular defence systems (for example, the HSR can potentially act as a complementary antioxidant system, as in response to oxidative stress redox enzymes are employed to detoxify RS whilst HSPs work to protect proteostasis which could be disrupted by protein interactions with RS and their by-products).

1.7 Implications of cell stress for immune signalling

As described previously, HSPs and REs provide vital protection for macromolecules against proteotoxic and oxidative stress. HSPs, as numerous and highly conserved molecular chaperones, have a variety of functions due to their ability to interact and form complexes with other molecules. HSPs play a variety of important roles in innate and acquired immune responses (Colaco *et al.*, 2013) and have been identified as key immunogens (i.e. molecules that stimulate immune responses) in the response against pathogens (Suzue *et al.*, 1997; Srivastava, 2002b; Wallin *et al.*, 2002; Osterloh and Breloer, 2008). HSP70 can stimulate human monocytes and macrophages to secrete cytokines, including IL-6, IL-10, IL-12 and

TNF-α (Ohashi *et al.*, 2000; Vabulas *et al.*, 2001). HSPs also play a role in the maturation of dendritic cells (DCs; specialised antigen presenting cells derived from monocyte precursors that activate and induce differentiation of naïve T-lymphocytes; Simones and Moser, 2010). HSPs bind to immature DCs and upregulate of a number of receptors including CD40, CD83, CD86 and CD91 which are markers of DC maturation (Basu *et al,*. 2000; 2001; Kuppner *et al.*, 2001; Calderwood *et al.*, 2007b; Binder, 2009; Colaco *et al.*, 2013). Gp96 (a member of the HSP90 family, expressed exclusively in the endoplasmic reticulum) is involved in the chaperoning, and in turn proper expression, of toll-like receptors (TLRs) which crucially sense and recognise microorganisms in antigen presenting cells (Liu *et al.*, 2010). Staron *et al.* (2010) showed mice with reduced gp96 expression are severely susceptible to bacterial infection due to compromised T- and B- cell development. This suggests gp96 can control immune responses to pathogens by controlling TLR binding (Binder, 2014). Binder (2014) considered HSPs, along with MHC (major histocompatibility complex), to be one of the major peptide binding families in immunology. HSP70 and HSP90 play vital roles in antigen presentation and cross-presentation. They bind and chaperone antigenic peptides to MHC class I molecules on the cell surface, where they can be presented to lymphocytes (Srivastava *et al.*, 1994; Tsan and Gao, 2009; Binder, 2014).

HSPs are also associated with numerous pathologies in which increased levels of chaperones occur in immune cells. In humans, sepsis significantly increased protein expression of HSP27, HSP70 and HSP90 in activated polymorphonuclear leukocytes (PMNLs) compared to healthy individuals (Hashiguchi *et al.*, 2001). The same study also reported increases in oxidative activity, specifically in PMNLs, along with enhanced HSP70 expression response to sepsis

and inhibition of apoptosis. This suggests that HSPs may not only regulate PMNL function in some disease states, but HSP expression in immune cells could be used as an indicator of immune system activation.

REs also play an important role in immunity, protecting cells from RS generated physiologically (i.e. phagocytes such as neutrophils and macrophages) to destroy pathogens (these can be toxic to cells surrounding the activated phagocyte and the phagocyte itself), whilst preventing oxidative stress, which is associated with a variety of pathologies, such as hypertension, sepsis, cancer, atherosclerosis and diabetes (Mühl *et al.*, 2011; Cheng *et al.*, 2013). RS can impair T-cell responses and decrease T-cell proliferation as a result of increased H_2O_2 production during chronic inflammatory conditions, such as rheumatoid arthritis (Otsuji *et al.*, 1996; Cemerski *et al.*, 2002). Oxidative stress can further impair immune cell function by inducing hypo-responsiveness in T-cells (Cemerski *et al.*, 2003).

RS affect protein structure, and cells exposed to moderate levels of H_2O_2 increase proteolysis rate (Grune *et al.*, 1995; 1996). Hypo-responsive T-cells are correlated with RS-induced structural protein alterations. Molecular chaperones, such as HSPs, may help protect protein structure against oxidative damage and maintain cell function during immune responses. Once activated, other immune cells, such as lymphocytes, are highly susceptible to oxidative-stress-induced cell death (Degasperi *et al.*, 2008). Therefore, when elements of the immune system are active, additional oxidative stressors (e.g. RS producing chemicals) that further challenge immune cells may lead to cell death and immune system dysfunction.

REs themselves play important intracellular roles in immune cell function and protection from RS. In humans and rats, mutations in genes encoding subunits of

Nox enzymes result in 'chronic granulomatous disease' (CGD). Mutations associated with CGD impact phagocyte Nox's ability to generate antimicrobial RS (i.e. phagocytes are unable to destroy some pathogens) leading to increased susceptibility to acute bacterial and fungal infections (Heyworth *et al.*, 2003). In the past, CGD was originally associated with GPx deficiency; for example, neutrophils of selenium deficient rats that were also GPx deficient presented defective microbicial activity and were more susceptible to chronic infection (Serfass and Ganther, 1975). Although no longer considered to be a driver of CGD phenotype (Newburger *et al.*, 1994), in some animal models (rats and humans) *in vivo* GPx deficiency still affects phagocyte function (McCallister *et al.*, 1980; Schulman *et al.*, 1980; Baker and Cohen, 1983; 1984; Cheng *et al.*, 2013) highlighting the importance of GPx activity in immunity.

CAT and SOD have been also implicated in the protection of leukocytes from phagocytic activity (Salin and McCord, 1975) and variation in these REs has been observed during a number of pathological states (Mühl *et al.*, 2011). Both Mn-SOD and Cu, Zn-SOD activities were reduced in neutrophils and lymphocytes of diabetic humans compared to healthy controls, suggesting that diabetics may be more vulnerable to infection and immune system dysfunction (Uchimura *et al.*, 1999). Lower SOD activity in polymorphonuclear leukocytes (PMNs) compared to healthy control subjects has been associated with inflammatory conditions such as acne, which is induced by *Propionibacterium acnes* bacterium (Kurutas *et al.*, 2005).

After analysing the various roles played by HSPs and REs in normal immune function and protection, cellular stress can certainly influence immune signalling. Immune cells can even be activated by cellular stress pathways (Muralidharan and Mandrekar, 2013) and it appears the two are closely linked.

1.8 Justification of experimental approach

1.8.1 Can mRNA expression profiles be used to predict functional responses to cellular stress?

Determination of mRNA expression profiles by quantitative real-time PCR (qRT-PCR) is a widely applied, extremely versatile, highly sensitive technique (Pfaffl, 2001; Bustin, 2002; Bustin et al., 2009). Applying qRT-PCR is ideal when working with limited tissue volumes, as the technique allows multiple genes of interest to be assessed simultaneously, depending on qRT-PCR plate size (plate sizes range from 48-384 well plates; Bustin, 2002). It is also affordable, intuitive and can be quickly and accurately learnt, with almost all institutions having qRT-PCR platforms from various manufacturers. qRT-PCR has a variety of applications within the medical and ecological communities. Identifying potential biomarkers is an important role this technique can play.

Discussions of findings in this thesis do not presume that an increased mRNA expression of a particular gene directly follows through to the proteome and metabolome. The correlation between RNA and protein expression ae generally not very good.The studies in this thesis aim to determine if, at the transcript level, I can detect changes in cellular defences in response to physiological (Chapter 3, 4 and 5) and experimental stressors (Chapter 6). This will be accompanied by measurements of tissue damage and whole-animal fitness measures, to attempt to distinguish between eustress and distress in this species. A more *complete* understanding of how a cell responds to a perceived stressor would require further cellular measurements of miRNA profiles, protein abundances (of the same transcripts) and associated metabolites, which is beyond the scope of this thesis.

Investigating relative expression and activity of HSPs and redox enzymes can provide a 'snap-shot' insight into the stress status of tissue from a particular life history state. However, it does not provide information on the functional consequences of that expression or activity; heightened levels of HSP/redox enzyme expression may protect the cells adequately, in which case the cells experience the stress as 'eustress'. Conversely, even if defences are heightened, they may not be adequate to protect the cells from the current, or future, insults. To answer such questions it is important to look for evidence of cellular damage or trade-offs at the tissue and whole animal levels. Therefore, malondialdehyde (MDA) will be measured.

1.8.2 Lipid peroxidation and malondialdehyde (MDA)

Lipid peroxidation is used as a biomarker of oxidative damage at the tissue level and is a result of the reaction between oxidants, including RS, and lipids (Ayala *et al.*, 2013). Lipids are key components of biological membranes that control cell function and maintain cell structure. The carbon-carbon double bonds of polyunsaturated lipids, which are present in cell membranes, are a primary target for RS. Hydrogen is abstracted from the adjacent carbon-hydrogen bond, forming the lipid radical (L$^\bullet$). This lipid radical reacts with oxygen to rapidly form a lipid peroxyl radical (LOO$^\bullet$). The chain reaction is continued when lipid peroxyl radicals (LOO$^\bullet$) abstract hydrogen from other lipid molecules generating a new lipid radical (L$^\bullet$) and lipid hydroperoxide (LOOH). Lipid hydroperoxides (LOOH) are the primary products of lipid peroxidation, but are unstable and their fragmentation yields secondary products such as malondialdehyde (MDA). MDA is a three carbon aldehyde, with a low molecular weight, and can be produced by various mechanisms. Unlike RS, which attack biomolecules close to their site of origin (Halliwell and Gutteridge,

1984), MDA can easily diffuse across membranes and covalently modify proteins far from its site of generation (Negre-Salvayre *et al.*, 2008).

MDA is a standard measure of lipid peroxidation and there are a variety of commercially available biochemical assay kits to ascertain MDA concentration in biological fluids and tissues.

1.9 Thesis aims

The aims of this thesis are: (1) to test the hypothesis that simultaneous breeding and fasting creates a trade-off that results in reduced cellular defences relative to pre-breeding females, and that this trade-off has negative tissue and whole animal level consequences. This will be done by investigating the differences in relative gene expression of HSPs and redox enzymes in the blubber of lactating-fasting and pre-breeding female grey seals to ascertain whether defences are altered. This will be accompanied by also measuring changes in lipid peroxidation to determine whether there is evidence for increased oxidative damage in lactating-fasting animals and/ or during the change from fat deposition to fat mobilisation (Chapter 3) (2) to investigate developmental changes in relative gene expression of HSPs and REs in the blubber of suckling and fasting grey seal pups and investigate changes in lipid peroxidation throughout the suckling period and the post-weaning fast to determine whether there is evidence for oxidative damage in animals with lower defences. I will also establish whether increased physiological stress (indicated by higher levels of HSPs or REs) or damage (indicated by lipid peroxidation) is associated with reduced mass gain or maternal mass transfer to the pup (maternal investment) during suckling or increased mass loss during fasting, which may be indicative of a trade-off between cellular defences and fitness (Chapter 4) (3) determine the relative gene expression

of HSPs and REs in circulating blood leukocyte populations of breeding adult females and in suckling and fasting pups and ascertain whether body mass and axial girth affect gene expression of HSPs and REs, because body condition may influence immune function in healthy wild animals. I will also ascertain whether blubber MDA concentration and circulating glucose can explain variation in the expression of HSPs and REs, because high glucose levels can negatively impact immune cells and increase susceptibility to infection, which may stem from altered expression of HSPs and REs (Chapter 5) (4) utilising an *in vitro* method I will investigate absolute gene expression of *Hsp70* and *Hsc70* in inner and outer blubber explants of suckling and fasting grey seal pups in response to the artificial GC, hydrocortisone (HC) and PCBs, to ascertain the ability to induce the HSR alone or in combination and determine whether other factors including morphometric data, sex and intrinsic concentrations of PCB congeners already sequestered in blubber can explain variation in gene expression data. I will also consider if is there a functional relationship between neuroendocrine and cellular stress responses in grey seals (Chapter 6) (5) to explore how using these biomarkers and identifying distressing conditions can help manage grey seal populations and predict how they may cope with environmental change.

Chapter 2.
General Methods

2.1 Field Techniques

2.1.1 Ethics Statement

All capture and handling procedures described in this thesis were carried out under Home Office project licence #60/4009 and conformed to the United Kingdom (UK) Animals (Scientific Procedures) Act, 1986. All research was approved by the University of St. Andrews Animal Welfare and Ethics Committee. All samples were taken by trained and licensed individuals who had been declared competent under European Union (EU) Directive 2010/63/EU on the protection of animals used for scientific procedures.

2.1.2 Lactating females and pups

2.1.2.1 Study Site

Where specified, studies presented in this thesis used samples obtained from free-ranging grey seals (both adult females and pups) on the Isle of May, Firth of Forth, Scotland (56° 11' 25" N, 02° 34' 25" W; Figure 2.1).
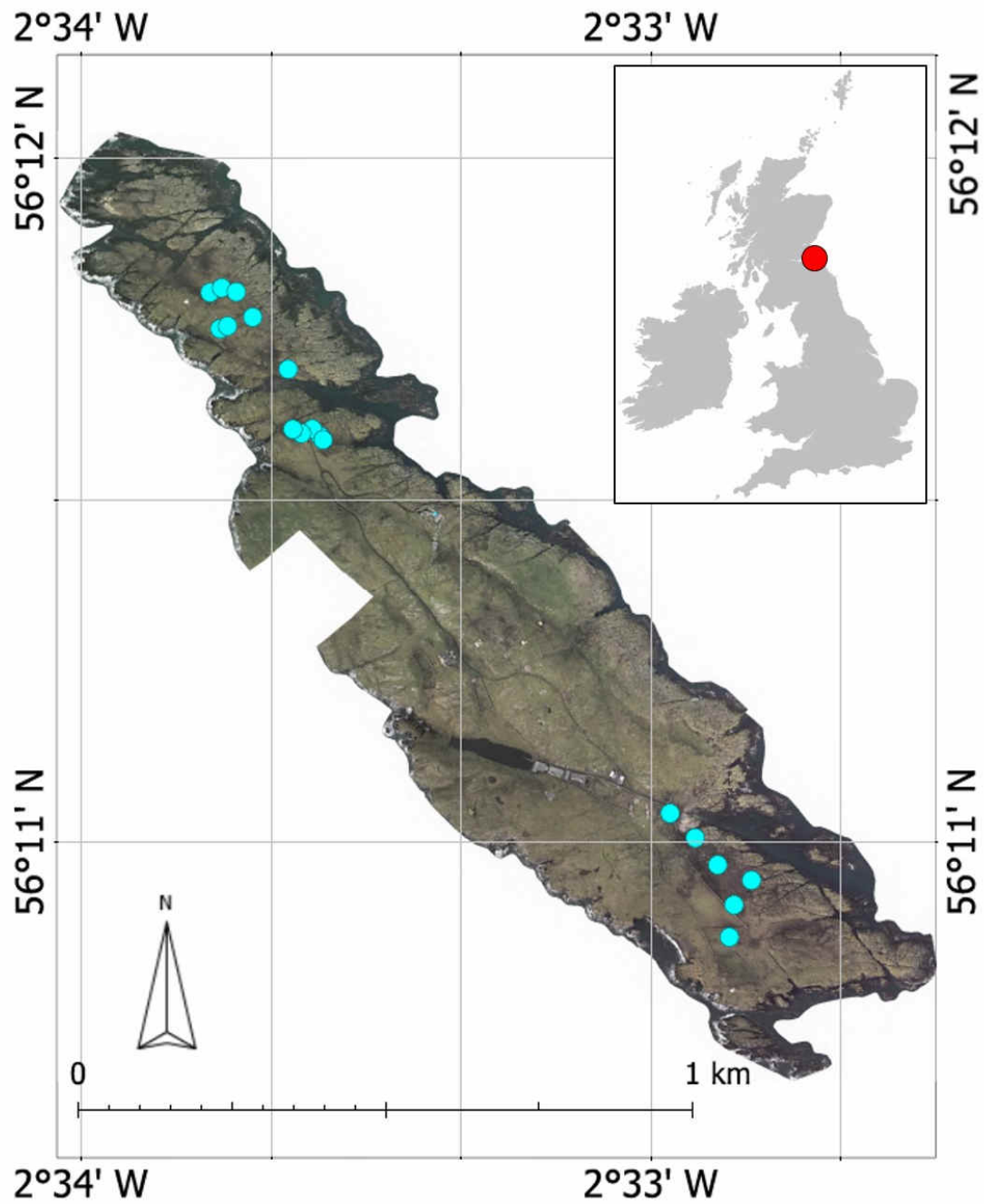
Figure 2.1. Map of the Isle of May (IoM) showing locations of animals captured during the studies presented in this thesis (Chapter 3, 4 and 5; light blue circles), with inset showing the IoM on a map of the UK and the Republic of Ireland; location is marked by the red dot. Credit to Izzy Langley for map-making assistance in Manifold® System 8.0 (http://www.manifold.net/index.shtml). Isle of May expanded aerial image courtesy of Chris Morris and Callan Duck.

2.1.2.2 Sampling regime

The studies were performed on the Isle of May in October to December 2013. Animals included in these studies were a part of a long-term study programme on grey seal breeding systems and physiology led by the Sea Mammal Research Unit (SMRU), UK. Females identified by either a brand or flipper tag were observed on the colony on a daily basis to record pup date of birth (Pomeroy *et al.*, 1999). Once date of birth had been determined, the mother-pup pairs were captured twice at EARLY and LATE lactation. The EARLY lactation capture was as close to day 5 of the suckling period as was practically possible.

Assuming grey seal pups gain 1.5-2.0 kg per day (Fedak and Anderson, 1982, Kovacs and Lavigne, 1986; Anderson and Fedak, 1987; Bowen *et al.*, 1992; Mellish *et al.*, 1999; Pomeroy *et al.*, 1999; Hall *et al.*, 2001) and are born on the Isle of May at 13-15 kg (Pomeroy *et al.*, 1999), five days after birth allowed the pup time to reach a sufficient body fat content and a mass of approximately 20kg. This minimum mass at capture was used because the removal of a blubber sample requires anaesthesia. General anaesthesia is administered as a mass-specific dose and a pup less than 20kg would require a volume of drug that is too small to administer in practical terms. A mass of 20kg was also indicative that the pup was feeding and had reached a stage in development at which it was robust enough to recover from the procedure. The LATE lactation capture was as close to day 15 of the suckling period as was practically possible. Most female grey seals wean their pups after approximately 18-21 days (Nordøy *et al.*, 1990; Nordøy and Blix, 1990; Reilly, 1991; Pomeroy *et al.*, 1999). Day 15 therefore meant females were unlikely to have departed the colony and ensured that pups were still suckling, but this also targeted a time when pups were expected to have almost tripled in body mass due to

gaining significant fat reserves in the form of subcutaneous blubber (Fedak and Anderson, 1982; Anderson and Fedak, 1987; Mellish *et al.*, 1999), and females were likely to have become very lean, having lost approximately 40% of postpartum body mass (Pomeroy *et al.*, 1999) and 61-84% of initial fat reserves (Fedak and Anderson, 1982).

Mother-pup pairs were observed daily where possible and their presence noted throughout the lactation period; such observations by researchers were made from a distance to avoid colony disturbance. Once the LATE lactation samples had been taken, the weaning date of the pup was established by observing the pair daily. If the pup was seen alone for two consecutive days, the weaning date was taken as the previous day, when the pup was first seen without the female in attendance (Bennett *et al.*, 2007, 2012; Pomeroy *et al.*, 1996, 1999). After the weaning date had been recorded, pups were recaptured at days representing the EARLY POST WEANING (~5 days after weaning) and LATE POST WEANING (~15 days after weaning) fast. Pups that are five days into the post weaning fast are mobilising fat and declining in rate of mass loss, losing approximately 0.50 kg day^{-1} (Worthy and Lavigne, 1987; Reilly, 1991; Bennett *et al.*, 2007). Pups later in the fast have a stable and low rate of mass loss (an average of 0.25 kg day^{-1}; Worthy and Lavigne, 1987; Reilly, 1991; Bennett *et al.*, 2007) despite continuing to mobilise fat, most likely as a result of a reduction in metabolic rate and protein conservation strategies (Nordøy *et al.*, 1990; Reilly, 1991). Although the length of the post weaning fast can vary from 10 days to >4 weeks (Reilly, 1991, Noren *et al.*, 2008, Bennett *et al.*, 2010), from approximately day 15 of the post weaning fast it is likely that pups will begin to leave the colony and go to sea.

2.1.2.3 Sample size

In 2013 a total of 18 mother-pup pairs were captured twice, at EARLY and LATE lactation (Table 2.1). 15 weaned pups were recaptured at two further time-points, with samples taken at both the EARLY and LATE POST WEANING fast (Table 2.1).

Table 2.1. Details of EARLY and LATE lactation captures and EARLY POST WEANING and LATE POST WEANING fast pup captures during 2013. At both EARLY and LATE lactation, the mean of the number of days after birth that the mother-pup pair capture took place is presented, ± standard deviation (SD). The mean number of days after weaning that the pup capture took place is presented for the EARLY POST WEANING and LATE POST WEANING fast, ± standard deviation. Sample size of mother-pup pairs captured at EARLY and LATE lactation and of pups at EARLY POST WEANING and LATE POST WEANING fast are also shown (*n*).

EARLY lactation (days)			LATE lactation (days)			EARLY POST-WEANING fast (days)			LATE POST-WEANING fast (days)		
Mean	SD	*n*	Mean	SD	*N*	Mean	SD	*n*	Mean	SD	*n*
6.60	±1.97	18	17.58	±0.71	18	5.00	±0.73	17	15.00	±0.80	15

2.1.2.4 Capture protocol

Mothers were anaesthetised with an intramuscular dose of Zoletil 100 (Virbac, Carros, France), at a dose of approximately 1.0 mL 100 kg^{-1} (1mg kg^{-1} scaled to 1.0 mL 100 kg^{-1}) estimated female mass (Baker *et al.*, 1990; Pomeroy *et al.*, 1996, 1999)

delivered using a pressurised projectile syringe that was aimed at the posterior lumbar muscles through a blow pipe (Baker *et al.*, 1990, Langton *et al.*, 2011).

At first capture pup sex was recorded and a tag (Dalton ID Systems, Henley on Thames, Oxon, UK) attached to the interdigital webbing of both rear flippers (Fedak and Anderson, 1982; Pomeroy *et al.*, 1996). For both mothers and pups, morphometric measurements including mass, nose-tail length and standard axial girth were obtained at EARLY and LATE lactation. Female mass was measured as described in Pomeroy *et al.* (1996). Pups were weighed at each capture in a tarpaulin bag using a load cell (± 0.2kg; Fedak and Anderson, 1982; Pomeroy *et al.*, 1996).

2.1.2.5 Blood samples

Before sampling, puncture areas were sprayed with the disinfectant Savlon (3% w/v cetrimide, 0.3% w/v chlorhexidine gluconate; Novartis, Horsham, UK) and immediately after with a terramycin ® spray (oxytetracycline, Pfizer Ltd.). Blood samples from adult females were taken by inserting a 20 gauge, 3.5 inch spinal needle into the extradural vein via an intravertebral space in the lower dorsal region above the pelvis (Harrison and Tomlinson, 1956; Geraci *et al.*, 1975; Gulland *et al.*, 2001; Sakamoto *et al.*, 2009). Pup blood was also taken from the extradural vein, using a 19 gauge, 2 inch needle or whichever needle size was most suited to the size of the animal (Bennett *et al.*, 2011; 2015). All blood was collected within one to two minutes from the time of needle insertion into ethylenediaminetetraacetic acid (EDTA) treated (plasma) vacutainers™ (Becton Dickinson, Cowley, Oxfordshire, UK) and immediately placed on ice. Before being placed on ice, EDTA-treated (plasma) vacutainers™ were inverted carefully to allow the mixing of the blood with the EDTA

anticoagulant. Plasma was collected at both EARLY and LATE lactation, from mothers and pups, and from pups at the EARLY POST WEANING and LATE POST WEANING fast. Figure 2.2 gives a visual representation of sampling regime.

2.1.2.6 Blubber biopsies

Pups were anaesthetised intravenously with a 0.01 mL dose of Zoletil 100 (Virbac, Carros, France; Bennett *et al.*, 2015). In addition to general anaesthesia, a subcutaneous injection of Lignol® (Dechra, Northwich, UK) was administered to both mothers and pups to minimise bleeding and to provide local anaesthesia to the skin and underlying tissue, which avoids whole-animal stress that is indicated by a significant increase in ventilation rate (Langton *et al.*, 2011). A small incision (approximately 1cm width) in the skin was made with a scalpel, and biopsies were then taken with a 6 mm diameter biopsy punch (Acu-punch, Acuderm, Schuco International, Watford, UK). The core was taken from the dorso-lateral pelvic area, and extended the full depth of the blubber layer. Mothers and pups were given an intramuscular dose of terramycin (1mL 10kg^{-1}; Pfizer Ltd.) to provide antibiotic cover to minimise infection risk at the biopsy site.

Blubber samples were placed in RNase free cryogenic vials and immediately frozen by immersion in liquid nitrogen. Samples were then stored at -80°C until they were analysed. One blubber sample was taken from mothers and pups at EARLY and LATE lactation. Two further blubber samples were also taken from pups, one at EARLY POST WEANING and one at LATE POST WEANING fast.
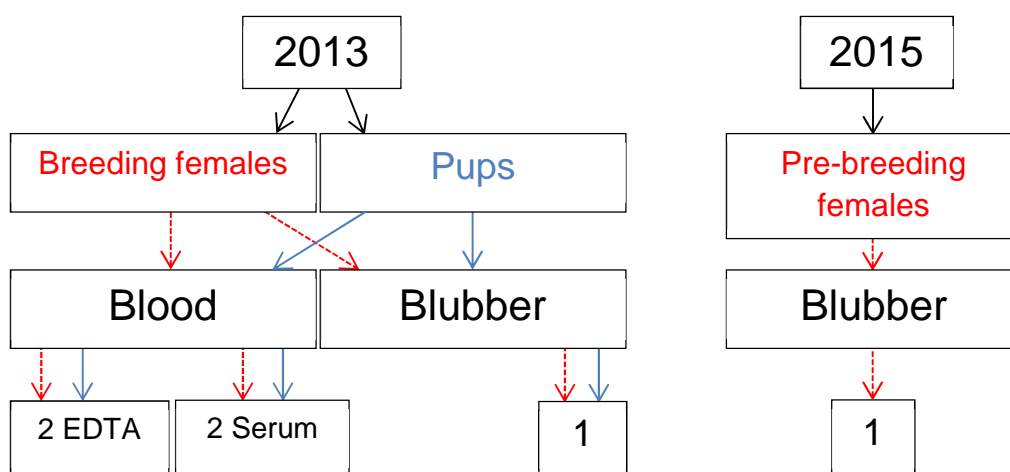
Figure 2.2. Schematic diagram to show blubber and blood samples taken and used in the studies presented in Chapters 3, 4 and 5. This includes breeding females and pups in 2013 and pre-breeding females in 2015. Adult females are represented by red, dotted arrows (↓) and pups are represented by complete blue arrows (↓). During 2013, the same samples were taken at all nutritional states (EARLY/ LATE lactation and the EARLY POST WEANING/ LATE POST WEANING fast).

2.1.2.7 Processing of blood samples

2.1.2.7.1 Plasma samples

A small volume of whole blood was removed from one EDTA-treated plasma vacutainer and used for blood smears and cell counts. Whole blood was diluted 1:10 with Baars fluid (saponin 0.25g, sodium citrate 3.5g, formalin 1mL, brilliant cresyl blue 0.1g; all chemicals obtained from Sigma Aldrich, Dorset, UK) to lyse red blood cells and allow WBCs to be counted using a haemocytometer counting chamber (Double Improved Neubauer, Weber Scientific International Ltd, UK; Figure 2.3; Table 2.2).
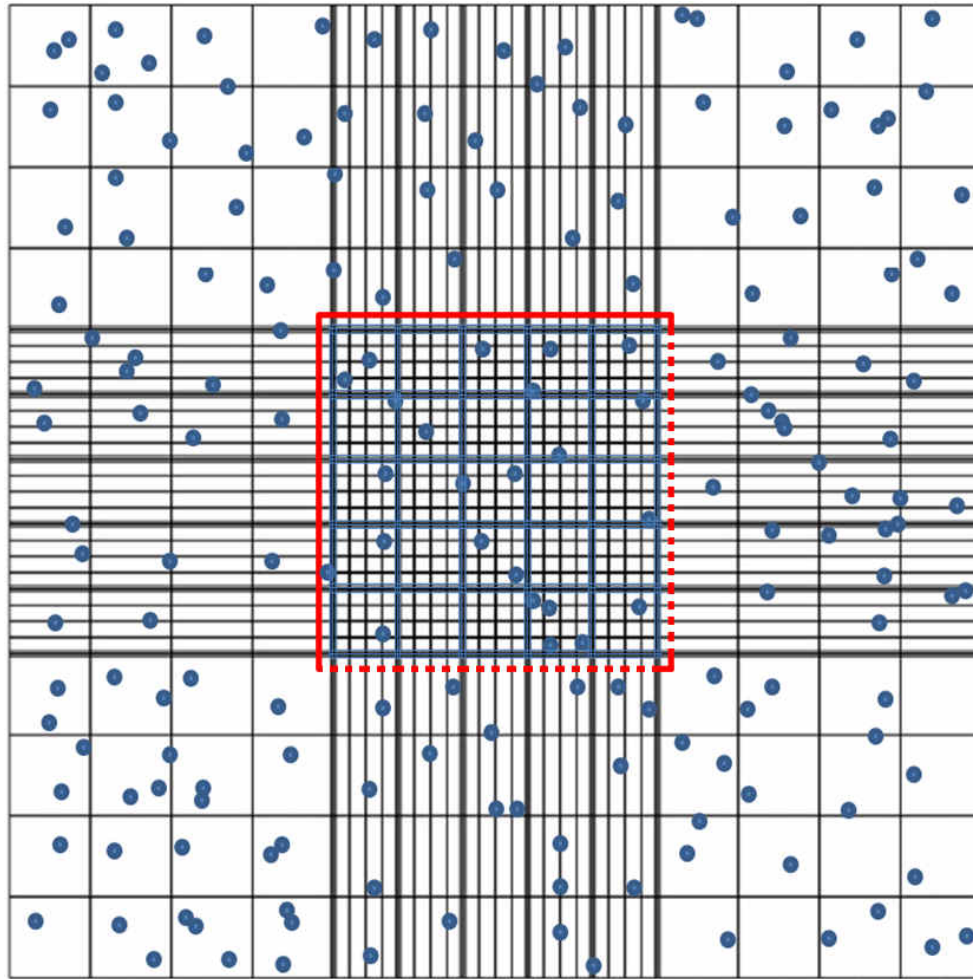
Figure 2.3. NOT TO SCALE Schematic image of a haemocytometer counting chamber, as viewed under a light microscope, when loaded with cells (blue filled circles) diluted in Baars fluid or stained with trypan blue (0.4%). Counting chamber was viewed at X10 magnification for counts. White blood cells were counted in the middle square (outlined in red) in both the upper and lower chambers of the haemocytometer, and the mean calculated. Cells were counted left to right in each small square and only counted within the triple lines (highlighted in blue) if they landed on the top and left (highlighted by complete red line) of the larger square.

2.1.2.7.2 Buffy coat (leukocyte) preparation

Vacutainers were centrifuged at 2000g for 15 minutes to separate the plasma, buffy coat (leukocytes; white blood cells) and red blood cells. Plasma was removed, being careful to not disturb the buffy coat, then carefully removed using a glass Pasteur pipette and aliquoted into 0.5mL microcentrifuge tubes. Tubes were stored at -80°C before analysis (e.g. glucose; samples stored at this temperature can be used for analysing a variety of proteins and short lived metabolites). The buffy coat was transferred into a 15mL sterile plastic centrifuge tube. Any remaining red blood cells were lysed using a potassium based lysis buffer (ammonium chloride 8.29g, potassium bicarbonate 1.0g, EDTA 0.037g, adjusted to pH 7.3 in 1L of ddH$_2$O; Sigma Aldrich, Dorset, UK), which was added directly to the buffy coat and inverted carefully. Once the red blood cells had lysed, the tube was centrifuged at 2000g for 10 minutes to pellet the white blood cells. The supernatant was discarded and the white blood cell pellet re-suspended in the same volume of lysis buffer, to ensure complete red blood cell lysis. The white blood cell suspension was again centrifuged at 2000g for 10 minutes and after the supernatant again removed. Further wash steps were included if the supernatant continued to appear particularly red in colour, indicating incomplete lysis of red blood cells, and continued until supernatant was clear.

2.1.2.7.3 White blood cell pellet for RNA extraction

After the final wash step, the white blood cell pellet was re-suspended in 1mL of lysis buffer, and then transferred to a 1.5mL RNase free microcentrifuge tube. This was centrifuged in a benchtop microcentrifuge at 2000g for 5 minutes. The

supernatant was removed and the remaining white blood cell pellet was immediately

flash frozen in liquid nitrogen. The sample was then stored at -80°C until analysis.

Table 2.2. Mean ± standard deviation cell populations of white blood cells from 2013 female and pup blood. Cell populations determined by cell counts after whole blood was treated with Baars fluid. All values expressed as $x10^7$. E.L, EARLY lactation; L.L, LATE lactation; E.P.W.F, EARLY POST WEANING fast; L.P.W.F, LATE POST WEANING fast.

	E.L Females (n = 18)	E.L Pups (n = 18)	L.L Females (n = 18)	L.L Pups (n = 18)	E.P.W.F Pups (n = 18)	L.P.W.F Pups (n = 15)
Cell numbers (mL^{-1})	1.39 ± 2.51	0.84 ± 0.30	1.5 ± 0.57	1.7 ± 0.96	0.92 ± 0.34	1.00 ± 0.28

2.1.3 Pre-breeding females

2.1.3.1 Study sites

Where specified, studies were performed using samples obtained from free-ranging pre-breeding grey seal females at Blakeney, Norfolk, England (52° 57' 58.32" N, 0° 57' 46.70" E) and Donna Nook, Lincolnshire, England (53° 28' 33.12" N, 0° 08' 27.02" E;) in early May 2015 (Figure 2.4). Adult females included in these studies were a part of a UK Department of Energy and Climate Change (DECC) Offshore Energy Strategic Environmental Assessment programme, tracking movements of grey seals that haul out on the UK coast of the southern North sea, led by the Sea Mammal Research Unit (SMRU), UK.
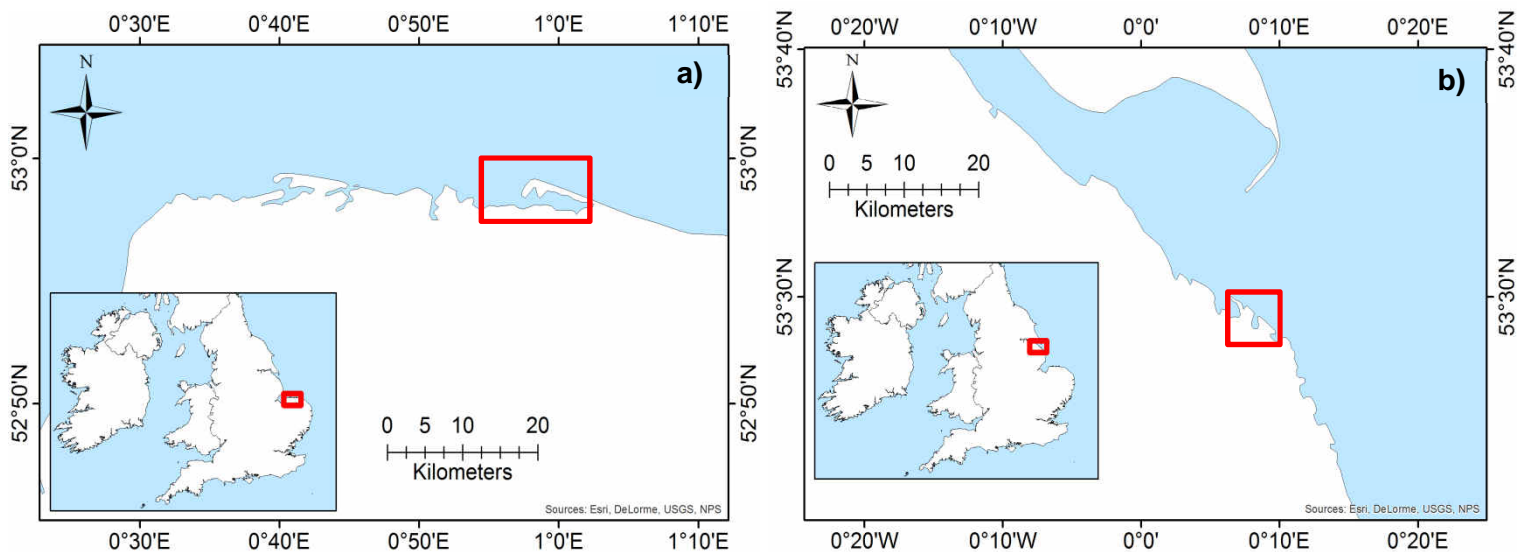
Figure 2.4. Maps showing the location of a) Blakeney, Norfolk, England and b) Donna Nook, Lincolnshire, England with inset showing both locations of the haul-out sites on a map of the rest of the UK and the Republic of Ireland, outlined in red. Expanded outlines of the coastal areas are depicted in the larger square, with red outlines showing the range across which study females were sampled. Where red outlines proceed into the blue water, this indicates sampled animals were hauled out on exposed sand banks at low tide. Credit to Rebecca Ross for map-making assistance in ArcGIS (Desktop Version 10, http://desktop.arcgis.com/en/).

2.1.3.2 Sampling regime and capture protocol

Individuals at Blakeney ($n = 5$) and Donna Nook ($n = 8$) were caught on or close to haul out sites either by hand using hoop nets or seine nets deployed from a rigid inflatable boat directly in front of the haul-out site (Jefferies *et al.*, 1993; McConnell *et al.*, 1999; Sharples *et al.*, 2012). Seals in this area normally haul-out onto intertidal sandbanks and these sites present large congregations of individuals, sometimes with groups of many hundreds, which increases the chances of catching an animal that meets study requirements (e.g. appropriate sex and body condition). The net formed a barrier whereby seals were trapped as they made their way to the water. The net was hauled to shore and captured animals transferred to smaller hoop nets for processing. Captured individuals were weighed immediately and a mass-dependent dose of Zoletil 100 (Virbac, Carros, France; approximately 1.0 mL/100 kg (1mg/kg scaled to 1.0 mL/100 kg) female mass; Baker *et al.*, 1990; Pomeroy *et al.*, 1996, 1999) given intravenously using a 20 gauge, 3.5 inch spinal needle into the extradural vein via an intravertebral space in the lower dorsal region above the pelvis (Harrison and Tomlinson, 1956; Geraci *et al.*, 1975; Gulland *et al.*, 2001; Sakamoto *et al.*, 2009). The puncture area was sprayed before the needle was inserted with the disinfectant Savlon (3% w/v cetrimide, 0.3% w/v chlorhexidine gluconate; Novartis, Horsham, UK) and after with a terramycin ® spray (oxytetracycline, Pfizer Ltd.). Mass outside of the breeding season can be exceptionally variable and a mass-dependent dose of Zoletil 100 is required. Masses cannot be estimated by eye, as is possible during the breeding season (S. Moss, pers comms), therefore animals would be vulnerable to being given a potentially fatal dose of Zoletil 100. Female mass was measured using a tripod as described in Pomeroy *et al.* (1996).

A tag (Dalton ID Systems, Henley on Thames, Oxon, UK) was attached to the interdigital webbing of the right rear flipper (Fedak and Anderson, 1982; Pomeroy *et al.*, 1996) and morphometric measurements including nose-tail length and standard axial girth were obtained for each female at capture.

2.1.3.3 Blubber biopsies

One blubber sample was taken from each adult female as described in section 2.1.2.6. A subcutaneous injection of Lignol® (Dechra, Northwich, UK) was administered and a small incision in the skin was made with a scalpel. Biopsies were then taken with a 6 mm diameter biopsy punch (Acu-punch, Acuderm, Schuco International, Watford, UK). The core was taken from the dorso-lateral pelvic area, and extended the full depth of the blubber layer. Females were given an intramuscular dose of terramycin (1mL 10kg^{-1}; Pfizer Ltd.). Blubber biopsies were wrapped in autoclaved, UV sterilised aluminium tin foil to identify in the inner and outer sections of the biopsy, before being placed in RNase free cryogenic vials and flash frozen in liquid nitrogen. Samples were then stored at -80°C until they were analysed. Figure 2.2 provides a visual representation of sampling regime for pre-breeding females and breeding females and pups.

2.2 Laboratory Techniques

2.2.1 Gene expression analysis

The relative expression of a number of stress responsive genes and those involved in tissue function and inflammation was tested and, when optimised satisfactorily (i.e. specific amplification with efficiency of between 90-110%), measured in fat tissue and white blood cells that were flash frozen in the field and in experimentally manipulated explants.

2.2.2 Reference gene selection

To ensure that the amount of target mRNA is reported as accurately as possible and for results to be comparable, careful template preparation and appropriate standardisation is required (Pfaffl, 2001). The relative quantification method (applied to qRT-PCR data in this thesis; Serazin-Leroy *et al.*, 1998) requires standardisation with another gene or genes, the reference, endogenous control, or house-keeping genes, the expression of which is assumed to be constant. However, some regularly used reference genes have occasionally been documented to be regulated (Foss *et al.*, 1998; Thellin *et al.*, 1999; Schmittgen *et al.*, 2000; Suzuki *et al.*, 2000; Bustin, 2002; Tricarico *et al.*, 2002). Therefore, before any gene is selected to act as a standard, a number of candidate reference genes should be considered and tested with the samples that are to be used in future qRT- PCR experiments, to determine if they are regulated under the different treatments or conditions of interest.

Two separate pieces of Excel spreadsheet based software were utilised to select the most stable reference genes; *NormFinder* (Andersen *et al.*, 2004; downloaded from http://moma.dk/normfinder-software) and *BestKeeper* (Pfaffl *et al.*,

2004). As the statistical algorithms employed by both these software's differ in their assumptions and methods, it is helpful to use both approaches to identify appropriate candidate genes (Expósito-Rodríguez *et al.*, 2008; Docimo *et al.*, 2013). *NormFinder* estimates standard deviation for each gene relative to the expression of all genes in the analysis and combines inter- and intra-group variation for each gene into a stability value. Genes with low standard deviations and, in turn, low stability values are deemed better candidates for reference genes (Andersen *et al.*, 2004). The *BestKeeper* software uses a different approach that calculates the *BestKeeper* index (BKI; a stability index), which is the geometric mean of the C_T values of the reference genes from all the samples and represents the highest level of stability as it includes every gene across all samples in the analysis. The standard deviation of the C_T values of each reference gene is also evaluated and only those that are below one are included in the BKI. The stability of each gene is assessed by its correlation with this index. *BestKeeper* relies on pair-wise correlation analyses to assess relationships and a high correlation between the BKI and the stability of the candidate endogenous control gene indicates a more stable and therefore more suitable gene (Pfaffl *et al.*, 2004).

In the studies presented in this thesis, six potential reference genes were considered for use under the different conditions of interest: cyclin A (CycA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein S9 (S9), ribosomal protein L8 (L8), ubiquitously expressed prefoldin-like chaperone (UXT) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ; primer details are shown in Table 2.3).

For the different conditions considered each of the candidate reference gene primer pairs were run in triplicate for all samples, using qRT-PCR as described in

Section *2.2.6 and 2.2.8*. The average cycle threshold (C_T) values were then input into both *NormFinder* and *BestKeeper* to ascertain the most suitable reference genes.

2.2.3 Primer Design

Primers pairs were designed to specifically amplify reference genes and a range of genes linked to cellular level stress (Table 2.3). Sequences of the most closely related Carnivore species (belonging to families Phocidae; Otariidae; Odobenidae; Ursidae; Mustelidae; Canidae) were obtained from the National Centre for Biotechnology Information protein database (http://www.ncbi.nlm.nih.gov/protein). Carnivore sequences were aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/; now Clustal Omega, http://www.ebi.ac.uk/Tools/msa/clustalo/) to identify the most conserved regions between species. Primers were designed against the conserved regions using Primer3 software (Koressaar and Remm, 2007; Untergrasser *et al.*, 2012) to have a melting temperature of 60°C.

Primers were synthesised by MWG Eurofins Operon (Ebersberg, Germany), Sigma Aldrich (Dorset, UK) or Integrated DNA Technologies (Leuven, Belgium).

2.2.4 Total RNA extraction from blubber tissue and white blood cell pellets

250-500mg of blubber tissue was weighed and white blood cell pellets were transferred into a 2mL homogenisation tube and homogenised in 1mL TRIzol® Reagent (Ambion, Life Technologies, Paisley, UK) on ice. Tissue and cell pellets were homogenised with a hand-held electric dispersing instrument (Disperser T10 basic ULTRA-TURRAX®, IKA®, Staufen, Germany). RNA was then extracted

according to the manufacturer's instructions. Briefly, the homogenised tissue-TRIzol mixture was left at room temperature for 5 minutes, to dissolve cell components, before the addition of 200µL chloroform (Sigma Aldrich Co., Dorset, UK). This was vortexed for 30 seconds and incubated at room temperature for 10 minutes, and then centrifuged at 13,000rpm in a benchtop centrifuge (Eppendorf 5415R Stevenage, UK). The aqueous phase was collected and 550µL cold isopropanol added to precipitate RNA, which was then mixed by inversion and incubated at -20°C for approximately 2 hours. After this time the mixture was centrifuged at 13,000rpm for 15 minutes at 4°C. The supernatant was removed and the RNA pellet was washed twice in 500µL cold 75% ethanol. The RNA pellet was air dried on the benchtop at room temperature for 5 - 10 minutes. The pellet was then resuspended in 15µL molecular grade water.

The concentration and purity of RNA was measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Basingstoke, UK). If required, RNA was further diluted to achieve a concentration of approximately 500ng/µL. Integrity was determined by gel electrophoresis (Figure 2.5). Approximately 200ng of total RNA loaded with a 6x Orange DNA loading dye (Thermo Scientific, Basingstoke, UK) was separated on a 1% agarose gel, stained with SYBR® Safe DNA gel stain (Invitrogen, Life Technologies, Paisley, UK). The 28S and 18S ribosomal bands visualised with a O'Generuler™ 50bp DNA ladder (Thermo Scientific, Basingstoke, UK) on a ImageQuant LAS 4000 (GE Healthcare Life Sciences, Hertfordshire, UK) imaging system and examined for degradation (Figure 2.5). RNA was stored at -80°C before cDNA synthesis.
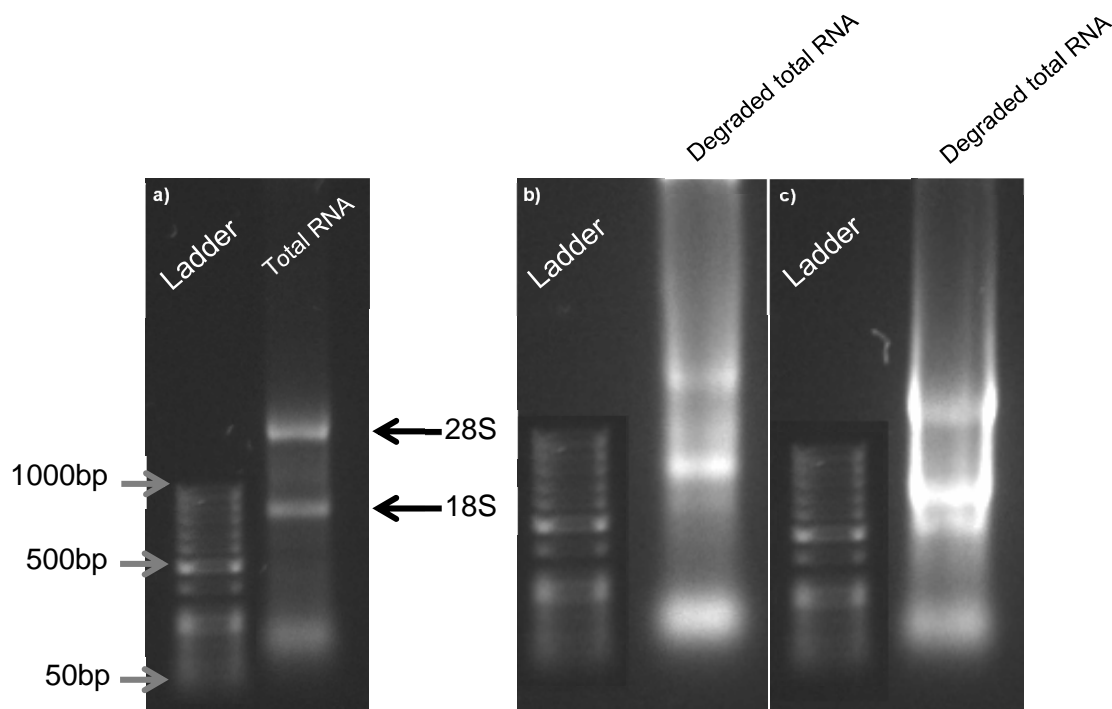
Figure 2.5. Examples of degraded and intact RNA from inner and outer blubber core samples. In these examples, approximately 1µg total RNA were run alongside O'Generuler™ 50bp DNA ladder (Thermo Scientific) on a 1% agarose gel. The 18S and 28S ribosomal RNA bands are clearly visible in image a), where the RNA is intact. Images b) and c) show degraded RNA appearing as a low molecular weight smear, with indistinct bands. Images b) and c) also indicate genomic DNA contamination, characterised by the high molecular weight smears, seen above both the 18S and 28S bands.

2.2.5 cDNA synthesis

For complementary DNA (cDNA) synthesis, 250-500ng of RNA was reverse transcribed using the QuantiTect Reverse Transcription kit (QIAgen, Manchester, UK), following the manufacturer's instructions. The quantity of RNA reverse transcribed was kept constant within the same experimental conditions to allow comparison between samples. A genomic DNA (gDNA) elimination reaction of 42°C for 2 minutes preceded reverse transcription to ensure RNA was total DNA-free.

2.2.6 Primer testing

To ensure primer pairs amplified one amplicon of the correct predicted size, each pair was used to amplify the specific gene from a pool of cDNA in a standard thermal cycler (Prime, Techne, Bibby Scientific, Staffordshire, UK) with a Taq polymerase chain reaction (PCR) core kit (QIAgen, Manchester, UK). Cycling conditions were as follows: initial denaturation of 94°C for 30 seconds, then 35 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 60°C and 1 minute extension at 72°C with a final extension step of 72°C for 1 minute. The PCR products were visualised on a 1% agarose gel stained with SYBR® Safe DNA gel stain (Invitrogen, Life Technologies) in Tris- borate EDTA buffer. Primer pairs that produced a single amplicon using standard endpoint PCR conditions were further tested to ascertain the annealing temperature and efficiency. This was ascertained using quantitative real-time PCR (qRT-PCR) including a melting curve analysis (StepOne software; Applied Biosystems) with the following cycling conditions: initial denaturation of 94°C for 10 minutes, then 40 cycles of 95°C for 15 seconds, 60°C annealing for 1 minute and finally 95°C for 15 seconds. Each primer pair was run in triplicate using a log serial dilution of pooled cDNA template (1:10, 1:100, 1:1000, 1:10000 or 1:2, 1:4, 1:8, 1:16, 1:32, 1:64), to determine the efficiency of the amplification. Efficiency (*E*) was calculated using Equation (1) (Pfaffl, 2001; Rasmussen, 2001; Liu and Saint, 2002; Tichopad *et al.*, 2003; Pfaffl *et al.*, 2004), where the slope of a linear regression model is fitted to the log-transformed data of the serial dilution, plotted against the corresponding C_T values.

$$E = 10^{-1/slope} \text{ (1)}$$

Primer pairs were required to have amplification efficiencies of between 90% and 110% (Bustin *et al.*, 2009; D'haene *et al.*, 2010; Taylor *et al.*, 2010).

2.2.7 Sanger sequencing to verify amplicon identity

To ensure gene specific primers were amplifying expected sequences, amplicon identity was verified via Sanger sequencing.

2.2.7.1 Gel extraction

Endpoint PCR using gene specific primers and pooled cDNA was performed using a PCR Taq Core kit (QIAgen, Manchester, UK) with conditions as described in section 2.2.6. PCR product was visualised on a 1% agarose gel, stained with SYBR® Safe DNA gel stain (Invitrogen, Life Technologies, Paisley, UK) and loaded with a 6x Orange DNA loading dye (Thermo Scientific, Basingstoke, UK). Once a single band of the correct size was confirmed, the DNA was extracted using a PureLink ™ Quick Gel Extraction Kit (Invitrogen, Life Technologies Ltd, Paisley, UK). The resulting purified DNA was subjected to an ExoSAP clean up reaction (described below in section 2.2.7.2). Samples were sent off for sequencing (Source BioScience Ltd, Nottingham, UK), however, not enough bases were called to allow a BLAST search to identify any matches.

2.2.7.2 ExoSAP clean-up reaction

Enzymatic clean-up of amplified PCR products was performed using exonuclease I of *Escherichia coli* (Exo; #EN0581; Thermo Scientific™, Thermo Fisher Scientific, Basingstoke, UK) and shrimp alkaline phosphatase (SAP; #EF0511; Fermentas Life Sciences, Thermo Fisher Scientific, Basingstoke, UK). Exo

removes excess primer (ssDNA containing a 3'-hydroxyl terminus) and SAP dephosphorylates nucleic acids, nucleotides and proteins (catalysing the release of 5'- and 3'- phosphate groups). The ExoSAP reaction purifies PCR products and prevents interference of excess primers and dNTPs in downstream applications e.g. DNA sequencing and cloning reactions.

1µL SAP, 0.5µL Exo and 0.5µL molecular H_2O was added to 5µL PCR product and then incubated at 37°C for 1 hour and 80°C for 15 minutes, to inactivate the enzymes.

2.2.7.3 Cloning of amplicons for sequencing

As the gel extraction protocol described in 2.2.7.1 failed to produce DNA that could be adequately sequenced, a cloning protocol was used.

Before the cloning protocol was initiated, a PCR was performed using pooled cDNA with the gene specific primers used in qRT-PCR. The amplified PCR products were treated using ExoSAP reaction (section 2.2.7.2) and the purified PCR products used directly in cloning reactions.

For this cloning protocol the avirulent laboratory *Escherichia coli* strain DH5α (Invitrogen, Thermo Fisher Scientific, Basingstoke, Hampshire, UK; hereafter referred to as *E. coli*) was used, stored at -80°C. This *E. coli* strain was suitable for direct transformation with the ligation product provided by the CloneJET™ PCR Cloning kit (#K1231; Thermo Scientific, Thermo Fisher Scientific, Basingstoke, UK), which was used here.

2.2.7.3.1 Agar plate and liquid media preparation

Luria-Bertani (LB; Bertani, 1951) agar plates were prepared using 32g of LB Agar (Lennox L Agar; Invitrogen, Thermo Fisher Scientific, Basingstoke, Hampshire, UK) powder dissolved in 1L of distilled H_2O, which was then autoclaved at 121°C for 15 minutes. Once the LB agar had cooled, plates were poured and left to solidify at room temperature before use. For the growth of transformants, LB agar plates were prepared with 50µg mL^{-1} ampicillin (Sigma Aldrich, Dorset, UK).

Liquid media was prepared using 20g LB Broth Base (Lennox L Broth Base; Invitrogen, Thermo Fisher Scientific, Basingstoke, Hampshire, UK) powder dissolved in 1L of distilled H_2O and autoclaved at 121°C for 15 minutes.

2.2.7.3.2 Preparation of working culture

10µL of stock *E. coli* cells were defrosted on ice and spread on LB agar plates (Lennox L Agar; Invitrogen, Life Technologies, Paisley, UK) using aseptic technique. Plates were then incubated at 37°C (SciQuip Incu – 50S Incubator, SciQuip Ltd, Shropshire, UK) for two days to resuscitate cells and produce colonies for the starter culture.

2.2.7.3.3 E. coli *starter culture*

A single colony was selected from the working culture and grown overnight in liquid media (LB Broth; Lennox L Broth Base; Invitrogen, Thermo Fisher Scientific, Basingstoke, Hampshire, UK) left in the incubator shaker at 37°C, 100rpm (shaking speed) overnight (SciQuip Constant Temperature Incubator Shaker, SciQuip Ltd, Shropshire, UK).

2.2.7.3.4 Exponential growth of E. coli cells for transformation

200μL starter culture was added to 10mL fresh liquid media and left one hour
15 minutes at 37°C, 100rpm to achieve exponential growth of *E. coli* cells (M. Emery,
pers. comm.) which are then ready for use in the transformation. Cells in the
exponential phase will continue to grow until the cell concentration exceeds the
capacity of the medium and are considered to be more genetically and
phenotypically stable, helping to ensure viability and increasing the likelihood of
vector insertion.

2.2.7.3.5 Blunting reaction and ligation reaction (vector preparation)

As the *Taq* DNA polymerase provided as part of the Taq polymerase chain
reaction (PCR) core kit (QIAgen, Manchester, UK) generates sticky ended DNA with
a 3'- overhang, a blunting reaction was required. The following reactions were
performed according to the manufacturer's instructions using the reagents provided
by the CloneJET™ PCR Cloning kit (#K1231; Thermo Scientific, Basingstoke, UK).
Briefly, the blunting reaction mixture was incubated at 70°C for five minutes then
chilled on ice. The ligation reaction mixture, containing the p.JET 1.2/ blunt Cloning
Vector (Thermo Scientific), was added directly to the blunting reaction mixture and
incubated at room temperature (22°C) for five minutes.

2.2.7.3.6 Calcium chloride (CaCl$_2$) transformation

Approximately 1.5mL of the exponential starter culture was transferred to a
2mL microcentrifuge tube and centrifuged at 7000 rpm for 2 minutes. The
supernatant was discarded, the cell pellet resuspended in 200μL ice-cold 50mM
CaCl$_2$ solution and placed on ice for 20 minutes. CaCl$_2$ desiccates cells making it

possible for them to survive exposure to low temperatures by preventing ice-crystal formation. The cell suspension was centrifuged at 7000 rpm for 2 minutes, supernatant discarded and the cell pellet gently resuspended in 100µL ice-cold 50mM $CaCl_2$ solution. This was transferred to a 1.5mL microcentrifuge tube containing 10µL ligation/blunting reaction mixture. The contents were mixed gently and placed on ice for 10 minutes. The mixture was then incubated at room temperature for five minutes and then on ice for 20 minutes. The cells were then heat-shocked at 42°C for exactly two minutes. 900µL liquid media was added, gently mixed and incubated at 37°C for one hour.

The transformed cells were then plated on duplicate ampicillin plates (50µg mL^{-1}) and incubated at 37°C for two days. After this time transformants (colonies) were counted on each plate to determine the frequency of transformation per µg of plasmid DNA used.

2.2.7.3.7 Colony PCR

One transformant (colony) was selected and incubated overnight at 37°C in liquid media containing 50µg mL^{-1} ampicillin. This was centrifuged at 10,000 rpm for 10 minutes and the supernatant discarded. The pellet was resuspended in 200µL liquid media and used directly in the endpoint PCR using pJET 1.2 Forward and Reverse primers, provided as part of the CloneJET™ PCR Cloning kit (#K1231; Thermo Scientific, Basingstoke, UK). Endpoint PCR utilised a Taq polymerase PCR core kit (QIAgen, Manchester, UK). Cycling conditions were as follows: initial denaturation of 95°C for 3 minutes, then 25 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 60°C and 30 second extension at 72°C with a final extension step of 72°C for 1 minute. The cells were prepared for long term storage

with the addition of 30% glycerol and flash frozen in liquid nitrogen before being transferred to -80°C.

The PCR product was analysed on an agarose gel to confirm the presence of the expected amplicon. The PCR product was treated with ExoSAP before being sent off for sequencing (Source BioScience Ltd, Nottingham, UK).

Table 2.3. Primer pair sequences, amplicon sizes and efficiencies of reference genes (*YWHAZ, L8, GAPDH, CycA, S9* and *UXT*) and genes of interest (HSPs and REs) used in qRT-PCR. "Δ" GAPDH published sequences taken from Beineke *et al.* (2004); "$\Delta\Delta$" ribosomal L8 published sequences taken from Tabuchi *et al.* (2006).

Gene	Primer Sequence 5'-3'		Amplicon size	Efficiency (%)
	Forward	Reverse		
YWHAZ	GAGGTTGCTGCTGGTGATGA	TCCGGGGAGTTCAGAATTTCG	170	91.61
L8$^{\Delta\Delta}$	GGTGTGGCTATGAATCCTGT	ACGACGAGCAGCAATAAGAC	126	113.26
GAPDH$^{\Delta}$	GCCAAAAGGGTCATCATCTC	GGGGCCATCCACAGTCTTCT	232	123.21
CycA	TCATCTGCACCGCCAAGAC	AAGCGCTCCATGGCTTCCAC	260	93.20
S9	ACATCCCGTCCTTCATTGTC	CAATCCTCCTCCTCGTCATC	157	101.44
UXT	CTCACAGAGCTCAGCGACAG	AGGTGTCTCCGGGAAATTCT	117	118.41
Hsp70	AAGATCACCATCACCAACGA	AAATCACCTCCTGGCACTTG	238	101.56
Hsc70	AATCAAGTTGCGATGAACCC	CCTGCCAGCATCATTCAC	139	102.90
Hsp90	TGGAGCGTCTTCGGAAG	TCTGGAAGTTCCAAGCCCT	139	99.24
Hsp40	CCTGGAATGGTTCAGCAAAT	GCCATCTTTCATGCCTTTGT	159	91.16
Hsp27	AGCTGACGGTCAAGACGAAG	GGCAGCGTGTATTTTCGAGT	112	97.32
GPx	TTGTCAACGTGGCCAGCTA	TGAGGCTGGGTAGGATTTCC	158	96.13
CAT	GGTAATTGGGATCTTGTTGG	ATGGTCTGGGACTTCTGG	140	101.42
SOD	CCTGGAGCCTCACATCAAC	TAGCTCTTCAGCCTGGGCT	127	98.63
GST	CCTCAAGGAGAGAACCCTGA	CTGGGCCATGTTAACCACTT	123	92.26
Nox4	AGCCTCCGCATCTGTTCTTA	CTTCTGGTTCTCCTGCTTGG	114	113.74

2.2.8 Quantitative real-time PCR (qRT-PCR)

For qRT-PCR, cDNA was diluted 1:10 with RNase free water and 4μL used as a template using 5μL iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hertfordshire, UK) and 0.5μL of each forward and reverse gene specific primers (Table 2.3). The qRT-PCR was performed in either a 48, 96 or 384 well plate with all samples run in triplicate, utilising the StepOne system (Applied Biosystems). The cycling conditions are reported above in section 2.2.7. A melting curve for each PCR was determined by reading the fluorescence emitted at each degree Celsius between 95 and 60°C. Raw C_T values generated by qRT-PCR reactions were inspected using StepOne™ Software version 2.3 (Applied Biosystems). Outliers within triplicate runs were removed from the analysis. For each gene the average threshold was calculated using thresholds from all qRT-PCR reaction plates. Each threshold was then set to this value to allow comparability across all plates. Therefore, each threshold for each gene was the same across all plates. Baselines were examined to ensure that they were similar for all genes across all plates. Any alterations made to the data were to ensure values in the study were comparable.

2.2.9 Glucose measurements

2.2.9.1 Glucose oxidase assay

Glucose concentration was determined in grey seal pups using the glucose oxidase method (Boehringer-Mannheim), according to Webster (1996). Before beginning the assay, 50μL volume plasma samples were deproteinated with 500μL ice cold 0.33 mol L^{-1} perchloric acid (PCA; Sigma Aldrich, Dorset, UK) and then centrifuged at 13,000rpm in a benchtop centrifuge (Eppendorf 5415R Stevenage, UK) for 20 minutes. The supernatant was removed and diluted a hundred fold with

ultra-pure water. Visibly haemolysed plasma samples were not included in the assay.

A 20 mmol L^{-1} glucose standard was prepared by dissolving 0.054g glucose (Sigma Aldrich, Dorset, UK) in 15mL ultra-pure water. Aliquots were taken and diluted to 1.25, 2.5, 5, 10 and 15 mmol L^{-1}; chosen to fully cover the range of glucose concentrations found in grey seal plasma (Hall, 1998). 500 μL of PCA was added to 50 μL of each standard, including a blank of ultra-pure water.

180 μL of a pre-made reagent to catalyse the oxidation of glucose (100 mL phosphate (NaH_2PO_4) buffer, 125 μg horseradish peroxidase, 5.0 mg glucose oxidase and 0.1 g 2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS); all chemicals obtained from Sigma Aldrich, Dorset, UK) and 20μL of blank, standard or sample was pipetted into a 96 well, flat bottomed microplate (Thermo Scientific™, Thermo Fisher Scientific, Basingstoke, UK) and allowed to mature for 15 minutes, turning a green/turquoise colour. Plates were read at 410 nm on a VERSAmax™ plate reader (Molecular Devices, Sunnyvale, California, USA). Blanks, standards and samples were run in duplicate.

A standard curve was constructed from the absorbance values of known glucose standards and the resulting equation was used to calculate glucose concentrations in samples.

2.2.9.2 RX Monza Clinical Chemistry Analyser glucose assay

Glucose was measured in plasma of breeding grey seal females using the RX Monza Clinical Chemistry Analyser (Randox Biosciences, County Antrim, UK) and associated Randox Glucose GOD-PAP GL 364 kit (Randox Biosciences, County

Antrim, UK), which determined glucose after enzymatic oxidation in the presence of glucose oxidase. The subsequent reaction of hydrogen peroxide with phenol and 4-aminophenazone (in the presence of peroxidase) forms a red-violet quinoneimine dye which acts as an indicator.

The RX Monza Clinical Chemistry Analyser was initially calibrated using a blank sample of ddH_2O. A reagent blank (buffer and glucose oxidase/4-aminophenazone/peroxidase reagent, described below, with no 'added' glucose) was also run. 10μL of two glucose standards, provided by the manufacturer (Randox Biosciences, County Antrim, UK), which represented the upper (15mM L^{-1}) and lower (6mM L^{-1}) extremes of expected glucose concentrations were then added to 1000μL of buffer (phosphate buffer, 0.1 mol L^{-1}, pH 7.0 and phenol, 11 mmol L^{-1}) and glucose oxidase (≥1.5 kU L^{-1})/4-aminophenazone (0.77mmol L^{-1})/peroxidase (≥1.5 kU L^{-1}) reagent and incubated at 37°C for 10 minutes. These standards were then read in duplicate. Duplicate readings were required to be within 10% (<0.5mM L^{-1}) or were re-run. Plasma samples were treated in the same manner as glucose standards. Blanks, standards and samples were read at 500nm and the absorbance of the standard ($A_{standard}$) was measured against the absorbance of the sample (A_{sample}) against the reagent blank, returning a glucose concentration in mM L^{-1} (mM L^{-1} = (A_{sample}/ $A_{standard}$) x standard concentration). Visibly haemolysed samples were not analysed.

Glucose standards were also read at the end of the assay for quality control purposes, to ensure machine and reagent function was adequate (i.e. instrument settings and light source, reagents within expiry date) and to confirm the experimental glucose concentrations obtained were accurate.

2.2.10 Malondialdehyde (MDA) concentrations

Lipid peroxidation (LP) was determined by measuring MDA concentrations in blubber tissue using a Lipid Peroxidation (MDA) Assay Kit (MAK085, Sigma Aldrich, Dorset, UK). Briefly, approximately 10 mg blubber tissue (made up of tissue remaining after RNA extractions) was homogenised on ice in 150µL MDA Lysis Buffer, 150µL perchloric acid and 3µL butylated hydroxytoluene (BHT; inhibits further lipid peroxidation), to lyse cells and precipitate protein, according to the manufacturer's instructions. Samples were then centrifuged at 13,000rpm in a benchtop centrifuge (Eppendorf 5415R Stevenage, UK) for 15 minutes to remove insoluble material. The fat layer above was carefully bypassed and 200µL supernatant was removed and filtered through a 0.20µM, regenerated cellulose, 4mm diameter syringe filter (Corning®, Corning Inc. Life Sciences, Massachusetts, U.S.A) as suggested by the manufacturer to eliminate sample turbidity.

A 0.1M MDA standard was prepared by diluting 10µL 4.17M MDA standard Solution in 407µL ddH$_2$O. This was further diluted (20µL 0.1M MDA standard solution in 980µL ddH$_2$O) to prepare a 2mM MDA standard solution. Aliquots were taken and diluted to prepare 4, 8, 12, 16 and 20nmol MDA standards.

600µL pre-prepared thiobarbituric acid (TBA) solution (TBA dissolved in 7.5mL glacial acetic acid (Sigma Aldrich) and made up to 25mL with ddH$_2$O) was added to all blanks, standards and the 200µL filtered samples which were incubated at 95˚C for 60 minutes to form the MDA-TBA adduct. This was then cooled to room temperature on ice for 10 minutes. 200µL reaction mixture was pipetted onto a 96 well, flat bottomed microplate (Thermo Scientific™, Thermo Fisher Scientific, Basingstoke, UK). The colorimetric product formed during the reaction of MDA with

TBA was read at 532nm on a VERSAmax^{TM} plate reader (Molecular Devices, Sunnyvale, California, USA). Blanks, standards and samples were run in duplicate.

Blank values were subtracted all readings to correct for background values. A standard curve was constructed from the absorbance values of known standards and the resulting equation was used to calculate MDA concentrations (nmol) of unknown samples.

2.2.11 Data analysis

qRT-PCR data were initially analysed using REST© 2009 (Pfaffl *et al.*, 2002). REST© or the relative expression software tool, calculates the relative quantification of each gene (normalised to the appropriate reference genes) and uses a Pair Wise Fixed Reallocation Randomisation Test © to test for significant differences between the two groups of gene data ('Samples (treated)' and 'Controls (untreated)') inputted into the software. However, as REST© 2009 only allows a comparison of two groups of data, there is no scope for further data exploration and the inclusion of other covariates that could explain variability in the relative expression pattern.

Linear mixed-effects models (LMEs) were used when there were more than two groups of data and to include covariates and look at time series and repeated measures. These statistical analyses were performed in R Studio (R 2.15.2; Ihaka and Gentleman 1996; R Core Development Team 2003).

The normalised expression values, derived from the ΔC_T method (described in Livak and Schmittgen 2001), were used in the analysis as the dependent variables. Independent variables included any variable related directly to the animals themselves e.g. sex, body mass, age that may influence gene expression, plus

additional covariates that had been measured i.e. glucose, MDA concentration, contaminant loads.

2.2.11.1 Maternal postpartum mass, pup weaning mass and expenditure estimates

Maternal postpartum mass (MPPM), percentage maternal expenditure (percentage of MPPM utilised by weaning), pup weaning mass and percentage mass transfer efficiency (total mass gain of pup/total mass loss of female x 100) were estimated as described previously by Pomeroy *et al.* (1999) in mother-pup pairs that had been captured at both EARLY and LATE lactation/ suckling and for which birth and weaning dates were known. This also provided daily pup mass gain rates ((estimated weaning mass – estimated birth mass)/ length of suckling period, in days OR (mass at LATE lactation - mass at EARLY lactation)/ time between EARLY and LATE suckling, in days) during suckling for all animals ($n = 18$). Daily mass loss rates ((mass at LATE POST-WEANING – mass at EARLY POST-WEANING)/ time between EARLY and LATE POST-WEANING, in days) for pups during the POST-WEANING fast were also calculated ($n = 18$). These estimates were included in LMEs as additional explanatory variables to determine if there was a trade-off between physiological stress markers and performance during lactation/suckling i.e. does gene expression of HSPs and REs have an effect on the lactation/suckling performance measures detailed above?

Chapter 3.

Blubber heat shock protein and redox enzyme mRNA abundance, and oxidative damage in female grey seals (*Halichoerus grypus*): support for the life-history-oxidative stress theory from an animal model of simultaneous lactation and fasting.

Abstract

The ability to respond adequately to stress is crucial to an organism's fitness and survival. Biological responses to stress are energetically costly. Therefore, when resources are limited, organisms face trade-offs in allocation of energy to stress responses and life-history tasks, such as reproduction. Adult female grey seals naturally experience resource limitation during lactation because they fast throughout the suckling period. I sought to understand the trade-offs in allocation of resources to cellular defences and reproduction faced by adult female grey seals. Here, I focussed on blubber tissue, which undergoes large changes in volume because it must supply fuel for metabolism and high fat milk during lactation and act as a lipid depot and insulating layer throughout the year. I used qPCR to measure the mRNA abundance of key cellular stress markers: heat shock proteins (*Hsp70*; *Hsc70*; *Hsp90*; *Hsp40*; *Hsp27*) and redox enzymes (*GPx*; *CAT*; *SOD*; *GST*; *Nox4*), in blubber of lactating ($n = 17$) and pre-breeding ($n = 13$) wild adult female grey seals. Malondialdehyde (MDA) concentration (a marker of lipid peroxidation) was used as an index of tissue-level damage. Measures of energy transfer, including mass loss rates and efficiency of mass transfer from mother to pup, were calculated as markers of performance during lactation that indicate consequences of whole-animal level trade-offs. There was no change in MDA concentration or gene expression of HSPs or the majority of redox enzymes, despite a 23% reduction in mass between early and late sampling points in the lactation-fasting period. This shows that animals maintain cellular defences despite high energetic costs of parturition and lactation. There was a 0.90 fold decrease in *Nox4* relative expression late in the lactation period, which could reflect a reduction in adipocyte turnover to reduce energy expenditure, or lower insulin sensitivity late in lactation. Higher levels of most HSP or

RE genes or MDA were not associated with female mass loss rates, reduced mass transfer from mother to pup, or lower pup weaning mass. SOD expression was inversely related to mass loss rate. Females with greater blubber oxidative stress during lactation did not experience reduced performance. Pre-breeding females showed significantly higher *Hsp90* and *Hsp27* mRNA abundance and lower expression of all redox enzymes and reduced MDA concentrations compared to lactating females. Pre-breeding individuals may rely on chaperone proteins *Hsp90* and *Hsp27* for production of adipokines or enzymes involved in lipid biosynthesis. These data suggest that lactating female grey seals experience greater oxidative stress compared to pre-breeding individuals, which provides support for the life-history-oxidative-stress theory. Our data suggest lactating females invest limited resources into pup-rearing at the expense of cellular defences, resulting in increased oxidative damage to macromolecules. Lactation may thus be a period of increased susceptibility to additional stressors in adult female grey seals, and this may be also be true of other species that face similar trade-offs.

3.1 Introduction

Biological responses to stress are energetically costly (Parsons, 1994; 2005; Segerstrom, 2007). However, the ability to mount an adequate stress response is crucial to fitness because it dictates whether an organism can survive and thrive during physiological challenges (Parsons, 2005; Blas *et al.*, 2007; Busch and Hayward, 2009). Stress responses alter metabolic demand and often divert resources from maintenance processes to deal with immediate requirements. Energy balance at the whole animal and cellular level is therefore altered. Organisms face trade-offs in allocation to energy-consuming biochemical and physiological cellular processes (Hardie and Hawley, 2001; Hardie, 2008; Romero *et al.*, 2009; Rabasa

and Dickson, 2016), particularly when resources are limited (Emlen *et al.*, 1998; Segerstrom, 2007; Guderley and Pörtner, 2010; Sokolova, 2013). By shifting relative investment in energetically expensive organism-level processes, such as reproduction or immune defences, fitness may be compromised. Stress therefore acts as a powerful selective force, shaping life histories and population dynamics (Hoffmann and Hercus, 2000; Lafferty and Holt, 2003; Pamplona and Constantini, 2011; Monaghan and Spencer, 2014).

Stress responses at all biological levels are adaptive because the organisms or cells 'normal' internal environment is temporarily or permanently altered to cope with the new conditions and regain homeostasis, a concept known as 'allostasis' (McEwen, 1998; McEwen and Wingfield, 2003). As described in detail in Chapter 1, 'allostatic load' is the sum of all the adjustments an individual has to make to meet a number of challenges. When the individual can meet the additional demands, it experiences eustress. If the energy requirements of all the adjustments exceed the capacity of the animal to replace that energy, then the individual experiences 'allostatic overload' and distress, which may result in reduced health, fitness and survival chances (Romero, 2004).

Stress responses at both the whole-animal and cellular level can be triggered by various stimuli or 'stressors' that disrupt homeostasis. Limited or excess nutrients or water, extreme temperatures, changes in pH gradients and cellular redox potential, high free radical and ion concentrations or hyper/hypoxic conditions can all produce stress responses at multiple levels (Adler *et al.*, 1999; Khaled *et al.*, 2001; Rodríguez-Vargas *et al.*, 2012). At the cell level, the interaction with and intensity of stressors acting on essential macromolecules, such as membrane lipids, proteins and DNA, is an important initiator of the cellular stress response (Kültz, 2003).

Stressors that overwhelm the ability of the cell to respond effectively can lead to deformation of or damage to these essential macromolecules (Kültz, 2003; 2005), which disrupts cell function and may damage cell integrity, with negative consequences at the whole-animal level.

Cellular stress may be proteotoxic and/ or oxidative in nature. The cellular stress response consists of a number of intracellular pathways that can be initiated depending on the nature and severity of the stressor. Stress responses provide an elevated level of protection for the cell as result of heightened availability of cellular defences, which temporarily makes cells more resistant to subsequent insults (Samali and Cotter, 1996; Samali and Orrenius, 1998; Samali *et al.*, 1999), including oxidative and proteotoxic stress, such that the cell experiences eustress.

To prevent or minimise the impacts of proteotoxic stress, protein homeostasis must be maintained by complex molecular interactions that ensure a balance between protein biosynthesis, folding, translocation, assembly, disassembly and clearance (Balch, 2008; Morimoto, 2008). Molecular chaperones, such as heat shock proteins (HSPs), and the stress-inducible response with which they are associated, the heat shock response (HSR), are important modulators of protein homeostasis. The HSR and HSPs are described in detail in Chapter 1, section 1.6.1. Briefly, the HSR is a protective mechanism that promotes cell survival and inhibits apoptosis by regulating the expression of inducible HSPs (Creagh *et al.*, 2000; Takayama *et al.*, 2003; Garrido *et al.*, 2001; 2006; Fulda *et al.*, 2010). The HSR, along with the HSPs, were so named because HSP synthesis was first observed in *Drosophila* during exposure to heat stress (Ritossa, 1962; Tissières *et al.*, 1974; Schlesinger, 1990). It has since been shown that HSP synthesis is increased in response to many different stressors, including proteotoxic and oxidative stress (Wheeler *et al.*, 1995; Ehrenfried

et al., 1996; Gasbarrini *et al.*, 1998; Martindale and Holbrook, 2002; Morimoto, 1998; 2008; Kalmar and Greensmith, 2009). HSPs are ubiquitous and have been found in the cells of every organism examined for their presence (Srivastava, 2002a; Li and Srivastava, 2004). HSPs are classified by molecular weight (kDa). Each HSP family has a different role within the cell and the families are highly functionally and structurally conserved across species (Ali and Banu, 1991; Feder and Hofman, 1999; Tutar *et al.*, 2006). HSP70 and HSP90 are ATP dependent and must bind and hydrolyse ATP to perform their chaperone function (Garnier *et al.*, 2002). Key elements of the cellular HSR are therefore energetically costly.

HSPs facilitate the proper folding of newly synthesised unfolded native proteins by binding to their hydrophobic regions and assisting in their import and translocation around the cell (Feder and Hofman, 1999; Hartl and Hartl-Meyer, 2002). HSPs also act as chaperones for a variety of transcription factors (Echeverria and Picard, 2010; Hayashida *et al.*, 2011; Kirschke *et al.*, 2014). During the stress response, HSPs aid misfolded and degraded proteins, either helping them retain their correct formation or regulating their removal from the cell to avoid the formation of cytotoxic aggregates (Parsell and Lindquist, 1993; Hartl, 1996; Fink, 1999).

The HSR is primarily controlled by heat shock transcription factors (HSFs; Åkerfelt *et al.*, 2010; Fujimoto *et al.*, 2010; Mahat *et al.*, 2016). In their inactive state HSFs exist as monomers in multi-subunit complexes, which include HSPs (Lee *et al.,* 2013). Upon HSP activation and dissociation from the complex, HSFs trimerise which allows them to enter the nucleus and bind to heat shock elements (HSEs), which induces the expression of HSPs (Williams and Morimoto, 1990; Xiao *et al.*, 1991).

Protein oxidation can occur as a result of oxidative stress, with modifications that lead to altered protein conformation and unfolding (Davies, 2016). Oxidative stress therefore increases the expression of inducible HSPs, including HSP70 and HSP27. HSP27 can itself also act as an 'anti-oxidant' by increasing intracellular glutathione (GSH) whilst lowering intracellular iron (Rogalla *et al.*, 1999; Arrigo *et al.*, 2005, Vidyasagar *et al.*, 2012), suggesting that the responses to proteotoxic and oxidative stress are interlinked.

The generation of reactive species (RS) is an unavoidable consequence of aerobic respiration and ATP production. Oxidative stress occurs when there is an imbalance between RS generation and the capacity of the cells intracellular machinery to remove them (Betteridge, 2000; Valko *et al.*, 2007). RS, which include superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^\bullet$), play a key role in normal cell signalling pathways (Thannickal and Fanburg, 2000; Hancock *et al.*, 2001; Veal and Day, 2011; Delaunay-Moisan and Appenzeller-Herzog, 2015). However, when present at high concentrations, RS attack macromolecules and cause widespread damage to lipids, DNA and proteins (Halliwell and Gutteridge, 1999; Marnett, 1999; Valko *et al.*, 2007; Birben *et al.*, 2012). The unpaired electron in RS attacks macromolecular double bonds, and can decrease the functional ability and subsequent survival of cells and tissues (Hancock, 1997; Forman and Torres, 2001). RS can also be generated by phagocytic cells (Babior *et al.*, 2002), in response to high glucose levels and free fatty acids (FFAs; Inoguchi *et al.*, 2000) and the induction of 'pro-oxidants' such as the nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidases (Noxs; Brandes *et al.*, 2014). High glucose levels and FFAs contribute to an elevated push

force through the electron transport chain (ETC) that can exceed the capacity of electron removal by oxygen and redox enzymes (Naudi *et al.*, 2012).

Defence mechanisms involved in detoxifying RS include a suite of essential redox enzymes such as the glutathione (GSH) peroxidases (GPxs), catalase (CAT), and the superoxide dismutases (SODs). The SODs catalyse the reaction that reduces highly reactive $O_2^{\bullet-}$ to the less reactive H_2O_2 and water. H_2O_2 is converted to water by the GPxs and CAT. GPxs decompose H_2O_2 slowly (turnover rate on the scale of a molecule per millisecond, dependent on GSH; Ng *et al.*, 2007) but have a much higher affinity for H_2O_2 than CAT (Nicholls, 1972; Antunes *et al.*, 2002; Pamplona and Costantini, 2011), which decomposes at a much higher rate (turnover rate of $10^6 \, sec^{-1}$; Chelikani *et al.*, 2005). Both enzyme families are therefore useful in different redox situations. GPxs are used to constantly decompose small amounts of H_2O_2, produced intracellularly by physiological processes. Conversely, CAT is most useful during increased H_2O_2 production or peroxide accumulation (Verkerk and Jongkind, 1992; Michiels *et al.*, 1994; Pamplona and Costantini, 2011), which can occur as a result of oxidative stress, experienced, for example, under hypoxic and inflammatory conditions.

Noxs are 'pro-oxidant' enzymes that catalyse the reduction of O_2 to generate RS, usually in the form of $O_2^{\bullet-}$ and H_2O_2, which are essential for cell signalling and metabolism (Goldstein *et al.*, 2005a; Chen *et al.*, 2012). Nox4 is one of the most abundant and widely expressed Nox isoforms (Ushio-Fukai *et al.*, 1996; Lyle *et al.*, 2009). Nox4 generates H_2O_2 constitutively, which suggests a role in resting cellular homeostasis (Miller Jr., 2009). Though first isolated in the kidney (Geiszt *et al.*, 2000), it is found in a variety of other tissues and cell types (Miller Jr., 2009),

including adipocytes (Mahadev *et al.*, 2004; Mouche *et al.*, 2007). Nox4 has been implicated in enhancing insulin signalling in adipocytes, where it generates H_2O_2 in response to insulin stimulation (Mahadev *et al.*, 2004), linking RS production and cellular stress with the control of energy balance.

Investigating relative expression and activity of HSPs and redox enzymes can provide a 'snap-shot' insight into the stress status of tissue from a particular life history state. However, it does not provide information on the functional consequences of that expression or activity; heightened levels of HSP/redox enzyme expression may protect the cells adequately, in which case the cells experience the stress as 'eustress'. Conversely, even if defences are heightened, they may not be adequate to protect the cells from the current, or future, insults. To answer such questions it is important to look for evidence of cellular damage or trade-offs at the tissue and whole animal levels.

Lipid peroxidation is used as a biomarker of oxidative damage at the tissue level and is a result of the reaction between oxidants, including RS, and lipids (Ayala *et al.*, 2014). Lipids are key components of biological membranes that control cell function and maintain cell structure (Ayala *et al.*, 2014). Lipid hydroperoxides (LOOH) are the primary products of lipid peroxidation, but are unstable and their fragmentation yields secondary products such as malondialdehyde (MDA; Ayala *et al.*, 2014). MDA is a three carbon aldehyde, with a low molecular weight. It can be produced by various mechanisms (Halliwell and Gutteridge, 1984; Ayala *et al.*, 2014). Unlike RS, which attack biomolecules close to their site of origin (Halliwell and Gutteridge, 1984), MDA can easily diffuse across membranes and covalently modify proteins far from its site of generation (Negre-Salvayre *et al.*, 2008). MDA is mutagenic in mammalian cell assays (Niedernhofer *et al.*, 2003) and carcinogenic in

rats, therefore it is essential that aldehyde formation driven by increased RS is controlled by cellular antioxidant mechanisms. MDA is a common measure of oxidative damage and a number of pathological states show elevated MDA levels, including diabetes (Kesavulu *et al.*, 2001; Grotto *et al.*, 2009).

Energy availability and budgets constrain life-history tasks such as reproduction, which is energetically demanding. The trade-off between survival and reproduction is a key feature of life history theory (Williams, 1966; Gadgil and Bossert, 1970; Stearns, 1992; Roff, 2002); animals cannot utilise or allocate limited resources to all life history components concurrently i.e. increased reproductive effort may result in reduced reproductive output in the future and/or increased mortality (Stearns, 1992; Pomeroy *et al.*, 1999; Roff, 2002; Hanssen *et al.*, 2005; Rivalan *et al.*, 2005). Mammals thus face an increased allostatic load during lactation and consequently a trade-off in energy allocation to maintenance and milk production may occur. Additional stressors experienced by females throughout reproduction, which includes pregnancy, parturition and suckling, may therefore be particularly problematic when resources are limited. It has been postulated that life-history trade-offs may be mediated by reactive species production and oxidative stress (Speakman *et al.*, 2015). The life-history-oxidative stress theory predicts that the investment in maintenance, protection and repair are lowered during reproduction, whereby there is an increase in oxidative damage and consequently negative effects on future survival and reproductive output.

Lactation is extremely energetically demanding and can increase energetic requirements by two to five-fold (Oftedal, 1984; Gittleman and Thompson, 1988; Prentice and Prentice, 1988). Maintaining positive energy balance is essential for animals that can feed during lactation, because adequate energy reserves can

determine reproductive fitness and survival probability of the individual and its offspring (Calow, 1979). As discussed in Chapter 1, section 1.1.2, most lactating female mammals normally increase food intake to meet their increased metabolic demand (Oftedal, 1984; Gittleman and Thompson, 1988; Prentice and Prentice, 1988). Some female mammals, including grey seals (*Halichoerus grypus*), fast during lactation (Iverson *et al.*, 1993, 1995; Mellish *et al.*, 2000) without any apparent detrimental effects. This unusual strategy is undertaken only by bears, phocid seals and baleen whales (Fowler *et al.*, 2014). Grey seals therefore represent an interesting and useful model in which to investigate the trade-off between allocation of limited resources to cellular defence and reproduction, and to test predictions of the life-history-oxidative stress theory.

Grey seal females haul out onto land to give to birth a single pup, which is intensively suckled for 18-21 days (Fedak and Anderson, 1982; Nordøy *et al.*, 1990; Pomeroy *et al.*, 1999) on milk consisting of 40-60% fat (Iverson *et al.*, 1993). To meet the metabolic requirements of lactation grey seal females rely on catabolism of fat derived from blubber (Fedak and Anderson, 1982; Anderson and Fedak, 1987; Reilly, 1991; Iverson *et al.*, 1993; 1995; Mellish *et al.*, 2000; Bowen *et al.*, 2001). The volume of blubber fat is dramatically reduced over the course of lactation. During lactation females lose approximately 40% of their initial mass (Pomeroy *et al.*, 1999) and 61% - 84% of their fat reserves (Fedak and Anderson, 1982; Baker *et al.*, 1995). Female grey seals wean their pups abruptly and then return to sea to forage. Since female grey seals experience such high energetic demands, they may have a limited ability to divert resources into cellular defence, and their prioritisation of cellular stress responses may be reduced.

Fasting, or prolonged food deprivation, is a stressful physiological state in some terrestrial mammals (Munck *et al.*, 1984; Sapolsky *et al.*, 2000; Wu *et al.*, 2004; Rocha *et al.*, 2008; Krause *et al.*, 2017). In animals not adapted to fasting, prolonged food deprivation can increase the expression of HSP70 (Ehrenfried *et al.*, 1996) and HSP90 (Gasbarrini *et al.*, 1998). Fasting also stimulates the production of oxidants in various tissues of non-fast adapted species, including rats, mice and humans (Mårtensson, 1986; Crescimanno *et al.*, 1989; Grattagliano *et al.*, 2000; Marczuk-Krynicka *et al.*, 2003; Sorensen *et al.*, 2006; Santos *et al.*, 2009), which depletes antioxidant reserves and may induce oxidative stress. In fast adapted species, such as the northern elephant seal (*Mirounga angustirostris*), there is no evidence to suggest that oxidant production is reduced during lactation and fasting (Sharick *et al.*, 2015; Crocker *et al.*, 2016). This is demonstrated by increased expression and activity of 'pro-oxidant' molecules, including Noxs and xanthine oxidase (XO), and raised levels of a suite of enzymes that detoxify RS (Vázquez-Medina *et al.*, 2010; Sharick *et al.*, 2015). Interestingly, lactating-fasting northern elephant seal females show no evidence of increased oxidative damage to macromolecules, including lipids, at late lactation relative to early lactation (Sharick *et al.*, 2015; Crocker *et al.*, 2016). There was also no increase in GSH production which may in part be due to high rates of energy expenditure required to meets the additional metabolic costs of lactation in the face of a limited resource. Vázquez-Medina *et al.* (2011b; 2011c) found that although adult hooded seals (*Cystophora cristata*) have increased superoxide radical and GSH production and higher levels of REs in muscle, relative to neonates and weaned pups, this was not associated with oxidative damage to macromolecules, suggesting that outside of the breeding season (without the additional energetic burden of the lactation-fast) adults are better

able to protect themselves from oxidative stress. The defences available to seals to protect against RS are much more substantial compared to terrestrial mammals (Zenteno-Savín *et al.*, 2002; Vázquez-Medina *et al.*, 2006; 2007). Compared to equivalent tissues (liver, lung, heart, kidney and muscle) from domestic pigs (*Sus scrofa*), ringed seal (*Pusa hispida*) tissues had a higher antioxidant capacity (Vázquez-Medina *et al.*, 2006; 2007). Higher antioxidant capacity, the importance of which is usually attributed to diving in seals, may play an essential role in protecting females during the lactation-fast. However, little is known about markers of oxidative stress and the defences employed by simultaneously lactating and fasting female phocids. There have been no studies that consider redox state in blubber, which is an especially important tissue for lactating-fasting females because it is the primary source of energy that supplies both metabolic fuel for maintenance and fat for milk synthesis. There are no cellular level studies considering RS defence and oxidative stress experienced by breeding female grey seals.

Outside of the breeding season, grey seal females must forage at sea and accumulate enough fat to provide them with the energy stores required for reproduction (Anderson and Fedak, 1987). Total body mass and fat content increase after the moult and prior to breeding (Sparling *et al.*, 2006), with the composition and amount of fat stored similar to that lost during breeding (Pomeroy *et al.*, 1999; Beck *et al.*, 2003; Sparling *et al.*, 2006). Deposition of large fat stores over a relatively short time period and radical seasonal changes in body mass and composition (Beck *et al.*, 2003) would be physiologically stressful for other species. This includes a range of health risks that are associated with obesity in rats, mice and humans, such as lack of vascularity, hypoxia and a high fat diet (Mokdad *et al.*, 2003; Kopelman, 2007).

Obesity sees the expansion of adipose tissue mass. Hypoxia occurs in enlarged adipocytes that are no longer in close proximity to vasculature (Trayhurn *et al.*, 2008; Goossens *et al.*, 2011; Trayhurn, 2013; Netzer *et al.*, 2015). Hypoxia in adipocytes disrupts tissue metabolism (low oxygen tension stimulates glucose use; Yin *et al.*, 2009) and overall function by altering adipokine (for example, adipocyte-specific hormones adiponectin and leptin, which play key roles in and insulin sensitisation) expression (Hosogai *et al.*, 2007). Altered adipocyte function leads to inflammation and changes in energy balance and insulin sensitivity (Wang *et al.*, 2007; Wood *et al.*, 2009; Ye, 2009; Trayhurn *et al.*, 2008; Trayhurn, 2013; Wang and Trayhurn, 2013). Hypoxia can also induce necrosis in 3T3-L1 adipocytes (Yin *et al.*, 2009), and inhibit adipogenesis and triglyceride synthesis (Yun *et al.*, 2002; Trayhurn *et al.*, 2008; Wood *et al.*, 2009). At the cellular level obesity leads to increased oxidative and endoplasmic reticulum (ER) stress (Furukawa *et al.*, 2004; Özcan *et al.*, 2004; Netzer *et al.*, 2015), which are key features of insulin resistance and type 2 diabetes (Özcan *et al.*, 2004; Grimsrud *et al.*, 2007). Evidence of adipose protein carbonylation (a marker of oxidative protein damage) has been described in adipocytes as a result of obesity-induced oxidative stress (Grimsrud *et al.*, 2007; Yuzefovych *et al.*, 2013). Grimsrud *et al.* (2007) found modification to enzymes involved in insulin sensitivity and the cellular stress response, including GPx and GST, in obese, insulin-resistant mice, which provides further evidence for a link between cellular stress signals in adipose tissue and control of energy balance.

Glucose can induce physiological stress. In pre-adipocytes under induced hyperglycemic/hyperinsulinemic conditions, Gupta and Tikoo (2012) observed direct regulation of stress responsive genes (including HSP90) and increases in oxidative stress regulating enzymes (including CAT). Fasting seals generally have high blood

glucose and higher endogenous glucose production (EGP) compared to mammals of a similar size (Keith and Ortiz, 1989; Nordøy and Blix, 1991; Champagne *et al.*, 2005 Houser *et al.*, 2012; 2013). Bennett *et al.* (2017b) found that higher glucose uptake was stimulated by higher glucose availability in blubber, which increased lactate production. The impact of glucose levels on expression of cellular defences in seals is unknown. However, Bennett *et al.* (2017b) established lower relative mRNA abundance of *11-β-hydroxysteroid dehydrogenase 1* (a metabolic gene involved in steroid metabolism) in response to a high glucose (25mM) environment, compared to untreated controls, in cultured grey seal adipose explants. This suggests glucose involvement in blubber cortisol sensitivity.

Investigation of the changes in cellular defences during the extreme mass changes experienced by adult grey seals throughout the year could provide useful insights into which life history states are most physiologically stressful for female grey seals and when they may be most vulnerable to other external sources of stress. This chapter aims to test the hypothesis that simultaneous breeding and fasting creates a trade-off that results in reduced cellular defences relative to pre breeding females, and that this trade-off has negative tissue and whole animal level consequences. Specifically, I aim to 1) investigate the differences in relative gene expression of HSPs and redox enzymes in the blubber of lactating and pre-breeding female grey seals to determine whether defences are altered during lactation and/ or during the change from fat deposition to fat mobilisation; 2) investigate changes in lipid peroxidation throughout the lactation period and compare this with females outside the breeding season to determine whether there is evidence for increased oxidative damage in lactating animals and/ or during the change from fat deposition to fat mobilisation; 3) ascertain whether body mass and axial girth can explain

variation in the expression of HSPs and redox enzymes to determine whether larger animals, and thus those with greater resources, have more robust defences; 4) investigate whether circulating glucose can explain variation in the expression of HSPs and redox enzymes and 5) establish whether increased physiological stress or damage is associated with a reduction in performance during lactation, indicated by mass loss rates, efficiency of transfer of mass from mother to pup and/ or weaning mass of pups.

3.2 Materials and Methods

3.2.1 Field and laboratory techniques

Blubber biopsies and plasma drawn into ethylenediaminetetraacetic acid (EDTA) coated vacutainers from the epidural sinus were obtained from adult female grey seals during early and late lactation in 2013, and during the post-moult fattening phase of May 2015 using methods described in detail previously (Chapter 2; General Methods; sections 2.1.2.1, 2.1.2.2, 2.1.2.3, 2.1.2.4, 2.1.2.6, 2.1.3.1, 2.1.3.2, 2.1.3.3). RNA was extracted from blubber and reverse transcribed as described in Chapter 2; General Methods; sections 2.2.4 and 2.2.5. qRT-PCR experiments were performed using gene specific primers (HSPs: *Hsp70*, *Hsc70*, *Hsp90*, *Hsp40*, *Hsp27*; and REs: *GPx*, *CAT*, *SOD*, *GST*, *Nox4*) including two or three reference genes, previously selected using *NormFinder* and *BestKeeper* softwares (see Chapter 2; General Methods; section 2.2.2), on a StepOne™ Real-Time PCR System (Applied Biosystems). Raw C_T values generated by qRT-PCR reactions were inspected using StepOne™ Software version 2.3 (Applied Biosystems). Additional quality control checks were performed on all experimental data, including examination of melt curves for single peaks, baselines for standardisation for all genes across all plates

and threshold values (Chapter 2; General Methods; section 2.2.8). For each gene the average threshold was calculated and each threshold was then set to this value to allow comparability across all plates.

Lactating female samples were run with the reference genes CYCA and S9; pre-breeding female samples were run alongside CYCA, L8 and S9, due to larger plate sizes (384 well) and a smaller sample set. The comparison between lactating females and pre-breeders used the same reference genes, despite the addition of L8 on pre-breeder qRT-PCR experimental plates.

3.2.1.1 Circulating glucose concentrations

Glucose was measured in plasma using glucose oxidase and the subsequent reaction of hydrogen peroxide with phenol and 4-aminophenazone (Barham and Trinder, 1972). The red-violet quinoneimine dye formed acts as an indicator. Standards and samples were read in duplicate on the RX Monza Clinical Chemistry Analyser (Randox Glucose GOD-PAP GL 364; Randox Biosciences, County Antrim, UK) as described in Chapter 2; General Methods; section 2.2.9.2. Visibly haemolysed samples were not analysed.

3.2.1.2 Malondialdehyde (MDA) concentrations

LP was determined by measuring MDA concentrations in blubber tissue. The colorimetric product formed during the reaction of MDA with thiobarbituric acid (TBA) was measured, as described in Chapter 2; General Methods; section 2.2.10. A standard curve was constructed from the absorbance values of known standards and the resulting equation was used to calculate MDA concentrations (nmol/µL) of unknown samples.

3.2.2 Maternal mass transfer and efficiency calculations

Calculations to estimate maternal mass at parturition, maternal mass loss rate and percentage maternal expenditure from the measured values at EARLY and LATE lactation are described in detail in Chapter 2; General Methods; section 2.2.11.1.

3.2.3 Statistical Analysis

Statistical analyses were performed in R software (R Version 3.4.2; Ihaka and Gentleman, 1996; R Core Development Team, 2012). A paired t-test was used to analyse the difference in mass and axial girth, circulating glucose and MDA concentrations of lactating females between EARLY and LATE lactation. Linear mixed effects models (LMEs) were used to investigate changes in Delta C_T for each GOI from EARLY to LATE lactation. EARLY lactation was used as the control condition to calculate delta C_T, relative gene expression and when fitting the intercept in statistical models. Models were fitted by maximum likelihood using the lme function, part of the nlme library, in R. Individual was included as the random effect (intercept only). State of lactation, body mass, daily rate of mass loss, axial girth and circulating glucose were included as fixed effects to explain variation in relative gene expression. Forward stepwise regression was used to find the most suitable model. Models in which fixed effects were included were then tested against the simpler model using the anova function in R. Residuals were plotted against fitted values and assessed for structure or pattern. For any particular variable (e.g. concentration of MDA, mRNA abundance) it is unlikely that we will know the value of the *population* level mean and standard deviation. Instead, we have a smaller sample from which we may estimate a population mean and standard deviation i.e. the sampling

distribution. During a hypothesis test, such as a T-test, a standardised value known as a T-value is calculated from the sample data, using the following equation, where x is the numerical value of an observation (e.g. MDA concentration), $\bar{x}$ is the mean of the sample size and s is the standard deviation of the sample (Fowler *et al.*, 1998; Matthiopoulos, 2011):

$$T = \frac{(x - \bar{x})}{s}$$

T is therefore the magnitude of the difference between two groups relative to the variance in the data. It is the difference between them in standard error units and the larger it is the less likely that the null hypothesis (that the groups are the same) is true. *T*-values are used when working with small sample sizes and/or the population variance is unknown, which are both cases here. *T*-values can be used to detect differences in the mean between two populations (i.e. hypotheses about the mean of a particular population are tested; this involves a null hypothesis, H_0, which states that the mean of the population is equal to the mean of the sample and the alternative hypothesis, H_1, which states that there is a difference between the means). *T*-distributions (probability distributions of *T*-values) are dependent on mean, standard deviation and sample size (*n*; however it is degrees of freedom (df; *n*-1) that determine *T* directly). Probability distributions of *T* can be constructed for any number of df. From a T-distribution it is possible to determine which *T*-values correspond to different levels of significance (i.e. $p = 0.05$), for either a one- or two-tailed test. *T*-values must be placed within the context of a known probability distribution. There is a probability associated with any given *T*-value; a *p* value, which is dependent on the significance level selected (in this case 0.05). This measure of significance represents the probability of an observation occurring as a

result of 'chance' or random error. Log likelihood, another output generated by LMEs, is itself an index of model fit and describes the probability, or 'likelihood', of obtaining the observed data, given the model. The likelihood ratio statistic, or L ratio, is a test statistic that is calculated using the following equation:

$$2log\frac{L_L}{L_S}$$

Where a larger model with l parameters and likelihood L_L and a smaller model with s parameters and likelihood L_S are considered, and the smaller model represents a linear restriction on the parameters (a linear subspace) of the larger model (Faraway, 2006). The L ratio compares the likelihood of two models, containing different factors, with each other using the Chi squared distribution (χ^2). The value is then compared to χ^2 to establish whether the inclusion of the additional parameter in the model significantly improves the fit to the data.

Marginal and conditional R^2 values were calculated for each of the model fits using the sem.model.fits function, part of the piecewiseSEM library, in R (Nakagawa and Schielzeth, 2013; Johnson, 2014). R^2 values can be used as a summary statistic for LMEs and describe the amount of variance explained by the model. Furthermore, as a model comparison tool, R^2 values offer an absolute value for the goodness-of-fit of a model, unlike an Akaike Information Criterion (AIC) value (Nakagawa and Schielzeth, 2013). Two R^2 values are generated to describe the variation explained by the fixed and random effects included in the model: marginal R^2 values represent the variance explained by fixed effects; conditional R^2 values represent the variance explained by fixed *and* random effects. For example: as individual was included as a random effect, LMEs with greater conditional than marginal R^2 values suggests random effects explain a proportion of the variation in addition to the fixed effects.

The greater the difference between marginal and conditional R^2 values, the greater individual level differences contribute to the overall model fit.

A separate model was fitted for each gene because no *a priori* assumptions were made that the genes would respond in a similar way to each other. I used a sequential Bonferroni method, ranking *p*-values by significance, to adjust for multiple comparisons. The 2015 pre-breeding female dataset (n = 13) represents different individuals from different locations compared to the 2013 lactating female dataset. Therefore, a student's t test was used to investigate whether there was a difference in relative expression of each GOI and MDA concentrations between lactating (mean value calculated for each gene) and pre-breeding females. When comparing MDA concentrations between lactating and pre-breeding females, one pre-breeding individual (58431) was considered an outlier and removed because the MDA value was 3.20 standard deviations above the mean.

3.3 Results

3.3.1 Redox enzyme gene expression during lactation

During LATE lactation the relative expression of *Nox4* was a significantly lower than EARLY in lactation (LME, *p* = 0.020; Table 3.1). There was no change in gene expression of the redox enzymes *GPx*, *CAT*, *SOD* and *GST* between EARLY and LATE lactation. With the exception of *Nox4*, individual improved the model fit to the data and explained a larger proportion of the variance than the fixed effects (Table 3.1).

Table 3.1. Model output for linear mixed effect models (LMEs) using stage of lactation (EARLY or LATE lactation) to explain variability in normalised gene expression of redox enzymes (Glutathione peroxidase, *GPx*; catalase, *CAT*; superoxide dismutase, *SOD*; glutathione-S-transferase, *GST*; and NADPH oxidase 4, *Nox 4*). Measured in wild lactating grey seal females in 2013. Significant difference where $p < 0.05$. Log2 relative mRNA abundance of LATE lactation compared to EARLY lactation ± standard deviation is also presented n (obs) = 34; n (individuals) = 17.

Gene	Marginal R^2	Conditional R^2	*T*	*P*	Log2 relative gene expression (± SD)
GPx	0.031	0.54	1.45	0.17	0.13 ± 1.19
CAT	0.0020	0.23	0.28	0.78	-0.066 ± 0.83
SOD	0.00019	0.40	0.098	0.92	-0.15 ± 0.90
GST	0.0024	0.27	-0.32	0.75	-0.066 ± 0.59
Nox4	0.16	0.28	-2.59	**0.020**	-0.73 ± 0.95

3.3.2 HSP gene expression during lactation

Despite the mass and girth loss reported in Section 3.3.8, females showed no significant change in relative expression of any of the HSP genes measured during the lactation period (LMEs, $p > 0.05$; Table 3.2). Additional fixed factors (i.e. glucose, body mass, axial girth and MDA concentration) in LMEs did not improve the model fit ($p > 0.05$). Individual improved the model fit and explained a larger portion of the variance in HSP mRNA abundance than fixed effects (Table 3.2).

Table 3.2. Model output for linear mixed effect models (LMEs) using stage of lactation (EARLY or LATE lactation) to explain variability in normalised gene expression of HSPs (heat shock proteins) and *Hsc70* (heat shock cognate 70). Measured in wild lactating grey seal females in 2013. Significant difference where $p < 0.05$. Log2 relative mRNA abundance of LATE lactation compared to EARLY lactation ± standard deviation is also presented. n (obs) = 34; n (individuals) = 17.

Gene	Marginal R^2	Conditional R^2	T	P	Log2 relative gene expression (± SD)
Hsp70	0.027	0.54	1.35	0.20	0.31 ± 1.27
Hsc70	0.021	0.74	1.57	0.13	0.37 ± 0.77
Hsp90	0.00057	0.75	-0.26	0.79	0.12 ± 0.66
Hsp40	0.018	0.69	1.37	0.19	0.20 ± 0.44
Hsp27	0.0030	0.26	0.36	0.72	0.083 ± 1.02

3.3.3 Redox enzyme gene expression in pre-breeding females compared to lactating females

All REs measured were significantly lower in pre-breeding females relative to lactating females (Students t-test, $p<0.05$; Figure 3.1). *GPx* was downregulated 2.11 fold (T test: $T = 6.15$; df = 16; $p <0.0001$); *CAT* 1.47 fold (T test: $T = 5.57$; df = 25; $p <0.0001$); SOD 1.19 fold (T test: $T = 5.18$; df = 18; $p <0.0001$); GST 1.40 fold (T test: $T = 3.86$; df = 23; p = 0.0005) and Nox4 1.17 fold (EARLY lactation T test: $T = 6.47$; df = 28; $p <0.0001$; LATE lactation T test: $T = 2.67$; df = 26; $p = 0.013$).
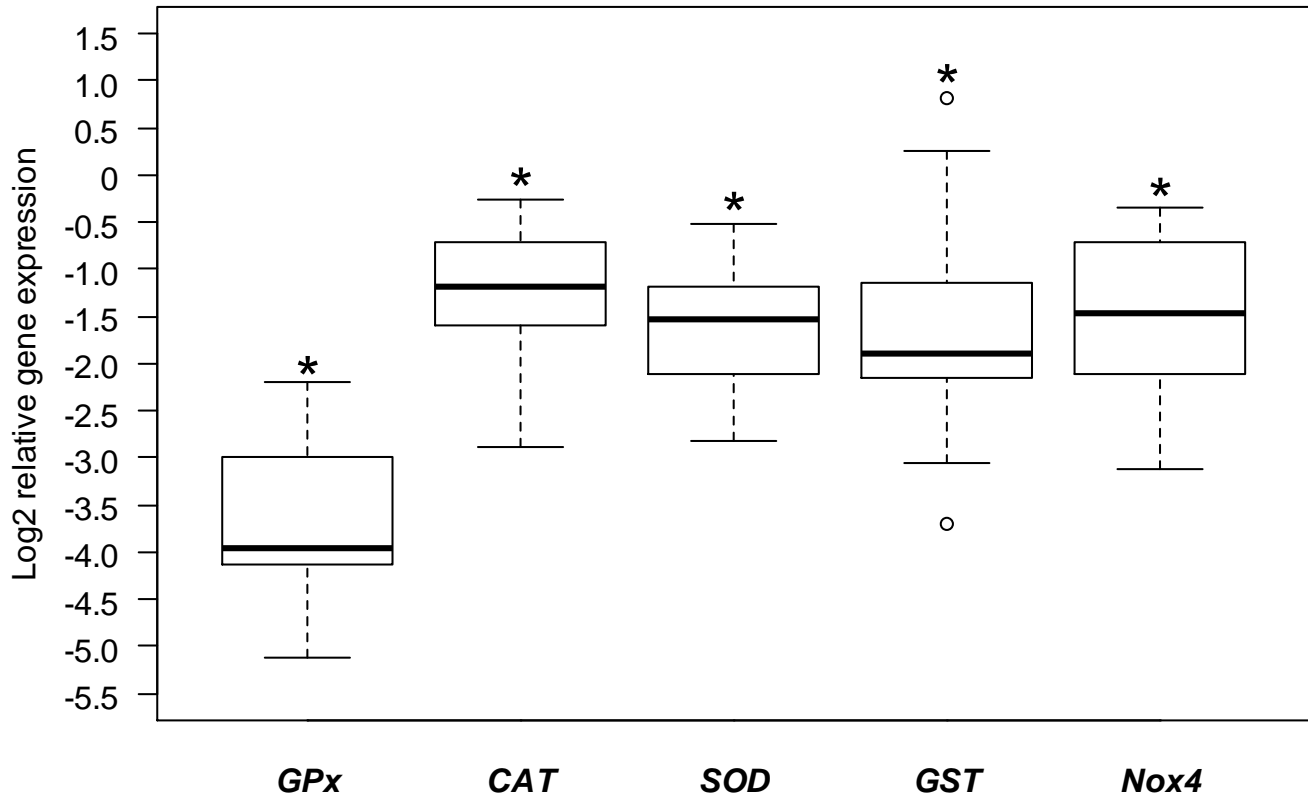
Figure 3.1. Boxplot of log2 relative gene expression for each redox enzyme gene measured, where 2015 pre-breeding female expression (*n*=13) is relative to 2013 lactating females. Minimum and maximum values, median and interquartile range (including the upper and lower quartiles) are presented. Glutathione peroxidase, *GPx*; catalase, *CAT*; superoxide dismutase, *SOD*; glutathione-S-transferase, *GST*; and NADPH oxidase 4, *Nox4*. The symbol "*" indicates mRNA abundance was significantly lower ($p < 0.05$) in pre-breeding females relative to lactating-fasting females.

3.3.4 Heat shock protein gene expression in pre-breeding females compared to lactating females

As there was no difference in relative expression of HSPs between EARLY and LATE lactation, pre-breeding females were compared to the mean relative expression of both EARLY and LATE lactation combined. There was a significant difference in relative expression of *Hsp90* and *Hsp27*, which were both higher by 1.82 fold (T test: $T = 2.83$; df = 28; $p = 0.0085$) and 2.31 fold (T test: $T = 3.77$; df = 28; $p = 0.0008$) respectively, in pre-breeding females compared to lactating, fasting females (Students t-test, $p < 0.05$; Figure 3.2). There was no difference in relative gene expression in blubber of *Hsp70*, *Hsc70* or *Hsp40* between pre-breeding females and females during the lactation period (Students t-test, $p < 0.05$; Table 3.3).

Table 3.3. Output from Students t-tests in which pre-breeding female and lactating female HSP relative expression data were compared. Log2 relative gene expression (where pre-breeding females are relative to lactating females) ± standard deviation. Abbreviations: "df", degrees of freedom; "*T*", t statistic.

Gene	df	*T*	*p*	Log2 relative gene expression (± SD)
Hsp70	17	1.22	0.24	0.74 (± 1.16)
Hsc70	21	-0.47	0.64	0.20 (± 0.50)
Hsp40	19	-1.35	0.19	-0.14 (± 0.42)
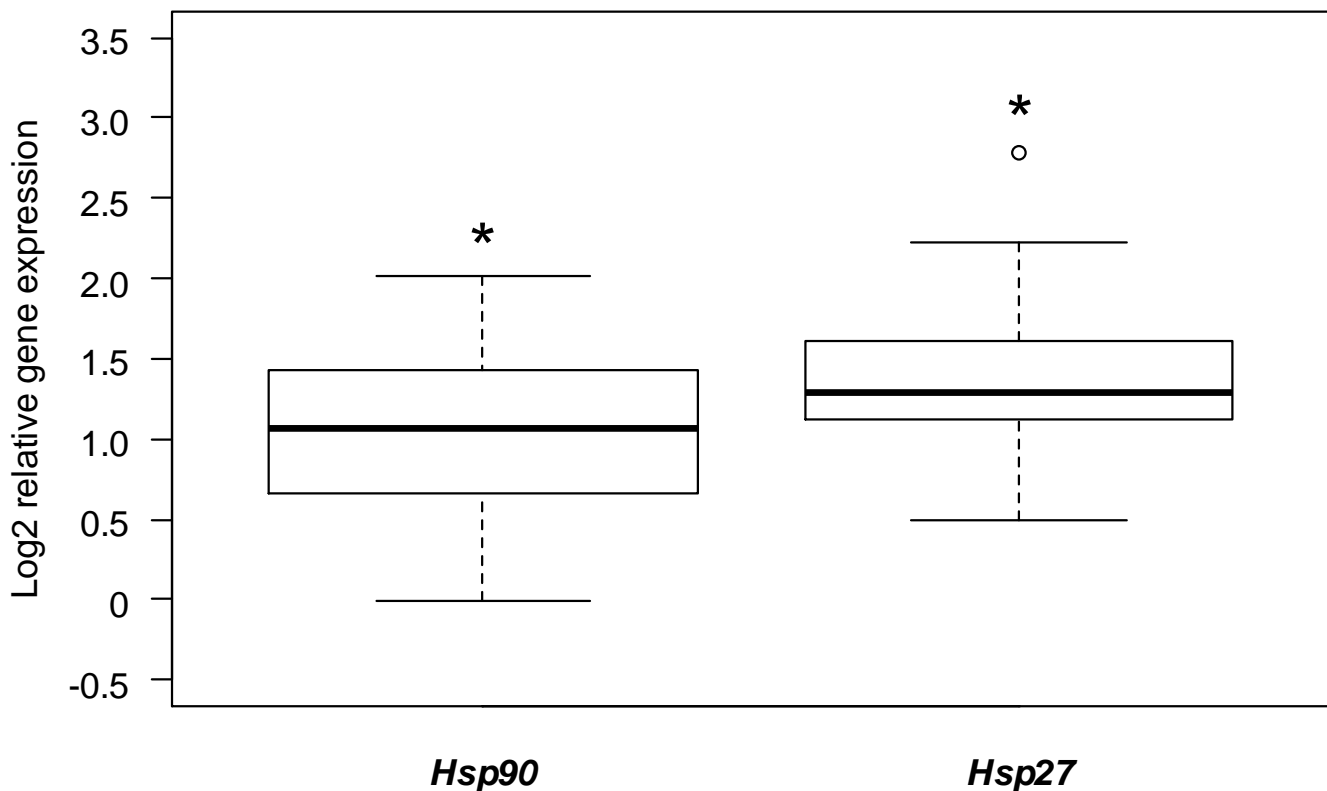
Figure 3.2. Boxplot of log2 relative gene expression of heat shock protein 90, *Hsp90*, and heat shock protein 27, *Hsp27,* where 2015 pre-breeding female expression is relative to 2013 lactating females. Minimum and maximum values, median and interquartile range (including the upper and lower quartiles) are presented. The symbol "*" indicates mRNA abundance was significantly higher ($p < 0.05$) in pre-breeding females relative to lactating-fasting females.

3.3.5 Lipid peroxidation: MDA concentration during lactation

There was no difference in MDA concentrations (nmol/µL) between EARLY and LATE lactation (Paired t-test, df = 11, T = 0.66, p = 0.53). The range and mean values are displayed in Table 3.4.

Table 3.4. Range and mean ± SD (*n*) values of MDA concentrations measured in blubber tissue of lactating wild grey seal females in 2013.

	Range		Mean ± SD (*n*)	
	EARLY lactation	LATE lactation	EARLY lactation	LATE lactation
MDA concentration (nmol/µL)	0.038 – 0.11	0.036 – 0.12	0.072 ± 0.024 (15)	0.066 ± 0.022 (13)

3.3.6 Lipid Peroxidation: MDA concentration in pre-breeding females

Pre-breeding females had significantly lower concentrations of MDA in their blubber compared to lactating females (Students t-test, df = 26, *T* = 5.76, *p*<0.0001; *n* (lactating) = 16; *n* (pre-breeding) = 12; Figure 3.3).
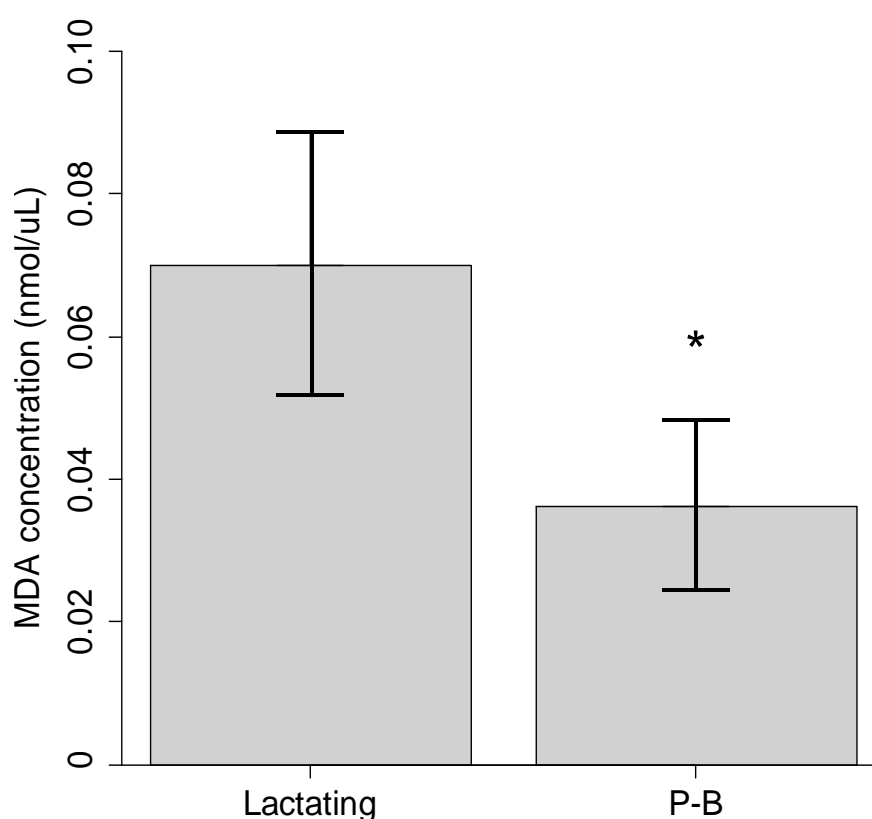
Figure 3.3. Mean (± SD) MDA concentration (nmol/µL) in blubber from lactating (Isle of May, Oct – Dec, 2013) and pre-breeding (P-B; Blakeney, Donna Nook, May 2015) females. The symbol "*****" indicates a significant difference (Students t-test, *p*<0.05).

3.3.7 Redox enzyme and heat shock protein gene expression and MDA

concentration during lactation

There was no relationship between normalised expression of REs and HSPs and MDA concentration (Table 3.4).

Table 3.4. Model output for linear mixed effect models (LMEs) using normalised gene expression of redox enzymes (glutathione peroxidase, *GPx*; catalase, *CAT*; superoxide dismutase, *SOD*; glutathione-S-transferase, *GST*; and NADPH oxidase 4, *Nox 4*)and heat shock proteins (HSPs) and heat shock cognate 70 (*Hsc70*) to explain variability in MDA concentration. Measured in blubber of wild lactating grey seal females in 2013. Significant difference where $p < 0.05$. n (observations) = 26; n (individuals) = 13.

Gene	Marginal R^2	Conditional R^2	T	p
GPx	0.018	0.83	-0.89	0.39
CAT	0.017	0.79	-0.91	0.38
SOD	0.0028	0.76	-0.31	0.76
GST	0.0051	0.78	-0.42	0.68
Nox4	0.0023	0.76	-0.36	0.73
Hsp70	0.011	0.77	-0.64	0.53
Hsc70	0.034	0.78	-0.98	0.34
Hsp90	0.0053	0.78	0.36	0.72
Hsp40	0.0087	0.76	-0.50	0.63
Hsp27	0.0058	0.78	0.52	0.61

3.3.8 Lactation duration, body mass and axial girth

Mean lactation duration was 20.16 ± 2.01 (SD) days (n = 18), ranging from 17-25 days.

At both EARLY and LATE lactation, the mean of the number of days after birth that the capture took place and morphometric data collected and blubber samples taken is presented in Table 2.1, Section 2.1.3.

There was a significant reduction in mass (paired t-test, df = 17, T = 20.52, p > 0.0001; n = 18) and axial girth (paired t-test, df = 17, T = 11.43, p > 0.0001; n = 18) between EARLY and LATE lactation. Over the 10.75 ± 2.02 (SD) days between EARLY and LATE lactation, females lost 23.20% ± 4.71 (SD) of their body mass and 12.62% ± 4.42 (SD) of their axial girth. Mean mass loss was 39.57 ± 8.18 (SD) kg and mean reduction in axial girth 18.69 ± 6.74 (SD) cm. Mean and range of mass and axial girth at EARLY lactation and LATE lactation are displayed in Table 3.5.

Table 3.5. Mean (± SD (n)) and range of body mass (kg) and axial girth (cm) at both EARLY and LATE lactation of 2013 female grey seals on the Isle of May.

	Range		Mean ± SD (n)	
	EARLY lactation	LATE lactation	EARLY lactation	LATE lactation
Mass (kg)	146.80 - 234.60	107 - 191.60	172.33 ± 22.28 (17)	133.16 ± 21.64 (17)
Axial girth (cm)	131 - 162	113 – 151	148.08 ± 8.26 (17)	129.39 ± 9.79 (17)

3.3.9 Effect of body mass and axial girth on RE and HSP gene expression

3.3.9.1 Redox enzymes

Axial girth and body mass were not significant covariates in LMEs for any of the RE genes measured and did not improve the models fit to the data (Table 3.6). The negative relationship between body mass and relative *SOD* expression approached significance (LME, $p = 0.064$; Table 3.6).

Table 3.6. Additional explanatory variables included as covariates in LMEs to explain variability in the relative gene expression of the redox enzymes (Glutathione peroxidase, *GPx*; catalase, *CAT*; superoxide dismutase, *SOD*; glutathione-S-transferase, *GST*; and NADPH oxidase 4, *Nox 4*). Each redox enzyme was modelled separately. Models in which body mass, axial girth, circulating glucose or MDA concentration were included were then tested against the simpler model with no additional covariates using anova.

		Covariate	
Gene		Body mass (kg)	Axial girth (cm)
GPx	Marginal R^2	0.13	0.13
	Conditional R^2	0.54	0.44
	L ratio	2.54	2.16
	P	0.11	0.14
CAT	Marginal R^2	0.054	0.035
	Conditional R^2	0.21	0.19
	L ratio	1.47	0.93
	P	0.23	0.33

SOD	Marginal R^2	0.13	0.053
	Conditional R^2	0.37	0.35
	L ratio	3.43	1.40
	P	**0.064**	0.24
GST	Marginal R^2	0.0038	0.052
	Conditional R^2	0.28	0.33
	L ratio	0.035	1.43
	P	0.85	0.23
Nox4	Marginal R^2	0.16	0.17
	Conditional R^2	0.27	0.33
	L ratio	0.11	0.40
	P	0.74	0.53

3.3.9.2 Heat shock proteins

Body mass or axial girth did not improve the models' fit to the data as neither covariate had a significant effect on HSP gene expression (Table 3.7).

Table 3.7. Additional explanatory variables included as covariates in LMEs to explain variability in the relative gene expression of the HSPs. Each HSP was modelled separately. Models in which body mass or axial girth were included were then tested against the simpler model form with no covariates using the anova function. Significant difference where $p < 0.05$.

Gene		Covariate	
		Body mass (kg)	Axial girth (cm)
Hsp70	Marginal R^2	0.10	0.091
	Conditional R^2	0.57	0.55
	L ratio	1.94	1.88
	p	0.16	0.17
Hsc70	Marginal R^2	0.025	0.021
	Conditional R^2	0.75	0.75
	L ratio	0.092	0.0073
	P	0.76	0.93
Hsp90	Marginal R^2	0.062	0.074
	Conditional R^2	0.78	0.73
	L ratio	1.45	2.29
	P	0.23	0.13
Hsp40	Marginal R^2	0.032	0.033
	Conditional R^2	0.71	0.68
	L ratio	0.32	0.41
	P	0.57	0.52
Hsp27	Marginal R^2	0.0040	0.0051
	Conditional R^2	0.26	0.27
	L ratio	0.026	0.060
	P	0.87	0.81

3.3.10 Body mass and axial girth of pre-breeding females

Range in pre-breeding female body mass was 80.8 kg to 150.4 kg. Range in axial girth was 127 – 141 cm. Mean values and details of 2015 study animal locations are displayed below in Table 3.8.

Table 3.8. Mean (± SD) mass (kg) and mean axial girth (cm) of 2015 pre-breeding females. Data are presented separately for each location, Donna Nook and Blakeney, and both locations combined, displayed in the 'Overall' column.

	Location		
	Donna Nook ($n = 8$)	Blakeney ($n = 5$)	Overall ($n = 13$)
Mean mass (kg)	127.83 ± 15.28 (SD)	100.68 ± 17.54 (SD)	117.38 ± 16.31 (SD)
Mean axial girth (cm)	134.25 ± 6.32 (SD)	120.0 ± 5.83 (SD)	128.77 ± 6.15 (SD)

3.3.11 Circulating glucose during lactation

There was no difference in circulating glucose concentrations between EARLY and LATE lactation (paired t-test, df = 14, T = 0.18, p = 0.86; n = 15; Table 3.9). EARLY lactation samples were reduced to n = 15 as two individuals had haemolysed plasma samples.

Table 3.9. Mean (± SD (n)) and range of glucose concentrations (mM) in plasma of adult female grey seals at EARLY lactation and LATE lactation.

	Range		Mean ± SD (n)	
	EARLY lactation	LATE lactation	EARLY lactation	LATE lactation
Glucose (mM)	7.51 – 8.91	6.85 – 11.49	8.23 ± 0.38 (15)	8.29 ± 1.01 (17)

3.3.12 Effect of circulating glucose on RE and HSP gene expression during lactation

Circulating glucose was not a significant covariate in LMEs for any of the RE or HSPs genes measured and did not improve the models fit to the data (Table 3.10, Table 3.11). The negative relationship between circulating glucose and normalised *CAT* expression approached significance (LME, p = 0.065; Table 3.10).

Table 3.10. Additional explanatory variable, circulating glucose, included as a covariate in LMEs to explain variability in the relative gene expression of the REs. Each RE was modelled separately. Models in which circulating glucose was included were then tested against the simpler model form with no covariates using the anova function. Significant difference where $p < 0.05$.

Gene		Covariate
		Circulating glucose (mM)
GPx	Marginal R^2	0.077
	Conditional R^2	0.39
	L ratio	1.11
	p	0.29
CAT	Marginal R^2	0.14
	Conditional R^2	0.20
	L ratio	3.42
	p	**0.065**
SOD	Marginal R^2	0.020
	Conditional R^2	0.46
	L ratio	0.39
	p	0.53
GST	Marginal R^2	0.012
	Conditional R^2	0.21
	L ratio	0.34
	p	0.56
Nox4	Marginal R^2	0.092
	Conditional R^2	0.14
	L ratio	<0.0001
	p	0.99

Table 3.11. Additional explanatory variable, circulating glucose, included as a covariate in LMEs to explain variability in the relative gene expression of the HSPs. Each HSP was modelled separately. Models in which circulating glucose was included were then tested against the simpler model form with no covariates using the anova function. Significant difference where $p < 0.05$.

Gene		Covariate Circulating glucose (mM)
Hsp70	Marginal R^2	0.089
	Conditional R^2	0.089
	L ratio	0.81
	p	0.37
Hsc70	Marginal R^2	0.10
	Conditional R^2	0.40
	L ratio	0.92
	p	0.34
Hsp90	Marginal R^2	0.091
	Conditional R^2	0.29
	L ratio	1.77
	p	0.18
Hsp40	Marginal R^2	0.094
	Conditional R^2	0.22
	L ratio	0.98
	p	0.32
Hsp27	Marginal R^2	0.069
	Conditional R^2	0.24
	L ratio	0.99
	p	0.32

3.3.13 Mass transfer and efficiency: performance during lactation

Mean and range of maternal mass at birth, maternal mass loss rate and expenditure are displayed in Table 3.12. There was a 100kg difference between lightest and heaviest females at parturition, a 1.8 fold difference in rate of mass loss over the mean 20.17 day lactation period and a 1.6 fold difference in maternal expenditure between females. Maternal mass did not predict the ability of the individual to invest in offspring, as the lightest females did not have correspondingly low mass loss rates or expenditures. Larger mothers generally produced larger pups.

Table 3.12. Mean (± SD (*n*)) and range of maternal postpartum mass (MPPM), mass loss rate during lactation, total mass loss expressed as a percentage of MPPM during lactation and pup weaning masses of 2013 female grey seals on the Isle of May.

	Range	Mean ± SD (*n*)
MPPM (kg)	169.6 – 269	198.40 ± 27.37 (17)
Maternal mass loss rate (kg day^{-1})	2.92 – 5.32	3.73 ± 0.59 (17)
Percentage maternal expenditure (%)	29.93 – 47.11	37.63 ± 4.22 (17)
Pup weaning mass (kg)	35.56 – 61.09	48.83 ± 7.55 (17)

3.3.14 Physiological stress markers and performance during lactation: no evidence for a trade-off

3.3.14.1 Maternal rate of mass loss

There was a negative relationship between *SOD* and rate of mass loss (a higher rate of mass loss was associated with lower *SOD* gene expression), which closely approached significance (Table 3.13). Gene expression of other REs and HSPs did not have a significant effect on efficiency of maternal mass transfer to pup (Table 3.13).

Table 3.13. Model output for linear mixed effect models (LMEs) using normalised gene expression of redox enzymes (Glutathione peroxidase, *GPx*; catalase, *CAT*; superoxide dismutase, *SOD*; glutathione-S-transferase, *GST*; and NADPH oxidase 4, *Nox 4*), HSPs and heat shock cognate 70 (*Hsc70*) to explain variability in maternal rate of mass loss measured in wild lactating grey seal females in 2013. Significant effect where $p < 0.05$. n (observations) = 17, n (individuals) = 17.

Gene	Marginal R^2	Conditional R^2	T	p
GPx	0.061	0.88	-0.95	0.35
CAT	0.064	0.88	-0.98	0.34
SOD	0.24	0.91	-2.12	**0.051**
GST	0.018	0.88	-0.51	0.61
Nox4 E.L	0.15	0.89	1.56	0.14
Nox4 L.L	0.0034	0.88	0.22	0.83
Hsp70	5.14×10^{-6}	0.88	0.0085	0.99
Hsc70	0.069	0.89	-1.02	0.32
Hsp90	0.061	0.88	-0.96	0.35
Hsp40	0.021	0.88	-0.55	0.59
Hsp27	0.039	0.88	-0.75	0.46

3.3.14.2 Efficiency of maternal mass transfer to pup

Gene expression of REs and HSPs did not have a significant effect on efficiency of maternal mass transfer to pup (Table 3.14).

Table 3.14. Model output for linear mixed effect models (LMEs) using normalised gene expression of redox enzymes (Glutathione peroxidase, *GPx*; catalase, *CAT*; superoxide dismutase, *SOD*; glutathione-S-transferase, *GST*; and NADPH oxidase 4, *Nox 4*), HSPs and heat shock cognate 70 (*Hsc70*) to explain variability in maternal mass transfer to pup. Measured in wild lactating grey seal females in 2013. Significant difference where $p < 0.05$. n (observations) = 17, n (individuals) = 17.

Gene	Marginal R^2	Conditional R^2	T	p
GPx	0.034	0.88	0.70	0.49
CAT	0.0098	0.88	-0.37	0.71
SOD	0.014	0.88	-0.45	0.66
GST	0.016	0.88	-0.48	0.64
Nox4 E.L	0.0019	0.88	0.16	0.87
Nox4 L.L	0.083	0.89	1.13	0.28
Hsp70	0.11	0.89	-1.29	0.21
Hsc70	0.047	0.88	-0.83	0.42
Hsp90	0.053	0.88	-0.89	0.39
Hsp40	0.096	0.89	-1.22	0.24
Hsp27	0.035	0.88	-0.71	0.49

3.3.14.3 Pup weaning mass

Gene expression of REs and HSPs did not have a significant effect on pup weaning mass (Table 3.15).

Table 3.15. Model output for linear mixed effect models (LMEs) using normalised gene expression of redox enzymes (Glutathione peroxidase, *GPx*; catalase, *CAT*; superoxide dismutase, *SOD*; glutathione-S-transferase, *GST*; and NADPH oxidase 4, *Nox 4*), HSPs and heat shock cognate 70 (*Hsc70*) to explain variability in pup weaning mass. Measured in wild lactating grey seal females in 2013. Significant difference where $p < 0.05$. n (observations) = 17, n (individuals) = 17.

Gene	Marginal R^2	Conditional R^2	T	p
GPx	0.020	0.88	0.54	0.60
CAT	0.012	0.88	-0.48	0.64
SOD	0.047	0.88	-0.84	0.42
GST	0.012	0.88	0.42	0.68
Nox4 E.L	0.0035	0.88	0.22	0.83
Nox4 L.L	0.0031	0.88	0.21	0.84
Hsp70	0.097	0.89	-1.23	0.24
Hsc70	0.11	0.89	-1.32	0.21
Hsp90	0.11	0.89	-1.29	0.22
Hsp40	0.16	0.90	-1.61	0.13
Hsp27	0.16	0.90	-1.63	0.12

3.3.15 Physiological damage and performance during lactation

Whilst MDA concentration was elevated in breeding females compared to pre-breeders, this measure did not have a significant effect on any lactation performance measures (Table 3.16).

Table 3.16. Model output for linear mixed effect models (LMEs) using MDA concentration to explain variability in lactation performance, indicated by mass loss rate, efficiency of maternal mass transfer to pup and pup weaning mass. Measured in wild lactating grey seal females in 2013. Significant difference where $p < 0.05$. n (observations) = 13, n (individuals) = 13.

Variable	Marginal R^2	Conditional R^2	T	p
Maternal mass loss rate (kg day^{-1})	0.020	0.88	0.46	0.66
Maternal mass transfer to pup (%)	0.0037	0.88	0.20	0.85
Pup weaning mass (kg)	0.16	0.90	1.38	0.20

3.4 Discussion

3.4.1 RE and HSP relative gene expression and MDA concentration during lactation

Nox4 was significantly reduced LATE in lactation compared to EARLY in the lactation period. However, despite radical changes in body mass and axial girth we saw no changes in relative expression of HSPs and other REs in lactating-fasting females.

The down regulation of *Nox4* could in part be a protective measure preventing RS production. However, LATE lactation is likely to be an energy-saving state in which energy-consuming pro-oxidant pathways are reduced. It has been suggested that RS production by Nox4 mediates adipocyte differentiation (Schröder *et al.*, 2009; Kanda *et al.*, 2011). Indeed, Kanda *et al.* (2011) found that in mesenchymal stem cells (MSC) increased levels of intracellular RS via Nox4 mediated adipocyte differentiation through cAMP response element-binding protein (CREB; considered to be an early promoter of adipocyte differentiation; Reusch et al., 2000). When Nox4 was inhibited by the Nox selective inhibitor apocynin, adipocyte differentiation was impaired. Furthermore, silencing the expression of Nox4 by RNA- interference resulted in significantly decreased lipid accumulation, compared to control MSCs which normally expressed Nox4, confirming the essential role that Nox4-derived RS production plays in adipocyte differentiation (Kanda *et al.*, 2011). Additionally, Mouche *et al.* (2007) have shown that *Nox4* is mostly present in the stromal fraction of mammalian adipose tissue, rather than the mature adipocytes themselves. A reduction in *Nox4* relative expression could therefore indicate that there are more mature, non-dividing adipocytes and fewer mitotic and differentiating pre-adipocytes

(cells in which Nox4 is highly expressed; Mouche *et al.*, 2007) in females at LATE lactation i.e. the process of adipocyte differentiation is reduced. This could be explored using histology and cell cycle markers. *Nox4* may thus be a poor marker of cellular oxidative stress in seals, but a useful indicator of adipocyte maturation.

Nox4 is linked with insulin signalling in adipocytes from other species (Mahadev *et al.*, 2004; Goldstein *et al.*, 2005b; Meng *et al.*, 2008; Schröder *et al.*, 2009). Nox4-derived RS are implicated in the stimulation of the insulin receptor (IR) signalling cascade which heightens insulin signal transduction, potentially via inhibiting cellular protein-tyrosine phosphatases (PTPases; Mahadev *et al.*, 2004). Nox4 is also highly expressed in insulin-sensitive adipocytes (Mahadev *et al.*, 2004); therefore reduced *Nox4*, seen here, may be a result of a decreased sensitivity to insulin. Insulin sensitivity has not been measured in grey seal females during lactation, in adipocytes or at the whole-animal level; Bennett *et al.* (2015) found no change in circulating insulin levels and here we report no change in circulating glucose in lactating-fasting female grey seals. Bennett *et al.* (2013) measured levels of phosphorylated insulin receptor (a marker of tissue insulin sensitivity) in liver of grey seal pups and found no difference between the fed and fasted state. In other phocid species, however, insulin sensitivity decreases over the lactation-fast, and is accompanied by a decrease in circulating insulin, most likely to facilitate lipolysis (Fowler *et al.*, 2008; Houser *et al.*, 2013), and a similar process may occur in breeding grey seal females. Tissues of various fasting seal species are reported to show insensitivity to insulin (Houser *et al.*, 2013). Insulin sensitivity could be measured in blubber from breeding females using methods that have already been validated in grey seals (Bennett *et al.*, 2013) and other phocid species (Viscarra *et*

al., 2011). It was not possible to perform such measurements here due to limited tissue availability.

Measures of tissue or whole-animal insulin sensitivity would also provide additional information regarding energy balance (Fowler *et al.*, 2008), which is vital to ensure maximum reproductive fitness (Calow, 1979). Interestingly, modulation of insulin sensitivity by the adipokine adiponectin is strongly involved in both metabolic regulation and control of inflammation (Fowler *et al.*, 2008).

HSP70 typically increases in expression and activity in response to fasting (Ehrenfried *et al.*, 1996; Heydari *et al.*, 1996; Zare *et al.*, 2011). However, an increase in HSP70 mRNA abundance was not observed here. Our data agree with Ehrenfried *et al.* (1996), who reported no change in mRNA levels of HSP27 in the stomach tissue of rats subjected to caloric restriction.

Adipocytes change size as they accumulate or mobilise stored energy. Adipocyte shrinkage experienced during weight loss has been reported to generate cellular stress by mechanical re-modelling of the cell membrane and the extracellular matrix, in both humans and mice (Bouwman *et al.*, 2009; Wang *et al.*, 2009; Mariman and Wang, 2010; Verhoef *et al.*, 2013). The extracellular matrix (ECM) is essential for tissue structure, whilst the protein composition and dynamics of the ECM are of crucial importance for cell function (Mariman and Wang, 2010). Shrinking adipocytes also change metabolic and inflammatory characteristics (Mariman, 2012; MacLean *et al.*, 2015). These phenomena, associated with the change in cell size, may alter the rates of synthesis and degradation of proteins and induce proteotoxic stress (Lloyd, 2013). Expanding adipocytes also experience cellular stress during weight gain, not only by mechanical strain on cell and tissue membranes (Bouwman *et al.*,

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2009; Wang *et al.*, 2009; Mariman and Wang, 2010; Verhoef *et al.*, 2013), but also by induction of both ER and oxidative stress (Gregor and Hotamisligil, 2007; Giordano *et al.*, 2013). Osmotic pressure (i.e. control of cell water content) and cell pH, also altered by changes in cell size, can disrupt cell function and homeostasis, inducing proteotoxic and/or oxidative stress responses (Kurz *et al.*, 2003; Koivusalo *et al.*, 2009; Kiehl *et al.*, 2011). Our data suggest that fat tissue of female grey seals does not experience higher proteotoxic stress during lactation whilst fasting, despite a postulated reduction in fat cell size, and suggest blubber cells are able to maintain protein homeostasis effectively irrespective of large size changes. However, the increased mRNA abundance of *Hsp90* and *Hsp27* in pre breeding females suggests these specific HSPs might be induced by increased cell size during fattening.

We may also expect a response to proteotoxic and oxidative stress during fasting because grey seal blubber contains anthropogenic toxins, including persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and heavy metals, such as mercury, which concentrate as the volume of blubber fat reduces during lactation (Debier *et al.*, 2003; Vanden Berghe *et al.*, 2010). However, although our data suggest increasing PCB concentrations previously seen in blubber as the fast progresses, PCB loads were not measured here. We are therefore unable to draw firm conclusions on the role of POPs in fasting females. Measurements of environmental pollutants may also provide an explanation for the large individual variability seen here; in all models conditional R^2 values were consistently high, suggesting other variables (i.e. unmeasured fixed effects), different on an individual-level (i.e. the random effects), are driving variation in the gene expression data. Therefore we recommend that, following this work, measurements of POP loads in blubber tissue are made or

experiments are performed whereby adipose tissue may be exposed to POPs and measurements of HSP and RE gene expression ascertained, to investigate whether contaminant exposure explains more of the variance in RE and HSP mRNA expression than natural stressors.

Minsky and Roeder (2015) found a direct link between metabolic regulation and the HSR. In various mouse tissues and cell lines, fasting (for approximately 24 hours) can suppress HSP gene expression via inhibition of HSF1 by peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) family members, compared to fed individuals. This may provide an explanation for the absence of a change in HSP gene expression observed here, because HSP expression may be inhibited during both early and late lactation. However, further study would have to elucidate if this mechanism is present in a mammal used to prolonged fasting conditions. The functional consequences of unchanged HSP cellular defences in blubber for tissue function and integrity during rapid fat loss need to be examined further. This could be done by measuring levels of protein damage using protein carbonylation as an index of oxidative protein damage which could be determined by an Oxyblot protein oxidation detection kit (by Western blot) method, that has already been validated in grey seal liver and blubber (Bennett *et al.*, 2014); measuring accumulation of unfolded proteins via a thiol probe, a tetraphenylethene- maleimide (TPE-MI) dye that labels unfolded proteome-free cysteines (Chen *et al.*, 2017). By assessing protein damage or negative changes in proteostasis these methods may answer further questions regarding the impact of unchanged HSP gene expression during lactation on the effectiveness of on protein protection. Further measures of adipose tissue function could be ascertained by: measuring relative mRNA abundance of genes (by qPCR) or circulating levels of hormones (by enzyme-linked

immunosorbent assays; ELISAs) related to adipose tissue function, for example the adipokines leptin and adiponectin (Bennett *et al.*, 2015), adipose triglyceride lipase (ATGL: has already been identified as an important lipase in phocids, regulating fat and energy balance, by both Fowler *et al.*, 2015 and Bennett *et al.*, 2017b), hormone sensitive or lipoprotein lipase (HSL: a key regulator of lipolysis, catalyses the hydrolysis of tri- and diglycerides of adipocytes which mobilises FFAs (Holm, 2003; Schweiger *et al.*, 2006); LPL: involved in hydrolysis of chylomicron triglyceride allowing delivery of FFAs to tissues and determines blubber FA uptake in grey seals by Iverson *et al.*, 1995), peroxisome proliferator activated receptor γ (PPARγ: abundant in adipocytes and an essential regulator of adipocyte differentiation, FA storage and glucose metabolism (Lefterova *et al.*, 2014)) and the classical cytokine interleukin-6 (IL-6: a major inflammatory mediator, secreted by adipose tissue; circulating levels are directly correlated with adiposity and insulin resistance (Shoelson *et al.*, 2006; Eder *et al.*, 2009)) and tumor necrosis factor α (TNF-α; pro-inflammatory cytokine secreted by adipose tissue that has been linked to insulin sensitivity in obese humans, rats and mice (Nieto-Vazquez *et al.*, 2008)).

Examining HSPs at the protein level is also important to see if there is a relationship between transcription and translation and additionally if the two are related in other tissues or systems, such as immune cells (leukocytes). The quantitative relationship between mRNA and protein abundances has been difficult to determine and is often reported to be poor, with only weak correlations found between the two intimately linked biological molecules (Maier *et al.*, 2009). We might expect that HSP mRNA is a better indicator of cellular stress than HSP protein because it provides an early signal that HSP transcription has been induced, whereas the protein-level may represent what is basally present in the cell.

The data here contrast with Sharick *et al.* (2015) who found up-regulation of anti-oxidant defences during breeding in northern elephant seals. However, oxidative defence markers were measured in plasma, not fat tissue; therefore direct comparisons with blubber may not be appropriate. There may be tissue differences in REs and antioxidant capacity (i.e. plasma antioxidants do not originate from or relate to blubber antioxidants), in addition to species level differences, related to differences in life history and diving behaviour (Bennett *et al.*, 2014). The Sharick *et al.* (2015) study suggests that the higher levels of a pro-oxidant (in contrast to the presented data) are countered by the up-regulation of anti-oxidant enzymes late in the lactation-fast period during breeding in this species. Since we found a reduction in *Nox4*, the grey seals here may not need to upregulate oxidative defences, perhaps because grey seals undertake a shorter lactation-fast than northern elephant seals.

There was no difference in MDA concentrations between EARLY and LATE lactation and therefore no increase in oxidative damage, therefore it can be inferred that oxidative defences appear to be adequate to protect fasting-lactating females from further RS damage. This matches the relative expression data as there was no increase in oxidative defences (GPx, CAT, SOD, and GST) LATE in the lactation-fast. This finding also agrees with Sharick *et al.* (2015) who found, despite increases in some oxidative defence activities late in the lactation-fast, there was no increase in lipid peroxidation measures (8-isoprostanes and 4-hydroxynoneal in plasma; different to that measured here) during the lactation-fast period in northern elephant seals. As both RE gene expression and MDA concentrations were lower in pre-

breeding females (section 3.4.2, below), this highlights the importance of comparing multiple life history states to be able to meaningfully interpret such data.

3.4.2 RE and HSP relative gene expression and MDA concentration in pre-breeding females compared to lactating females

Relative abundance of HSP90 and HSP27 were both significantly higher in pre-breeding than in lactating females. HSP90 regulates sterol regulatory element-binding proteins (SREBPs), which are crucial transcription regulators of genes responsible for lipid biosynthesis and metabolism (Kuan *et al.*, 2016). As pre-breeding females are laying down blubber fat, HSP90 may be upregulated in response to increased lipid biosynthesis and the subsequent demand for SREBPs. HSPs may also be essential in chaperoning newly synthesised proteins in highly secretory, active cells (Pechan, 1991; Liu and Chang, 2008).

Pre-breeding females have significantly lower relative mRNA abundance of redox enzymes in blubber compared to lactating individuals. Heightened defences against oxidative stress indicated during lactation-fasting, could correspond with elevated RS production and this is supported by the higher MDA levels. Fasting does induce oxidant production in other species, such as humans, rats and mice (Di Simplicio *et al.*, 1997; Sorensen *et al.*, 2006). The higher RE expression and MDA concentrations suggest fasting, lactating females face increased oxidative stress compared to the pre-breeding state and this provides support for the life-history-oxidative stress theory. These data suggest that grey seal females are less well protected during lactation and an increase in oxidative stress occurs before day 5 of lactation, when we took our first sample. The onset of the trade-off in cellular defences must occur at the start of lactation, even before any decrease in body

mass or fat stores. Mellish *et al.* (1999) showed that milk production, including milk fat content, increased rapidly immediately after parturition and by day 5 of lactation milk fat content was an average of 49%, compared to 35% at day 0. One suggestion, therefore is that the trade-off occurs as the investment in milk fat production increases.

There is a body of mammalian literature that both supports and contradicts oxidative damage during lactation/reproduction in rats, mice, hamsters and humans (Al Jothery *et al.*, 2016). As suggested by Al Jothery *et al.* (2016) this could in part be due to the wide selection of tissues and assays used in experimental designs. Further tests, including other markers of oxidative damage (for example protein carbonylation), could be used to confirm increased oxidative damage in lactating females and provide extra support for the life-history-oxidative-stress theory. Exposure to additional stressors, particularly those that are oxidative in nature, experienced during the lactation-fast might be detrimental during this period. Potentially, the pressure of oxidative stress may terminate lactation which could mitigate the effects of RS and preventing further oxidative tissue damage, whilst limiting the trade-off between current and future reproductive fitness. There is no clear evidence of this in other lactating mammals, however most research does not specifically aim to answer this question. My data also highlight the importance of testing multiple life-history states. If the focus of this study was only the lactation-fast it would have appeared that these individuals were not experiencing increased oxidative stress.

3.4.3 No evidence of reduced performance during lactation

Despite increased RE gene expression and oxidative damage to lipids, compared to pre-breeding females, there was no reduction in performance during lactation; higher gene expression or MDA concentrations did not result in smaller pups at weaning, a greater mass loss rate over the course of the lactation-fast or reduced maternal mass transfer. The unchanged RE gene expression and MDA concentrations reported during the lactation-fast may further support this finding and suggests that breeding grey seal females allocate more energy to pup-rearing than to cellular defences, and this represents a trade-off which results in oxidative damage to lipids as levels of REs do not provide sufficient protection. The oxidative damage experienced by lactating-fasting females may be at a sustainable level that will not compromise current survival or future reproductive fitness, or the individuals that 'skip' breeding years, as reported by Pomeroy *et al.* (1999), may be a product of RS damage and the resultant tissue recovery required.

These findings also highlight the importance of linking oxidative stress measurements to functional outcomes such as reproductive performance to investigate their real world consequences.

3.4.4 Distinguishing true stress effects from other aspects of natural grey seal physiology

Another challenge to be considered as part of identifying stress in a natural system, is being able to clearly distinguish between the effects of exogenous stress and the effects of natural physiology, which may influence stress measurements. For example, MDA is a by-product of prostaglandin biosynthesis (Vaca *et al.*, 1988) and can be used as a measurement of this process (Smith *et al.*, 1976). Prostaglandins

are acidic lipids, derived from aracadonic acid in a reaction catalysed by cyclooxygenase, which also is involved in the synthesis of thromboxane (Pettipher, 1998). Prostaglandins can be enzymatically synthesised by most mammalian cell types in response to various chemical, mechanical or immunological stimuli (Pettipher, 1998). However as they are not secreted by a particular gland and appear to only have a local action (due to a short biological half-life), they are not strictly considered hormones but may be referred to as 'local hormones' (Andersson, 2008). Prostaglandins are markedly increased at parturition in uterine tissue and throughout lactation (O'Brien, 1995) as they play an important role in cervical ripening, uterine contractions and milk ejection (Vorherr, 1979; O'Brien, 1995). Prostaglandins also play a role in regulating reproductive cycles by control of luteal activity; prostaglandins can re-initiate oestrus by terminating the luteal phase (which occurs after ovulation) by causing lysis of the corpus luteum (Bradecamp, 2007; Andersson, 2008). We know very little about prostaglandin synthesis in female seals which are simultaneously lactating and fasting. MDA may therefore naturally be raised in lactating females and the increased concentrations recorded in comparison to pre-breeding females unsurprising. This makes distinguishing between eustress and distress more challenging. In this case however, MDA measurements were taken in adipose tissue (rather than measuring circulating MDA) and a concurrent increase in RE mRNA abundance was observed. I would suggest that increased MDA in blubber is not simply an artefact of pregnancy as pre-breeding females would also have high levels of MDA (though unconfirmed, we are assuming most a pregnant). What we see here is the result of an event that happens after birth. In lactating-fasting females oxidative stress is occurring, but also during this time animals are not experiencing reduced performance: therefore, is it a problem?

Here I attempt to establish whether eustress can be distinguished from distress in grey seals. I have identified the lactation-fast as a life-history state in which grey seal females experience increased RE mRNA abundance and oxidative damage to lipids (increased MDA concentrations), when compared to pre-breeding females i.e. they appear to be experiencing oxidative stress. However, increased MDA concentrations may be an artefact of natural reproductive biology, which highlights the challenges of discriminating stress from natural processes. Furthermore, this poses additional questions regarding the distinction of eustress and distress. Though increased cellular defences (REs) do not appear to protect cells from the effects of oxidative damage, whole-animal level measures directly related to lactation performance are not impacted. So are lactating-fasting females actually experiencing distress? Blubber itself may be a sacrificial tissue, where cellular defences are raised to offer functional protection (but not enough to prevent oxidative damage) and resources are instead diverted to other tissue and cell types. It therefore seems that simply experiencing stress in one tissue type is not enough to induce distress at the whole animal level. However, the increased susceptibility of blubber as a sacrificial tissue means that lactation may be a life history state in which animals are more prone to experiencing distress because they are already at the limits of what the tissue can defend. To truly determine whether the lactation-fast is a life-history state in which animals experience distress, it would be interesting to map the recovery of blubber as females transition from a breeding to pre-breeding state; if heightened defences result in eustress we may expect cells to be protected against additional stressors experienced in the future. It would be interesting to establish when RE gene expression returns to levels consistent with pre-breeders. It would also be interesting to investigate whether increased RE defences in blubber enable

breeding females to better cope with additional oxidative challenges (e.g. adipocyte differentiation and food intake and digestion when they return to sea). This hypothesis would need further investigation by repeating measurements of REs and MDA concentrations in other tissue types and at different stages of the annual reproductive cycle. Measurements in other tissue types would allow me to better investigate whether the animal really does experience distress or if blubber is actually sacrificial. It would also be interesting to artificially elevate REs and HSPs to see if they make tissue more robust. It would be possible to present lactating-fasting female blubber with an extra oxidative challenge to see if they can in fact raise defences. Additional concurrent measurements at the protein and activity level (enzymes) would further verify these suggestions.

3.4.5 Methodological considerations

3.4.5.1 Confounding factors: lactating-fasting vs pre-breeding females

The pre-breeding female samples were collected in 2015 from a different location (Isle of May, Scotland for the lactating females vs Blakeney/Donna Nook, east England for the pre-breeders). However, based on published data on seal movements, it is likely that the seals in this study are from the same overall population (Thompson *et al.*, 1991; McConnell *et al.*, 1999; Klimova *et al.*, 2014). Additionally, there are likely other localised differences in where these animals forage and their prey species. The difference in sampling year may also be a confounding factor. It is also likely that most of the pre-breeding females were pregnant, based on fecundity and fertility rates in grey seals (see SCOS). The trade-offs observed here therefore represent a comparison between the cost of lactation and the cost of pregnancy. Further work could compare costs experienced by non-

breeding (not pregnant) and breeding females. It would be possible to find out if the pre-breeding individuals were pregnant by conducting progesterone analyses on plasma, serum or urine depending on sample availability (Raeside and Ronald, 1981; Greig, 2002; McKenzie *et al.*, 2005). An ultrasound can also be performed, though was not conducted here. Physical examination can determine the presence of a vaginal plug; however individuals must be far enough along into the pregnancy for a vaginal plug to be observed and the absence of a vaginal plug is not a definite negative. In other marine mammals it has also been possible to determine pregnancy by analysing progesterone concentration in blubber (humpback whales; dolphins; Trego *et al.*, 2013; Clark *et al.*, 2016). Reproductive status could then be included as a covariate in LMEs to determine if this has any influence on gene expression in pre-breeding females, and to allow further distinguishing of pregnant and non-pregnant categories, which would help further test the life history oxidative stress theory.

3.4.5.2 Variation in qRT-PCR data

Large variations resulting in large standard deviations (see Figures 3.1, 3.2 and Tables 3.1, 3.2, 3.3) were observed in qRT-PCR data, both between and within individuals. This variation could be explained by the presence of multiple cell types present in the blubber and contributing to total RNA. Other cell types include resident blood (white blood cells or immature red blood cells) trapped in blubber tissue at the time of sampling (which results in increased RNA yield), vasculature (capillaries that provide a blood source for skin/pelage; Strandberg *et al.*, 2008), and macrophages and B-cells (Armani *et al.*, 2010) that have infiltrated the tissue. The high degree of gene expression variability, including in reference genes, highlights the need to

better understand blubber structure and composition in grey seals (Strandberg *et al.*, 2008; Iverson, 2009).

3.4.5.3 Measures of lipid peroxidation: MDA concentrations

It has recently been suggested that measuring MDA may not be the most useful method to measure lipid peroxidation, because the reaction used can itself result in MDA formation. Therefore, it is difficult to be certain if the values obtained are truly physiological (Cobley *et al.*, 2017). However, the MDA generated as an artefact during the methodological process is likely to be constant, such that the final measurement is the artefact plus physiological levels. As a result, the actual levels may be artificially high, but differences between groups will still reflect differences in physiological MDA. If this MDA assay is used in the future it would be sensible to include a background MDA generation value from control samples, which would improve the robustness of the assay and allow for the analysis to be baselined.

It would be interesting to ascertain the level or type of stress to which female grey seals would have to be exposed to upregulate HSPs, to help answer questions regarding their use as cellular stress markers in this species. Such experiments would also be informative in terms of better understanding the capacity for this species to buffer stress at the cellular level during vulnerable life history stages. This could include exposure to external stressors such as disturbance or disease (Twiss *et al.*, 2012a; 2012b). Experimentally, cells could be exposed to lipopolysaccharide to stimulate bacterial infection. Statistically, power analysis would be a logical step to see what sample size would be needed to produce a specific effect size during different life history stages. Our data show that a large fold change in gene expression would have to occur to detect induction of many of the cellular stress

responses here. Given that individual explained much more of the variance in the gene expression data than any fixed effects, perhaps individual-level differences dictate cellular stress responsiveness. It would be informative to collect data classifying behavioural type (i.e. proactive or reactive; Twiss *et al.*, 2012a; 2012b) of individuals to see whether whole animal individual characteristics link with cellular physiology.

In summary, there was no change in mRNA abundance of any of the HSPs or REs measured here over the course of the lactation-fast in breeding female grey seals. However, there was decreased expression of *Nox4* late in lactation which may reflect an energy-saving measure by reducing energy consuming pro-oxidant pathways; reflect a shift in the proportion of mature to differentiating adipocytes or result from decreased insulin sensitivity. In pre-breeding females there was an increase in the expression of *Hsp90* and *Hsp27*, compared to breeding individuals, potentially as a result of their role in regulating lipid biosynthesis proteins and chaperoning large volumes of newly synthesised proteins. Interestingly, the data here provide support for the life-history-oxidative-stress theory as both RE gene expression and MDA concentrations were higher in fasting, lactating females, compared to the pre-breeding state. This is the first time such a comparison has been made in this species and highlights the potential vulnerability of breeding grey seal females if they are exposed to further sources of oxidative stress. Despite increases in RE gene expression and MDA concentrations, there was no evidence for a reduction in performance during lactation; mothers with higher RE gene expression or MDA concentrations did not produce smaller pups, lose mass more rapidly or reduce the mass they transferred to their offspring. This represents a trade-off as breeding grey seal females allocate more energy to pup-rearing than

cellular defences, resulting in oxidative damage to lipids. The need to repair tissues or regain antioxidant capacity may help explain the observation that female grey seals that invest heavily in a pup in a given year often do not return to breed the next year.

Understanding how grey seals respond to natural and anthropogenic physiological stress at a cellular level will help identify the conditions that these animals experience as distress, the states during which they are most vulnerable to additional anthropogenic stress, and could help predict and mitigate against individual, population and species level responses to environmental change.

Chapter 4.

Blubber heat shock protein and redox enzyme mRNA abundance and oxidative damage are higher during the post-weaning fast and associated with increased mass loss rates in grey seal pups.

Abstract

Biological responses to stress are energetically costly. Early life stress exposure can negatively impact on fitness if resources are diverted away from essential developmental processes. Grey seal pups experience rapid physiological changes in fat tissue during development as they transition from rapid fat gain during suckling to fat mobilisation during fasting. Here I investigated: a) developmental profiles of key cellular stress markers, heat shock proteins (HSPs) and redox enzymes (REs), in blubber of grey seal pups during suckling and the post weaning fast; b) the effect of morphometric measures, circulating glucose and sex on HSP and RE gene expression in these animals; c) trade-offs in resource allocation to cellular defences and maintenance of body mass, as an index of energetic costs; and d) the relationship between blubber lipid oxidative damage, measured using MDA concentration, and gene expression of key cellular stress defences. *Hsp90*, *CAT*, *SOD* and *Nox4* abundance increased up to 3 fold during suckling. There was no change in MDA concentration or mRNA abundance of other *HSP* genes during suckling. Gene expression changes were not associated with daily mass gain rate, suggesting increased cellular defences in suckling pups did not occur at the expense of growth or fat deposition. During the post weaning fast there was a further increase in *SOD, Nox4* and *Hsp90* and increases in *GPx* in some individuals. MDA concentration increased late in the post weaning fast, when *CAT* and *GST* abundance fell. These data suggest elevated defences late in fasting were insufficient to prevent lipid peroxidation, and that *CAT* and *GST* may be particularly important for adequate blubber protection for this life history stage. Higher mass loss rate was associated with greater abundance of *Hsp90*, *Hsc70*, *SOD*, *Nox4* and *CAT*, suggesting that increased defences require greater investment of limited resources,

or that pups with higher energy expenditure require greater cellular defences. Importantly, individual explained more of the variance in mRNA abundance than nutritional state, which could relate to intrinsic factors such as pollutant, pathogen or parasite loads. These data suggest that developmental or nutritional changes in cellular defences may impact on the ability to cope with additional stressors, to which pups may be more vulnerable later in their post-weaning fast.

4.1 Introduction

Stress acts as a strong selective force and plays an important role in survival probability, recruitment to the breeding population and shaping life histories (Weber, 1990; Weber and Diggins, 1990; Hoffmann and Hercus, 2000; Pamplona and Constantini, 2011; Dantzer *et al.*, 2014). Mounting an adequate biological stress response during physiological challenges is required to ensure survival (Parsons, 2005; Blas *et al.*, 2007; Busch and Hayward, 2009), but is energetically costly (Parsons, 1994; 2005; Segerstrom, 2007). Development presents a range of whole-animal and cell-level physiological challenges, in addition to increased energetic requirements for growth, organ development, immune system maturation and co-ordinated responses to novel environmental stimuli.

Stress responses can divert resources from processes crucial to post-natal development to deal with the immediate requirements of coping with the 'stressor', which can lead to trade-offs in energy allocation at the whole animal and cellular levels (Hardie and Hawley, 2001; Segerstrom, 2007). Exposure to stress early in life can negatively impact current and future fitness (Blas *et al.*, 2007; Busch and Hayward, 2009; Costantini *et al.*, 2012) if investment in energetically expensive processes, such as growth and immune system development, is altered. However,

during early development low levels of exposure to stressors, such as chemical/toxic agents (e.g. heavy metals and reactive species) and ischemia, can be beneficial, stimulating an adaptive resistance, which leaves the organism better able to deal with similar challenges later in life (Kristensen *et al.*, 2003; Calabrese and Blain, 2005; Costantini *et al.*, 2012). The biphasic response to an environmental agent, whereby low doses are beneficial or have a stimulatory effect whereas higher doses are toxic or inhibitory, is known as hormesis (Mattson, 2008). Heat shock proteins (HSPs) and redox enzymes (RE) form a key part of the hormetic response to physiological stress by preventing proteotoxic or oxidative damage to macromolecules, such as membrane lipids, proteins and DNA, and thus help to maintain cell integrity and function (Kültz, 2003; 2005).

HSPs, discussed in detail in the Chapter 1, section 1.6.1, are ubiquitous molecular chaperones, expressed in all cells (Ali and Banu, 1991; Feder and Hofman, 1999). Classified by molecular weight (KDa), the HSP families (70kDa, 90kDa, 40kDa and 27kDa HSPs, amongst others) are responsible for protein quality control and turnover, including regulating the proper folding and conformation of newly synthesised proteins, dissolution of protein complexes, and the degradation of unstable or incorrectly folded proteins (Feder and Hofman, 1999; Hartl and Hartl-Meyer, 2002; Balch *et al.*, 2008; Morimoto, 2008; Hartl *et al.*, 2011; Hetz and Glimcher, 2011). HSPs are essential not only as a mechanism to survive a range of cellular stresses, but for normal growth and development as a result of their chaperone function (McMillan *et al.*, 1998; Morimoto, 1998; Sørensen *et al.*, 2003; Morimoto, 2008). High rates of protein synthesis and turnover occur during postnatal development (Goldspink and Kelly, 1984), which increases the requirement for effective maintenance of protein homeostasis and the prevention or minimisation of

proteotoxic stress (Walsh *et al.*, 1997; Balch *et al.*, 2008; Morimoto, 2008). HSPs are elevated during development to prevent proteotoxic stress and cope with environmental change (Walsh *et al.*, 1997).

The heat shock response (HSR) is a protective mechanism that promotes cell survival and inhibits apoptosis by regulating the expression of heat shock proteins (HSPs). The HSR has been implicated in organ development and maintenance in mice, rats and humans, and is of particular importance in maintaining cell/tissue function and developmental trajectory (Rupik *et al.*, 2011), especially just after birth (Walsh *et al.*, 1997; Takaki *et al.*, 2006; Åkerfelt *et al.*, 2010). In mice, the HSR has also been linked to postnatal growth and protection during inflammatory responses in a non-limiting manner i.e. heat shock factor 1 (*Hsf1*) deficient mice experienced suppressed post-natal growth, were less likely to survive to adulthood (dependent upon genetic background) and suffered increased mortality during pathological challenges (Xiao *et al.*, 1999). In *Drosophila*, mutant HSC70 proteins caused lethality in cells and subsequent developmental defects as a result of accumulation of misfolded proteins (Elefant and Palter, 1999). Nollen *et al.* (1999) showed that overexpression of HSP70 directly contributed to increased stress-tolerance of O23 hamster fibroblasts. However, stressors that induce HSP70 expression have consequences for cell growth and function (Feder *et al.*, 1992; Krebs and Feder, 1997) because HSP70 and HSP90 require ATP to perform their chaperone function (Garnier et al., 2002) and the HSR is therefore energetically costly. In addition, an imbalance of the chaperones available can impact a diverse range of cellular processes (perhaps due to their interactions with a range of transcription factors; Morimoto, 2008), including those directly involved with development, such as cell growth and proliferation (Pechan, 1991; Feder *et al.*, 1992). In *Drosophila*, Hsp70

overexpression slows cell growth (Krebs and Feder, 1997) and proliferation which resumes only when cytoplasmic Hsp70 is inactivated by sequestration into intracellular granules (Feder *et al.*, 1992).

HSPs are also upregulated in response to oxidative stress (Wheeler *et al.*, 1995; Ehrenfried *et al.*, 1996; Gasbarrini *et al.*, 1998), which occurs as a result of an imbalance between reactive species (RS) generation and RS defence mechanisms (Betteridge, 2000; Valko *et al.*, 2007). RS are produced during aerobic metabolism, by phagocytic cells during respiratory bursts and by 'pro-oxidant' enzymes, such as the nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidases (Noxs; Bedard and Krause, 2007). Noxs play a role in immune defence against pathogens, are involved in the tissue repair functions of myofibroblasts and fibrogenesis and generate hydrogen peroxide (H_2O_2) required for TGF-β1-induced myofibroblast differentiation, extracellular matrix (ECM) production and contractility (Geiszt and Leto, 2004; Bedard and Krause, 2007; Hecker *et al.*, 2009). RS are crucial components of normal cell signalling pathways (i.e. nitric oxide (NO), which is a multifunctional signalling molecule involved in immune defence, neuronal communication and regulation of the cardiovascular system; Murad *et al.*, 1978; 1979; Ignarro *et al.*, 1987; Garthwaite and Boulton, 1995; Lane and Gross, 1999; Thannickal and Fanburg, 2000; Blaise *et al.*, 2005; Bryan *et al.*, 2009). However, when present at high concentrations, and depending upon their reactivity, RS attack macromolecules and cause widespread damage to lipids, DNA and proteins (Valko *et al.*, 2007; Rains and Jain, 2011). Damage to lipids and proteins by RS leads to formation of lipid hydroperoxides (including malondialdehyde; MDA) and carbonylated proteins, respectively, which can be measured to assess oxidative damage. Oxidative damage to lipids and other biomolecules threatens cellular

homeostasis and alters protein function, increasing protein degradation, promoting

apoptosis and inflammatory responses (Dröge *et al.*, 2002; Kohen and Nyska, 2002;

Valko *et al.*, 2007).

Essential REs include the glutathione peroxidases (GPxs), catalase (CAT), and

the superoxide dismutases (SODs) which catalyse reactions that detoxify the RS

superoxide anion ($O_2^{\bullet-}$), H_2O_2 and the hydroxyl radical ($OH^{\bullet}$), respectively. REs

provide crucial protection against oxidative damage and ensure healthy development

in an oxygen rich environment. REs and oxidative stress are discussed in detail in

Chapter 1, sections 1.4 and 1.6.2.

Oxidative stress occurs during post-natal development at the cellular level as a

result of local changes in oxygen and fuel availability and increased growth rate

(Monaghan *et al.*, 2009; Nussey *et al.*, 2009; Metcalfe and Alonso-Alvarez, 2010). A

number of tissue types, including brain, heart, skeletal muscle and lung of mice and

rats, experience oxidative stress during early post-natal development, with

concomitant increases in essential REs. Various studies investigating oxidative

stress during early post-natal development have found evidence that suggests an

increase in RS generation, for example in response to increased growth rate

(Monaghan *et al.*, 2009; Nussey *et al.*, 2009). Therefore, early postnatal

development represents a time of increased vulnerability to oxidative stress.

Grey seal (*Halichoerus grypus*) pups face extreme physiological challenges

during early development. They take on 40-60% fat milk, gain 1.5 - 2.5 kg d^{-1} and

triple in body mass during their 18-21 day suckling period, predominantly through the

accumulation of subcutaneous blubber (Fedak and Anderson, 1982; Anderson and

Fedak, 1987; Mellish *et al.*, 1999). Both a high fat diet and excessive nutrients,

similar to those experienced by pups during the intensive suckling period, can induce oxidative stress and inflammation in other mammals, including humans and rats (Chang and Chuang, 2010; Conceição *et al.*, 2013). In humans, rapid accumulation of fat tissue through hypertrophy causes RS production through hypoxia. High fuel availability causes pseudohypoxia, whereby increased free fatty acids (FFAs) and glucose levels provide an elevated push force through the electron transport chain (ETC) that exceeds the capacity of electron removal by REs (Naudi *et al.*, 2012). RS are thus produced in response to high glucose levels and FFAs (Inoguchi *et al.*, 2000). Suckling pups frequently have elevated blood FFAs and glucose levels (Schweigert, 1993; Hall, 1998; Sakamoto *et al.*, 2009; Bennett *et al.*, 2013; 2015), which are associated with oxidative tissue damage in non-fast adapted species. Therefore, adipose tissue may be susceptible to RS and oxidative damage even though it has a low metabolic rate.

Pups are weaned abruptly when the mother returns to sea to feed and enter a post-weaning fast of 10 days to > 4 weeks (Reilly, 1991; Noren *et al.*, 2008; Bennett *et al.*, 2010). Fasting pups are completely reliant on the fat reserves laid down whilst suckling, before they go to sea and begin to forage for themselves. Fasting itself is a physiologically stressful state in non-fast adapted species. Since their resources are limited, it may be difficult for fasting pups to sustain energy consuming stress responses.

Compared to terrestrial mammals, seals have higher antioxidant defences (Vázquez-Medina *et al.*, 2007). Previously, Bennett *et al.* (2014) showed that suckling grey seal pups have higher protein abundance of HSP70 and tissue activity of CAT and SOD than fasting pups. The higher protein abundance of HSP70 could have been due to changes in the stress-inducible or constitutive isoform (HSC70).

Suckling pups did not have greater protein carbonylation, another index of oxidative tissue damage, in either liver or blubber which suggests that increased levels of cellular defences were adequate to prevent protein damage. Vázquez-Medina *et al.* (2010) found increases in GPx, CAT, SOD and Nox4 protein expression in muscle of Northern elephant seal (*Mirounga angustirostris*) pups over the post-weaning fast. However, plasma and muscle levels of inflammatory markers, thiobarbituric acid reactive substances (TBARS; a marker of lipid peroxidation (LP), an index of oxidative damage) and protein carbonyls did not increase. This again suggests that, despite increases in 'pro-oxidant' activity and protein expression, 'anti-oxidant' enzyme cover was adequate to offset potential oxidative damage.

This chapter aims to better understand cellular defences in blubber tissue of grey seal pups. Specifically, I aim to a) investigate developmental changes in relative gene expression of HSPs and REs in the blubber of suckling and fasting grey seal pups; b) investigate changes in LP throughout the suckling period and the post-weaning fast to determine whether there is evidence for oxidative damage in animals with lower defences; c) ascertain whether body mass, axial girth, circulating glucose and pup sex can explain variation in the expression of HSPs and REs; d) establish whether increased physiological stress (indicated by higher levels of HSPs or REs) or damage (indicated by MDA) is associated with reduced mass gain or maternal mass transfer to the pup (maternal investment) during suckling or increased mass loss during fasting, which may be indicative of a trade-off between cellular defences and fitness; e) ascertain whether my data at the gene expression level corroborate findings in suckling and fasting grey seal pups and fasting Northern elephant seal pups at the protein and tissue activity level.

4.2 Materials and Methods

4.2.1 Field and laboratory techniques

Blubber biopsies and plasma drawn into EDTA coated vacutainers from the epidural sinus were obtained from grey seal pups during EARLY and LATE suckling and EARLY and LATE in the POST-WEANING fast in 2013, using methods described in detail previously (Chapter 2; General Methods; sections 2.1.2.1, 2.1.2.2, 2.1.2.3, 2.1.2.4, 2.1.2.6, 2.1.3.1, 2.1.3.2, 2.1.3.3). RNA was extracted and reverse transcribed as described in Chapter 2; General Methods; sections 2.2.4 and 2.2.5. qRT-PCR experiments were performed using gene specific primers (HSPs: *Hsp70*, *Hsc70*, *Hsp90*, *Hsp40*, *Hsp27*; REs: *GPx*, *CAT*, *SOD*, *GST*, *Nox4*) including two reference genes, *CYCA* and *S9*, previously selected using *NormFinder* and *BestKeeper* softwares (see Chapter 2; General Methods; sections 2.2.1, 2.2.2, 2.2.8).

4.2.1.1 Circulating glucose concentrations

Glucose was measured in deproteinised plasma using glucose oxidase (Bennett *et al.*, 2017) as described in Chapter 2, General Methods, Section 2.2.9.1. Visibly haemolysed samples were not analysed.

4.2.1.2 Malondialdehyde (MDA) concentrations

LP was determined by measuring MDA concentrations in the blubber tissue that remained after RNA extractions. The colorimetric product formed during the reaction of MDA with thiobarbituric acid (TBA) was measured, as described in Chapter 2; General Methods; section 2.2.10. A standard curve was constructed from

the absorbance values of known standards and the resulting equation was used to calculate MDA concentrations in samples.

4.2.2 Pup birth and weaning mass calculations

Calculations to estimate pup mass at birth and weaning, pup daily mass gain/loss rate and maternal mass transfer to pup were calculated from the measured values at EARLY and LATE suckling and EARLY and LATE POST-WEANING fast are described in detail in Chapter 2; General Methods; section 2.2.11.1 (Pomeroy *et al.*, 1999; Bennett *et al.*, 2015).

4.2.3 Statistical Analysis

Statistical analyses were performed in R software (R Version 3.4.2; Ihaka and Gentleman, 1996; R Core Development Team, 2012). Linear mixed effects models (LMEs) were used to investigate changes in Delta C_T for each gene of interest (GOI), circulating glucose and MDA concentrations from EARLY to LATE suckling and EARLY to LATE in the POST-WEANING fast. LMEs were used as there were more than two groups of data and to include covariates, look at time series and repeated measures. Where appropriate, Delta C_T data were log transformed for use in LMEs when the raw data did not have a normal distribution and to improve the fit of the model, which was assessed by inspection of residual plots. EARLY suckling was used as the control condition to calculate delta C_T and relative gene expression. In order to investigate the differences between each of the suckling and fasting time-points, models were run with alternate time points captured in the intercept (EARLY SUCKLING , LATE suckling and EARLY to LATE in the POST-WEANING fast). Models were fitted by maximum likelihood using the lme function, part of the nlme library in R. Maximum likelihood is a common approach used to estimate the

parameters of a model so that the observed values are most probable. It finds the estimate of each parameter that maximises the probability of observed values of y over all possible combinations of values for the different parameters to be estimated in the model. We only need the mean and variance of a sample, assuming a normal distribution of y, to produce a maximum likelihood estimate from the population. Individual was included as the random effect. Developmental state (either feeding or fasting), body mass, axial girth, sex and circulating glucose were included as fixed effects to explain variation in relative gene expression. Forward stepwise regression was used to find the most suitable model. Models in which fixed effects were included were then tested against the simpler model using the anova function in R, as described in Chapter 3. Residuals were plotted against fitted values and assessed for structure or pattern. Marginal and conditional R^2 values were calculated for each of the model fits using the sem.model.fits function, part of the piecewiseSEM library, in R (Nakagawa and Schielzeth, 2013; Johnson, 2014). R^2 values can be used as a summary statistic for LMEs and describe the amount of variance explained by the model. Furthermore, as a model comparison tool, R^2 values offer an absolute value for the goodness-of-fit of a model, unlike an Akaike Information Criterion (AIC) value (Nakagawa and Schielzeth, 2013). Two R^2 values are generated to describe the variation explained by the fixed and random effects included in the model: marginal R^2 values represent the variance explained by fixed effects; conditional R^2 values represent the variance explained by fixed *and* random effects. For example: as individual was included as a random effect, LMEs with high conditional R^2 values suggests fixed effects are not explaining a large proportion of the variation. Model outputs including L ratio, *T* value and *p* values are presented and are described in more detail in Chapter 3, section 3.2.3. A separate model was

fitted for each gene because no *a priori* assumptions were made that the genes would respond in a similar way to each other. I used a sequential Bonferroni method, ranking *p*-values by significance, to adjust for multiple comparisons between genes. LMEs in which daily rate of mass gain and maternal mass transfer to pup were included as a covariates used only suckling data (EARLY – LATE); daily rate of mass loss was used in post weaning fast (EARLY – LATE) data analysis only. LMEs were used to investigate the difference in mass and axial girth between the different time points.

4.3 Results

4.3.1 HSP gene expression during suckling and the post-weaning fast

Log *Hsp90* mRNA abundance increased 3.04 fold during suckling and was 3.40 and 1.99 fold higher EARLY and LATE in the POST-WEANING fast, relative to EARLY suckling, respectively (LME; Figure 4.1; Table 4.1). There was no change in log *Hsp90* mRNA abundance during fasting relative to LATE suckling.

The 1.40 and 1.37 fold lower log *Hsp70* abundance LATE in the POST-WEANING fast, relative to EARLY and LATE suckling respectively, approached significance (LME; Figure 4.1; Table 4.1). There was no change in log gene expression of *Hsp70* during suckling or fasting. Relative to EARLY suckling, there was no change in log gene expression of *Hsc70*, *Hsp40* or *Hsp27* during suckling or the POST-WEANING fast (LME; Figure 4.1; Table 4.1). There was no change in expression of any HSPs during fasting (EARLY – LATE POST-WEANING).
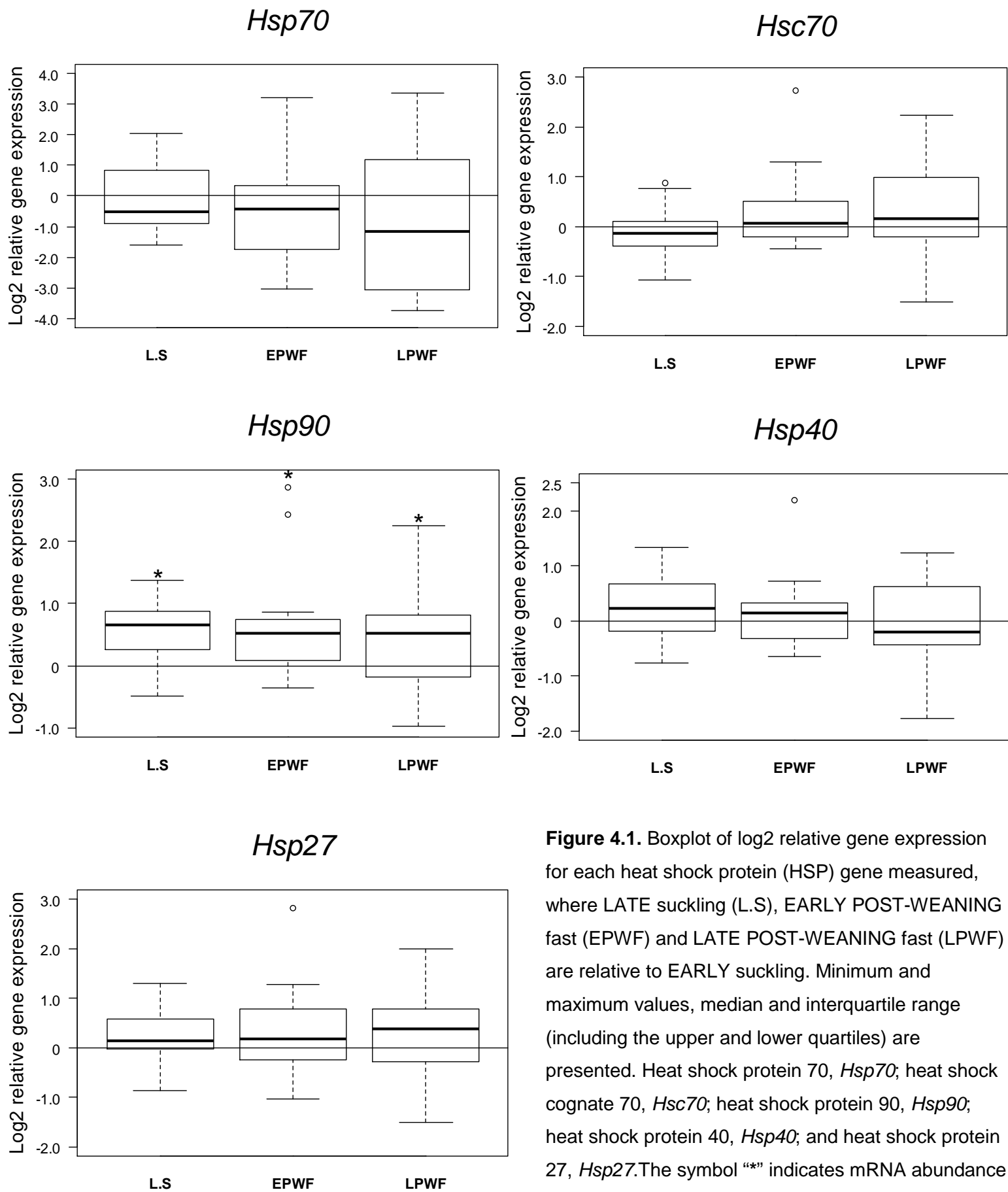
Figure 4.1. Boxplot of log2 relative gene expression for each heat shock protein (HSP) gene measured, where LATE suckling (L.S), EARLY POST-WEANING fast (EPWF) and LATE POST-WEANING fast (LPWF) are relative to EARLY suckling. Minimum and maximum values, median and interquartile range (including the upper and lower quartiles) are presented. Heat shock protein 70, *Hsp70*; heat shock cognate 70, *Hsc70*; heat shock protein 90, *Hsp90*; heat shock protein 40, *Hsp40*; and heat shock protein 27, *Hsp27*.The symbol "*" indicates mRNA abundance was significantly higher or lower (*p* < 0.05) in pups LATE in the suckling period or fasting pups relative to pups EARLY in the suckling period.

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Table 4.1. Model output for linear mixed effect models (LMEs) that explain variability in log relative gene expression of HSPs (heat shock proteins) and *Hsc70* (heat shock cognate 70), where LATE suckling (L.S), EARLY POST-WEANING fast (EPWF) and LATE POST-WEANING fast (LPWF) are compared to EARLY suckling (intercept); measured in suckling and fasting wild grey seal pups in 2013. Bold font indicates a significant difference where $p < 0.05$. n (observations) = 59, n (individuals) = 15.

Gene	Marginal R^2	Conditional R^2	L.S T	L.S p	EPWF T	EPWF p	LPWF T	LPWF p
Log *Hsp70*	0.075	0.19	-0.11	0.92	-0.96	0.34	-1.96	**0.060**
Log *Hsc70*	0.054	0.36	-0.30	0.77	1.40	0.17	1.28	0.21
Log *Hsp90*	0.15	0.41	3.10	**0.0035**	3.38	**0.0016**	2.22	**0.032**
Log *Hsp40*	0.030	0.29	0.93	0.36	0.53	0.60	-0.50	0.62
Log *Hsp27*	0.038	0.23	0.97	0.34	1.60	0.12	1.01	0.32

Individual improved the model fit and explained a larger portion of the variance for all HSPs. Neither body mass, axial girth, sex, circulating glucose, or MDA concentration, included as covariates in LMEs, improved any model's fit to the data and did not help explain variability in relative gene expression of any HSPs (Appendix 2, Table A2.1).

When suckling and fasting were modelled separately, neither rate of mass gain nor maternal mass transfer to pup during suckling was related to gene expression of any HSPs (Appendix 2, Table A2.2). However, mass loss rate was positively related to expression of log *Hsc70* and log *Hsp90* during fasting (Table 4.2). The best models to explain variation in log *Hsc70* and log *Hsp90* expression during fasting included nutritional state and mass loss rate (Table 4.2).

Table 4.2. Further explanatory variables included as covariates in LMEs to explain variability in log relative gene expression/ relative gene expression of HSPs during suckling and fasting. Each HSP was modelled separately. Time-point represents nutritional state (i.e. EARLY or LATE suckling or EARLY or LATE POST-WEANING fast). Models in which body mass gain rate, maternal mass transfer to pup or mass loss rate were included were then tested against the simpler model with no additional covariates using the anova function. Bold font indicates $p < 0.05$.

Gene	Model	Marginal R^2	Conditional R^2	L ratio	p
Log *Hsc70*	**Time-point + Mass loss rate (during fasting; kg day^{-1})**	**0.24**	**0.55**	**5.41**	**0.020**
	Time-point * Mass loss rate (during fasting; kg day^{-1})	0.24	0.55	0.26	0.61
Log *Hsp90*	**Time-point + Mass loss rate (during fasting; kg day^{-1})**	**0.29**	**0.41**	**6.61**	**0.010**
	Time-point * Mass loss rate (during fasting; kg day^{-1})	0.31	0.56	2.21	0.14

4.3.2 RE gene expression during suckling and the post-weaning fast

Relative to EARLY suckling, log *GPx* mRNA abundance was 1.24 and 1.42 fold higher EARLY and LATE in the POST-WEANING fast, respectively; *CAT* was significantly higher at LATE suckling (1.30 fold) and EARLY in the POST-WEANING fast (1.24 fold); log *SOD* and log *Nox4* were significantly higher at all time-points relative to EARLY suckling; *GST* was significantly lower LATE in the POST-WEANING fast relative to EARLY suckling (1.28 fold; Table 4.3; Figure 4.2). Log *GPx* and log *SOD* were increased during fasting. Conversely, *CAT* and *GST* were decreased during fasting. There was no change in log *Nox4* mRNA abundance during the POST-WEANING fast.

Marginal and conditional R^2 values indicate that fixed factors (i.e. nutritional state) in LMEs are not as important as random factors when explaining variation in the data for most REs, with the exception of *GPx* and *GST*. Individual improved the model fit and explained a larger portion of the variance than fixed effects for all REs (Table 4.3).

GPx

CAT

SOD

GST

Nox4
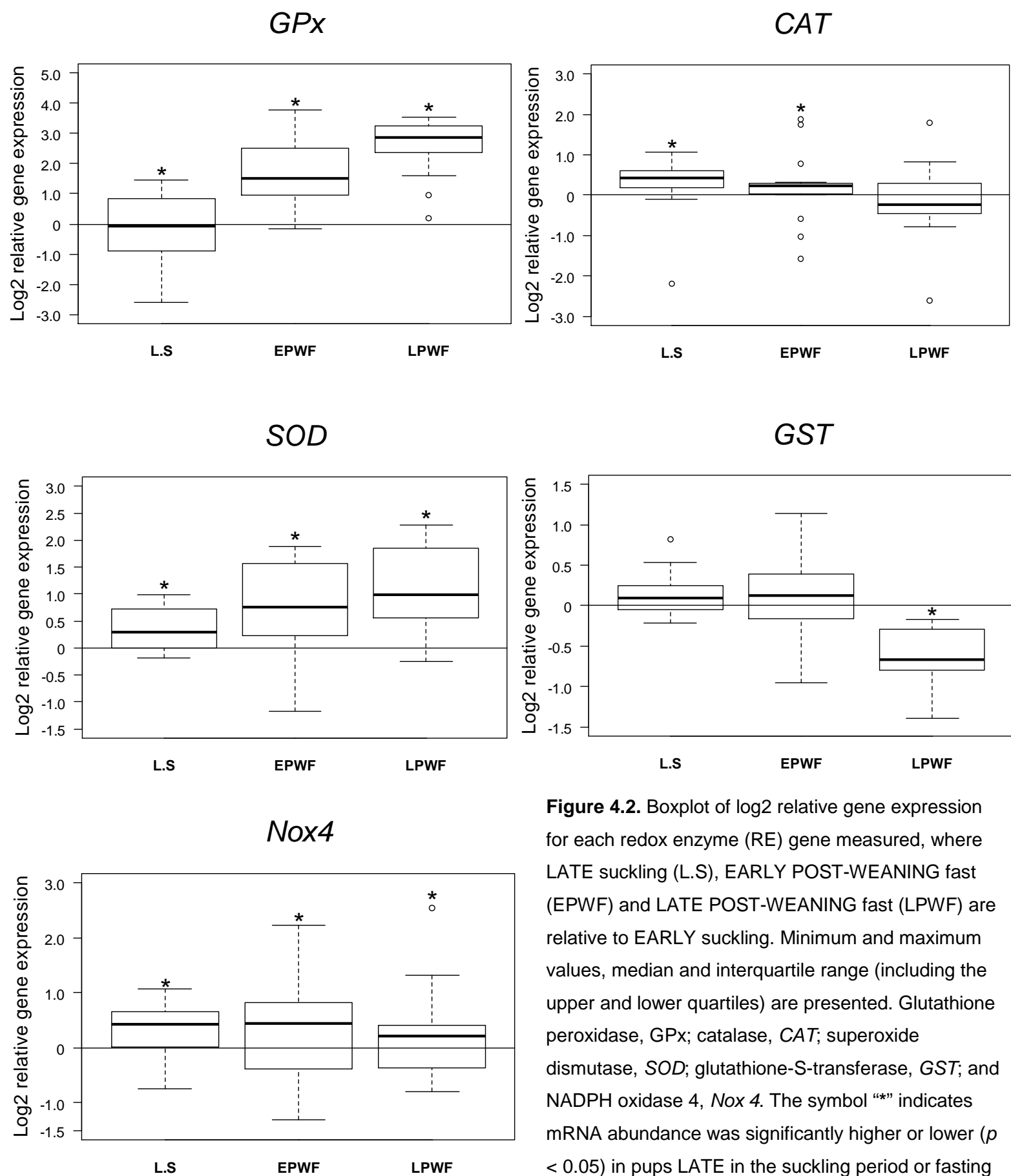
Figure 4.2. Boxplot of log2 relative gene expression for each redox enzyme (RE) gene measured, where LATE suckling (L.S), EARLY POST-WEANING fast (EPWF) and LATE POST-WEANING fast (LPWF) are relative to EARLY suckling. Minimum and maximum values, median and interquartile range (including the upper and lower quartiles) are presented. Glutathione peroxidase, GPx; catalase, *CAT*; superoxide dismutase, *SOD*; glutathione-S-transferase, *GST*; and NADPH oxidase 4, *Nox 4*. The symbol "*" indicates mRNA abundance was significantly higher or lower (*p* < 0.05) in pups LATE in the suckling period or fasting pups relative to pups EARLY in the suckling period.

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Table 4.3. Model output for linear mixed effect models (LMEs) that explain variability in relative mRNA abundance/log relative mRNA abundance of redox enzymes (glutathione peroxidase, *GPx*; catalase, *CAT*; superoxide dismutase, *SOD*; glutathione-S-transferase, *GST*; and NADPH oxidase 4, *Nox 4*), in which LATE suckling (L.S), EARLY POST-WEANING fast (EPWF) and LATE POST-WEANING fast (LPWF) are compared to EARLY suckling (intercept); measured in suckling and fasting wild grey seal pups in 2013. Bold font indicates a significant difference where $p < 0.05$. n (observations) = 59, n (individuals) = 15.

Gene	Marginal R^2	Conditional R^2	L.S T	L.S p	EPWF T	EPWF p	LPWF T	LPWF p
Log *GPx*	0.47	0.83	-0.55	0.59	6.72	**<0.0001**	9.72	**<0.0001**
CAT	0.11	0.53	2.72	**0.0095**	2.17	**0.036**	-0.17	0.86
Log *SOD*	0.23	0.69	4.03	**<0.0001**	5.15	**<0.0001**	5.74	**<0.0001**
GST	0.26	0.39	1.29	0.20	1.25	0.22	-2.95	**0.0052**
Log *Nox4*	0.096	0.36	2.22	**0.032**	2.40	**0.021**	2.29	**0.027**

The best models to explain variation in *CAT* and log *SOD* expression included nutritional state and body mass (Table 4.4). The best model to explain variation in *GST* expression included nutritional state and axial girth (Table 4.4). Increased *CAT* and log *SOD* expression were associated with increased body mass. Increased *GST* expression was associated with increased axial girth. In the model explaining variation in *CAT* expression, the interaction between nutritional state and body mass was on the cusp of significance (Table 4.4). In log *SOD* and *GST* models, the interactions between nutritional state and body mass and nutritional state and axial girth were not significant (Table 4.4). Neither sex, circulating glucose, nor MDA concentration, included as covariates in LMEs, improved any model's fit to the data and did not help explain variability in relative gene expression of any REs (Appendix 2, Table A2.3). The addition of sex and MDA concentration as covariates in log *GPx*

models and *GST* models, respectively, approached significance (Appendix 2, Table A2.3).

Table 4.4. Additional explanatory variables included as covariates in LMEs to explain variability in the relative gene expression/ log relative gene expression of the REs. Each RE was modelled separately. Time-point represents nutritional state (i.e. EARLY or LATE suckling or EARLY or LATE POST-WEANING fast). Models in which body mass, axial girth, circulating glucose, sex or MDA concentration was included were then tested against the simpler model with no covariates using the anova function. Bold font indicates $p < 0.05$.

Gene	Model	Marginal R^2	Conditional R^2	L ratio	*p*
CAT	**Time-point + Body mass (kg)**	**0.19**	**0.65**	**5.00**	**0.025**
	Time-point * Body mass (kg)	0.24	0.72	7.57	**0.056**
Log *SOD*	**Time-point + Body mass (kg)**	**0.39**	**0.77**	**12.62**	**<0.0001**
	Time-point * Body mass (kg)	0.39	0.78	2.76	0.43
GST	**Time-point + Axial girth (cm)**	**0.34**	**0.49**	**6.45**	**0.011**
	Time-point * Axial girth (cm)	0.37	0.52	3.53	0.32

When suckling and fasting were modelled separately, neither rate of mass gain nor maternal mass transfer to pup during suckling was related to gene expression of any REs (Appendix 2, Table A2.4). However, mass loss rate was positively related to expression of *CAT*, log *SOD* and *Nox4* during fasting (Table 4.5). Increased expression of these genes was associated with increased rates of mass loss.

During fasting, the best model that explained variation in *CAT* mRNA abundance was an interaction between nutritional state and mass loss rate (Table 4.5). Variation in *Nox4* mRNA abundance was best explained by a model including nutritional state and mass loss rate; the interaction between state and mass loss rate was not significant (Table 4.5). The best model that explained variation in log *SOD* mRNA abundance was an interaction between nutritional state and mass loss rate with the addition of body mass (Table 4.5). There was a positive relationship between mass loss rate and body mass; there was an increased rate of mass loss with increased body mass.

Table 4.5. Further explanatory variables included as covariates in LMEs to explain variability in the relative gene expression of REs during suckling and fasting. Each RE was modelled separately. Models in which body mass gain rate, maternal mass transfer to pup or mass loss rate was included were then tested against the simpler model with no covariates using the anova function. Bold font indicates $p < 0.05$.

Gene	Model	Marginal R^2	Conditional R^2	L ratio	p
CAT	**Time-point + Mass loss rate (during fasting; kg day^{-1})**	**0.012**	**0.62**	**10.56**	**0.0012**
	Time-point * Mass loss rate (during fasting; kg day^{-1})	**0.29**	**0.77**	**4.83**	**0.028**
Log *SOD*	Time-point + Mass loss rate (during fasting; kg day^{-1})	0.012	0.62	10.56	0.0012
	Time-point * Mass loss rate (during fasting; kg day^{-1})	**0.13**	**0.81**	**10.40**	**0.0013**
	Time-point * Mass loss rate (during fasting; kg day^{-1}) + Body mass (during fasting; kg)	**0.32**	**0.81**	**4.33**	**0.038**
Nox4	**Time-point + Mass loss rate (during fasting; kg day^{-1})**	**0.36**	**0.80**	**8.18**	**0.0042**

| Time-point * Mass loss rate (during fasting; kg day^{-1}) | 0.37 | 0.79 | 0.24 | 0.63 |

4.3.3 Suckling and post-weaning fast duration, pup body mass and axial girth

The mean duration of suckling ($n = 18$) was 20.17 ± 2.01 (SD) days, ranging from 17-25 days. At EARLY and LATE suckling and EARLY and LATE POST-WEANING fast, the mean number of days after birth/weaning that the capture took place and morphometric data collected and blubber samples taken is presented in Table 2.1, Section 2.1.3.

There was a significant difference in body mass between suckling and fasting pups (LME, $p<0.0001$), with the exception of mass loss between LATE suckling and the EARLY post weaning fast, which approached significance (LME, $p = 0.076$). Pups exhibited a mean 19.01 ± 4.97 (SD) kg, or 1.74 ± 0.22 (SD) fold, increase in mass between EARLY and LATE in the suckling period and a mean 4.97 ± 2.76 (SD) kg (11.28 ± 6.33 (SD) % body mass) decrease in mass between the EARLY POST-WEANING and LATE POST-WEANING fast (LME, $p<0.0001$). There was a mean 1.74 ± 1.94 (SD) kg decrease in mass between LATE suckling and the EARLY post weaning fast. There was no relationship between body mass and weaning date i.e. pups that suckled for longer did not have a higher mass than those that weaned immediately after LATE suckling.

Axial girth followed the same pattern; there was a significant difference between suckling and fasting pups (LME, $p<0.0001$), with the exception of change in axial girth between LATE suckling and the EARLY post weaning fast (LME, $p = 0.73$). Pups exhibited a mean 21.61 ± 6.63 (SD) cm, or 1.29 ± 0.11 (SD) fold, increase in axial girth between EARLY and LATE in the suckling period and a mean

5.62 ± 2.84 (SD) cm (5.72 ± 3.02 (SD) %) decrease in axial girth between the EARLY POST-WEANING and LATE POST-WEANING fast (LME, $p = 0.0030$).

Individuals were not monitored in between EARLY and LATE POST WEANING or after the collection of the LATE POST WEANING fast sample, so the exact departure date and duration of the land-based post weaning fast is not known. Using known dates we can estimate mean minimum fast duration, suggesting the post weaning fast lasted an average of 14.0 ± 1.13 (SD) days or beyond (calculated from the weaning date to the date of the LATE post-weaning fast capture) for all but three individuals. These three were assumed to have departed the colony after EARLY post weaning fast sampling, an average of 4.0 ± 1.73 (SD) days after weaning, as they were not found approximately ten days later, for the LATE post-weaning fast sample to be taken. Some pups may have left for sea on the day of the EARLY/ LATE post weaning fast capture or continued fasting for an unspecified amount of time and moved to an inaccessible area where they could not be located on the colony.

Table 4.6. Mean (± SD (*n*)) and range of body mass (kg) and axial girth (cm) at both EARLY and LATE suckling and the EARLY POST-WEANING and LATE POST-WEANING fast. Mean (± SD (*n*)) and range of birth mass (kg), mass change from birth to weaning (kg), rate of mass gain (kg day^{-1}), rate of mass loss (kg day^{-1}) and maternal mass transfer to pup (%) is also presented for 2013 wild grey seal pups from the Isle of May, Scotland. "M:F" indicates the proportion of pups that were male (M) or female (F).

	Range				Mean ± SD (*n; M:F*)			
	EARLY suckling (age 4-10 days)	LATE suckling (age 15-19 days)	EARLY POST-WEANING fast (age 18-30 days)	LATE POST-WEANING fast (age 31-40 days)	EARLY suckling	LATE suckling	EARLY POST-WEANING fast	LATE POST-WEANING fast
Mass (kg)	19.2 – 31.0	31.6 – 56.4	29.8 – 56.0	29.4 – 50.6	26.0 ± 3.27 (18; 11:7)	45.01± 5.92 (18; 11:7)	43.28 ± 6.64 (18; 11:7)	39.23 ± 6.40 (15; 8:7)
Axial girth (cm)	66 - 85	89 - 108	82 - 108	82 - 113	76.28 ± 5.38 (18; 11:7)	97.88 ± 4.91 (18; 11:7)	96.55 ± 6.96 (18; 11:7)	93.93 ± 7.96 (15; 8:7)
Birth mass (kg)	10.72 – 24.40				15.83 ± 3.34 (18; 11:7)			
Weaning mass (kg)	35.56 – 61.09				48.90 ± 7.33 (18; 11:7)			
Mass change (birth to weaning; kg)	18.92 – 45.59				33.90 ± 7.53 (18; 11:7)			
Rate of mass gain (EARLY to LATE suckling; kg day^{-1})	0.88 – 2.56				1.81 ± 0.45 (18; 11:7)			
Rate of mass loss (EARLY to LATE POST-WEANING fast; kg day^{-1})	0.20 – 1.38				0.49 ± 0.26 (15; 8:7)			
Maternal mass transfer to pup (total pup mass change, birth-	28.17 – 55.10				44.72 ± 7.46 (17; 10:7)			

weaning, as a %
of total maternal
mass change,
post-partum –
weaning)

Mean and range of pup mass at birth, pup mass gain rate and mass loss rate are displayed in Table 4.6. There was a 13.68 kg difference between lightest and heaviest pups at birth, and a 25.53 kg difference at weaning. There was a 2.9 fold difference in rate of mass gain during suckling and a 6.9 fold difference in rate of mass loss over the 9.87 ± 1.19 (SD) days (n = 15) between early and late in the post-weaning fast. Pups with larger pre-weaning masses did not experience a greater overall maternal mass transfer (LME: marginal R^2 = 0.014, conditional R^2 = 0.014, df = 13, T = 0.63, p = 0.54). However, rate of mass gain during suckling was positively associated with higher percentage maternal mass transfer efficiency (LME: marginal R^2 = 0.25, conditional R^2 = 1.0, df = 13, T = 2.67, p = 0.019).

4.3.4 Circulating glucose

There was no change in plasma glucose concentration during the suckling period or the post weaning fast (Table 4.7; 4.8). There was no change between suckling and the EARLY POST WEANING fast (Table 4.7; 4.8). The decrease in plasma glucose concentration from EARLY suckling to LATE in the POST WEANING fast approached significance (Table 4.7; 4.8); there was a significant decrease in plasma glucose concentration from LATE suckling to LATE in the POST WEANING fast (Table 4.7; 4.8).

Table 4.7. Model output for linear mixed effects models (LMEs) that explain variability in circulating glucose concentration (mM) in which the intercept is varied to compare all nutritional states to one another. E.S, EARLY suckling; L.S, LATE suckling; EPWF, EARLY POST-WEANING fast; LPWF, LATE POST-WEANING fast; measured in suckling and fasting wild grey seal pups in 2013. Bold font indicates a significant difference where $p <$ 0.05. n (observations) = 66, n (individuals) = 18.

Glucose concentration (mM)	Marginal R^2	Conditional R^2	T	p
E.S vs L.S	0.034	0.63	-0.28	0.77
EPWF vs LPWF	0.034	0.63	-0.68	0.50
E.S vs EPWF	0.034	0.63	1.13	0.26
L.S vs EPWF	0.034	0.63	1.42	0.16
E.S vs LPWF	0.034	0.63	1.76	0.084
L.S vs LPWF	0.034	0.63	-2.04	**0.048**

Table 4.8. Range and mean ± SD (n) values of circulating glucose (mM) measured in plasma of suckling and fasting wild grey seal pups in 2013.

	Range				Mean ± SD (n)			
	EARLY suckling	LATE suckling	EARLY post-weaning fast	LATE post-weaning fast	EARLY suckling	LATE suckling	EARLY post-weaning fast	LATE post-weaning fast
Glucose concentration (mM)	7.88 – 10.97	6.84 – 10.09	7.12 – 9.11	7.05 – 11.07	9.34 ± 0.90 (17)	8.57 ± 0.83 (18)	7.91 ± 0.56 (18)	8.19 ± 1.01 (15)

4.3.5 Lipid peroxidation

There was a significant increase in MDA concentration between suckling and the LATE POST WEANING fast (LME, $p<0.05$; Figure 4.3). There was no change in MDA concentrations between suckling and EARLY in the post-weaning fast (Figure 4.3). Appendix 2, Table A2.5 and A2.6 and Figure A2.1 consider correlations between lipid peroxidation and mRNA abundance.
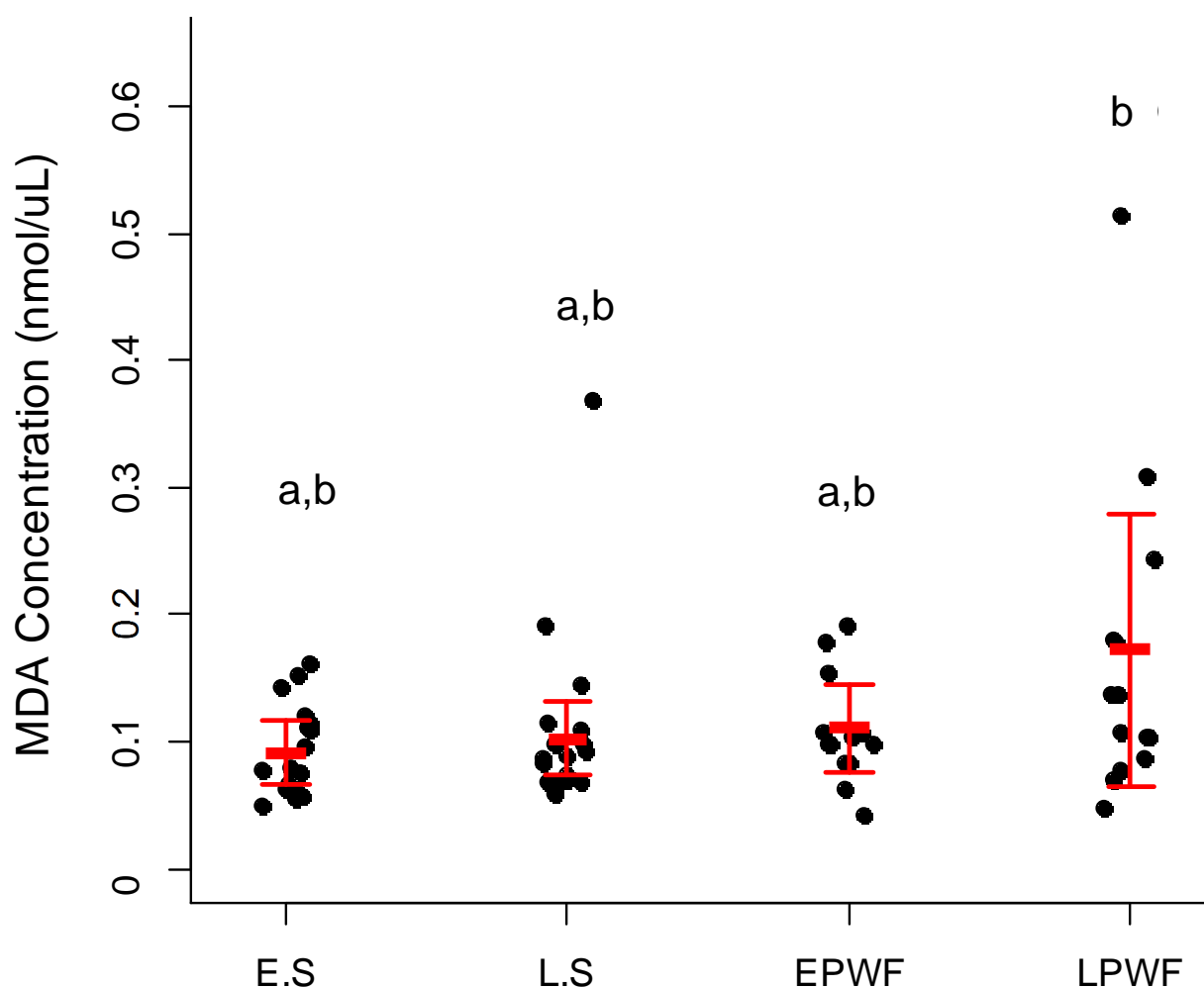
Figure 4.3. Mean (± SD) MDA concentration (nmol/µL) of suckling and fasting grey seal pups (Isle of May, Oct – Dec, 2013) in red. Abbreviations: "E.S", EARLY suckling (n = 17); "L.S", LATE suckling (n = 17); "EPWF", EARLY POST WEANING fast (n = 13); "LPWF", LATE POST WEANING fast (n = 14).

4.4 Discussion

4.4.1 Developmental and nutritional changes in HSP and RE gene expression

The present study shows the developmental profiles of a range of HSPs and REs in blubber of grey seal pups, during suckling and the post-weaning fast.

HSPs provide essential protection to intracellular proteins under a range of physiologically stressful conditions, and are associated with early post-natal development, exposure to a high fat diet and under fasting conditions. Despite intake of a high-fat diet, rapid fat deposition and mass gain (up to 2.56 kg body mass per day) grey seal pups did not increase HSPs during suckling, with the exception of log *Hsp90*. Increased mRNA abundance of *Hsp90* during suckling may be attributed to its role in the regulation of sterol regulatory element-binding proteins (SREBPs) which regulate lipid biosynthesis and metabolism genes (Kuan *et al.*, 2016; described in Chapter 3, Section 3.4.2). During suckling, pups rapidly lay down blubber fat; therefore HSP90 may be upregulated as a result of increased demand for SREBPs due elevated rates of lipid biosynthesis. This finding suggests for the theory that *Hsp90* plays an important role in lipid biosynthesis in grey seals, as in other animals. We may expect pups to be susceptible to proteotoxic stress at this time as a result of rapid protein turnover and synthesis (Anderson and Fedak, 1987; Iverson *et al.*, 1993), requiring increased chaperone presence (Craig *et al.*, 1994; Wiederkehr *et al.*, 2002; Whitesell and Lindquist, 2005). HSP90 alone may fulfil this requirement during suckling or other HSPs are present tonically. Furthermore,

HSP90 regulates both the stability and function of a number of protein kinases (Marcu *et al.*, 2002) and modulates the unfolded protein response (UPR; initiated in response to the accumulation of unfolded proteins in the endoplasmic reticulum (ER), an organelle central to lipid and protein synthesis and modification) in various mammalian cell lines (Marcu *et al.*, 2002). Protein kinases are enzymes essential for regulation of the biological activity of proteins. They phosphorylate specific amino acids, which causes activation or inhibition of enzyme function, making them critical regulators of almost all cellular processes. HSP90 stabilises the transmembrane protein kinase inositol-requiring enzyme 1 alpha (IRE1-α), which acts as an ER stress sensor (Marcu *et al.*, 2002). Increased mRNA abundance of *Hsp90* in suckling pups may thus be as a result of heightened requirement for stable and/or functioning protein kinases necessary to modify and activate large volumes of newly synthesised proteins during rapid development. Increased protein synthesis, protein accumulation and requirement for multiple post-translational protein modifications (i.e. phosphorylation, ubiquitination, methylation, lipidation and nitrosylation) are potential causes of ER stress and processes that are likely increased in suckling grey seal pups. Therefore, upregulation of *Hsp90* may also be due to its role in sensing ER stress and modulating the UPR (Marcu *et al.*, 2002), which, like the HSR, is essential to protect cell function by maintaining efficiency of protein production and upholding the secretory capacity of developing tissues. No measurements of ER stress were made here. However, it would be useful to measure initiators of the UPR in developing grey seal pups, such as IRE1, protein kinase R-like ER kinase (PERK) and activating transcription factor 6 (ATF6). These are ER transmembrane proteins, bound to the ER chaperone immunoglobulin

binding protein (BiP) in their inactive state and disassociate when activated by ER stress (Oslowski and Urano, 2011).

A high fat diet and fat gain, associated with obesity, induce cellular stress. In adipose tissue, HSP27 protein expression has been shown to increase in rats fed a high fat diet (Joo *et al.*, 2010). However, blubber HSP27 expression did not change in suckling pups. HSP27 may be immediately elevated as pups begin to suckle or its function is not needed as these conditions are considered 'normal'.

CAT, *SOD* and *Nox4* increased during suckling. Some studies suggest that CAT and SOD increase (at the gene and protein-level and in terms of enzymatic activity) in response to a high-fat diet (Marczuk-Krynicka *et al.*, 2009; Rindler *et al.*, 2013; Jarukamjorn *et al.*, 2016; Piao *et al.*, 2017), which may provide an explanation for significant increases seen whilst suckling. For developing grey seal pups *CAT* (and the mechanism by which it detoxifies H_2O_2) may be more important than *GPx*. *Nox4* is upregulated in adipose tissue of mice in response to a high fat diet, leading to increased RS production (Matsuzaw-Nagata *et al.*, 2008), which may also explain the current findings. *Nox4* may also be elevated as a result of increased adipogenesis. Chapter 3 identified a study by Mouche *et al.* (2007) who showed that *Nox4* is mostly present in the stromal fraction of mammalian adipose tissue, rather than mature adipocytes. Increased *Nox4* relative expression during suckling could therefore indicate that there are more mitotic and differentiating pre-adipocytes (cells in which Nox4 is highly expressed; Mouche *et al.*, 2007) in females at LATE lactation i.e. the process of adipocyte differentiation is heightened. As suggested in Chapter 3, this could be explored using histology and cell cycle markers, and further supports *Nox4* as a useful indicator of adipocyte maturation in grey seals.

We may expect the rate of protein turnover and synthesis during fasting to be much lower than suckling, because resources are limited and animals reduce metabolic costs when fasting (Reilly, 1991; Bennett *et al.*, 2007), which should lower the requirement for chaperones. However, contrary to expectation, *Hsp90* gene expression increased further during fasting. Increases in relative gene expression also occurred in *Hsc70*, *GPx*, *SOD* and *Nox4* during fasting, suggesting that pups experience greater physiological stress during fasting than during suckling. MDA concentration increased significantly LATE in the POST-WEANING fast, which suggests oxidative damage to lipids occurred despite increases in the gene expression of some REs. These data are following the same logic as in females, that later in fast is a time when pups may be more vulnerable to stress. Also the LATE POST-WEANING fast (day 15) sample was actually not that 'late' compared to how long pups can actually fast for (considering an average 10 day to > 4 week fast duration; Reilly 1991; Noren *et al.*, 2008; Bennett *et al.*, 2010). Therefore, it would be interesting to sample pups much later in the post-weaning fast to see if this continues to increase. Additionally, this could provide support for the speculation that oxidative stress plays a role in the termination of the fast.

HSP90 and HSP27 (but not HSP70) protein expression was increased in the gastrointestinal tract of piglets as a result of fasting (Grongnet and David, 2003; Lallès and David, 2011). At the protein level, there was an increase in HSP expression (ER associated 78 kDa glucose-regulated protein (GRP78), a member of the HSP70 family) in liver of rats after 48 hours of fasting. Peroxisome proliferator-activated receptor (PPAR) plays an important role in fatty acid (FA) oxidation during fasting and adipocyte function (Kersten *et al.*, 1999; Christodoulides and Vidal-Puig, 2010). Nguyen *et al.* (2013) have identified HSP90 as a regulator of PPAR, forming a

complex that facilitates the survival of adipocytes. HSP90 monitors PPAR activity and in turn FA oxidation (Sumanasekera *et al.*, 2003). Hence the increase in *Hsp90* seen here could help maintain adipose tissue and modulate use of lipids during the fast.

HSP70 and HSP27 were elevated in subcutaneous adipose tissue by fat loss during periods of reduced calorific intake in humans (Roumans *et al.*, 2016). The HSP increase associated with fasting in liver greatly improved transplant recipient survival (when compared to rat transplant recipients of livers from fed rats; Nishihara *et al.*, 1998) and subsequent protection from ischemic injury. This suggests that fasting-induced cytoprotective HSPs can provide increased protection against future challenges. These heightened defences may be particularly important after weaning and when pups begin to feed on fish (a high fat, solid food diet) and adopt a diving lifestyle (experiencing ischemia/reperfusion). Potential trade-offs in energy allocation (i.e. increased energetic requirement for ATP dependent HSPs during a period of limited resources) may be offset if these increased HSPs provide future protection and increase survival probability when pups go to sea (provided HSP production can be sustained at the time). However, *Hsp90* only increased EARLY and LATE in the POST-WEANING fast relative to EARLY suckling and did not increase over the course of the POST-WEANING fast; *Hsc70* increased only EARLY in the POST-WEANING fast and remained constant as the fast progressed. Additionally, it is important to distinguish between inactive HSPs present in the cytoplasm and HSPs that are actually being used by the cell. Here, I have only measured mRNA abundance, so the increase in *Hsp90* and *Hsc70* seen might be a reduction in protein degradation of the mRNA for those defences (albeit quiescent) rather than transcription of newly made HSPs mRNA.

The increased expression of HSP genes in some individuals during suckling and fasting may provide protection against RS protein damage and oxidative stress (Ahn and Thiele, 2003; Fittipaldi *et al.*, 2014). *GPx*, *SOD* and *Nox4* mRNAs increased further or increased during fasting, suggesting physiological stress increases in some individuals. Increased RE expression during fasting also matches the chemistry behind RS generation. A fasted state, where we may assume metabolism and ATP production to be low, in fact results in increased mitochondrial RS production (Murphy, 2009).

Individual regulation of *SOD* and *Nox4* is more variable than that of *GPx*. *Nox4* is a RS producing enzyme. RS production can be an important factor regulating RE expression and activity (Warner *et al.*, 1996; Dalton *et al.*, 1999; Franco *et al.*, 1999; Turpaev, 2002). Since increased *Nox4* results in increased RS production and thus oxidative stress, the increase in some REs and oxidative damage, seen late in the fast may occur to counteract the pro-inflammatory effects of Nox4, which may account for the lack of correlation between *Nox4* mRNA abundance and MDA concentrations. Changes in *Nox4* expression may also result from adipocyte differentiation, which is discussed above in more detail in the context of Mouche *et al.* (2007).

GST was decreased LATE in the POST-WEANING fast. It is unclear why *GST* would significantly decrease, as this enzyme provides support to *GPx* and plays an essential role in RS defence. Down-regulation of *GST* has been linked to increased RS production and oxidative damage in the intestines of mice in response to fasting (van den Bosch *et al.*, 2007) and in adipocytes of obese, insulin resistant humans and mice (in comparison to lean individuals; Curtis *et al.*, 2010). Oxidative damage in both cases was measured via detection of protein carbonylation.

Decreased *GST* gene expression LATE in the POST-WEANING fast may contribute to the increase in oxidative damage to lipids (MDA concentration). GSTs catalyse the detoxification of xenobiotics and aldehyde products of lipid peroxidation, such as MDA, by glutathione conjugation (Sies, 1999). Therefore, the decrease in GSTs also removes a defence against MDA, which again may explain the increase in oxidative damage to lipids later in the fast.

GST expression levels are directly dependent upon glutathione presence, which typically decreases during fasting in mice, rats and humans (Sies, 1999). Both GPx and GST require glutathione as an essential co-factor, therefore it may not be possible to sustain two glutathione dependent enzymes during fasting, conserving energy and resources. Indeed, *GPx* appears to become more important during fasting, when *CAT* expression appears to decline.

Measurement of glutathione could facilitate interpretation of these findings. Lower levels of glutathione during fasting could support the theory that only one glutathione dependent enzyme can be supported when resources are limited. Bennett *et al.* (2014) found higher glutathione levels in blubber of suckling grey seal pups compared to fasting pups. However, northern elephant seal pups experience increases in glutathione content and biosynthesis in both muscle and blood constituents (red blood cells and plasma) over the course of their post weaning fast (these data were not compared to suckling individuals; Vázquez-Medina *et al.*, 2011a). GST activity increases in muscle in fasting elephant seal pups, which is contrasted by the reduction in *GST* gene expression reported here in blubber. This may mean, as suggested for females, that blubber is a sacrificial resource.

MDA concentration also did not explain variation in gene expression of any REs or HSPs overall. Higher *CAT* expression EARLY in the POST-WEANING fast was correlated with higher levels of oxidative damage to lipids whilst, conversely, increased *Hsc70* and *Hsp27* expression at LATE suckling was related to decreased MDA concentration. Moghimian *et al.* (2014) found lower MDA concentrations were related to increased *Hsp70* and *Hsp27* in cardiac muscle of mice exposed to exercise stress. This provides further support for the concept that HSPs provide protection for tissues exposed to oxidative stress. Additionally, suckling pups may benefit from additional RS protection in the form of dietary derived 'anti-oxidants', such as vitamin E (Debier *et al.*, 2002), which could explain why oxidative damage is worse during fasting. Although there was increased expression of some REs and HSPs, MDA concentration increased significantly LATE in the POST-WEANING fast, suggesting that higher defences at this point were not sufficient to protect lipids from oxidative damage. Increased oxidative damage to lipids identifies the post weaning fast as a life history state potentially vulnerable to additional stressors.

Age (stage of development) can be a factor that explains patterns in HSP and RE expression and activity. A number of studies have measured RE activity during development in a variety of tissue types. In brain tissue, Mavelli *et al.* (1982) found GPx activity to be constant from birth in rats up to 60 days old. In human lung tissue there was no clear change in GPx activity, from 1 week before birth up to two days post-partum (Fryer *et al.*, 1986). At the protein level, HSP90 expression decreased in brain and kidney of rats during post-natal development (from 1 to 20 days old) though HSP90 levels in both tissues were comparable on post-natal day 1 (highest in kidney, there after). HSC70 was abundant throughout post-natal development and did not vary over the 20 days in brain but decreased in kidney tissue. HSP70

increased in brain and kidney up to day 20, with higher protein abundance in kidney (D'Souza and Brown, 1998). HSF1 protein levels increased in brain up to 98 days of age in rats, but declined in kidney tissue to undetectable levels after post-natal day 30 (Morrison *et al.*, 2000). It had been suggested that HSPs are expressed during development in a 'stage-specific' pattern (Franklin *et al.*, 2005). Here, it is difficult to disentangle development from the transition from suckling to fasting. It may be possible to ascertain if fasting itself is driving the changes in gene expression by feeding pups that would otherwise be fasting (Bennett *et al.*, 2007) or by making further comparisons with pups that have started to feed naturally.

It is unclear whether the patterns of gene expression seen during suckling and fasting will be experienced as 'eustress' and better equip animals to fight future challenges. Some of the changes seen could be a result of maturation, such that if stresses are encountered earlier, the system is not yet 'ready'. An increase in a stress response does not mean distress and no stress response may mean exhaustion. Therefore, knowing to what extent animals are able to respond to a challenge would help to work out how closely they operating to physiological limits. An experimental approach is needed to assess this (see Chapter 6). My data suggest that pups are potentially more likely to experience 'distress' if additional challenges are encountered during the post weaning fast, a time when they are already increasing gene expression of a number of HSPs and REs and face increased oxidative damage to lipids.

As discussed in Chapter 3, section 3.4.4 it is important to consider the challenges of distinguishing the effects of stress from the natural developmental biology of this species, which will also help define the distinction between eustress and distress. Chapter 3, section 3.4.4 highlighted the potential of MDA concentration to be

influenced by an artefact of pregnancy and lactation, the prostaglandins, whose concentrations fluctuate before and after birth in both mothers and their offspring (Shibuya *et al.*, 1986) and whose synthesis produce MDA as a by-product. Interpretation of results may then attribute increased MDA concentration to stress, rather than a consequence of natural processes. Following this presumption, MDA concentrations seen in pups during different developmental states may also be elevated as a result of prostaglandin synthesis. Prostaglandins can increase during the post-natal period to ensure proper development. In rat brain, prostaglandins have been shown to modulate cerebral development and inhibition can lead to altered complex social and sensory behaviours (Dean *et al.*, 2012). As prostaglandins also mediate brain function, synthesis and expression of prostaglandin receptors is high in developing brain tissue (Dean *et al.*, 2012). Prostaglandins are also known to modulate immune system development in murine neonates, in part, by inducing the maturation of T-cells and inhibiting splenic lymphocytes (Shibuya *et al.*, 1986). Despite this, I would argue that the MDA concentrations measured here in adipose tissue are not simply the artefact of prostaglandin synthesis. The significantly increased MDA concentrations LATE in the POST-WEANING fast are accompanied by increased expression of REs, which are associated with increased rates of mass loss during the post-weaning fast. Adipocytes can produce various prostaglandins (Rahman, 2018), however increased levels are associated with increasing adiposity and obesity (such as the suckling period where MDA concentrations remain constant), rather than a fasted state where adipose tissue is being reduced (such as LATE in the POST-WEANING fast). As the post-weaning fast progresses, it is likely that pups may begin to experience distress that may be a consequence of their reproductive strategies. However, it is certainly important to consider if the

measurements taken as part of a study can be interpreted as measuring true stress, rather than life-history artefacts. If we assume our data are presenting natural changes, then to see if anything is contributing additional load on the system we need to account for natural history/development state. It would be better to choose a marker that is stable or predictable over time so that elevations or reductions can be definitely attributed to a stressor. By running reference genes for relative gene expression analysis I potentially have data that could be used to explore this. Reference genes are tested under all experimental conditions to ensure that they are not regulated. This might be an avenue worth exploring however I would need to consider the physiological interactions of my reference and genes of interest.

4.4.2 Trade-off between gene expression and mass changes

Higher body mass was associated with higher *SOD* expression. Pups with higher *SOD* mRNA abundance gained mass faster during suckling and lost mass more slowly during the post weaning fast. These data support previous work showing SOD activity was positively correlated with blubber thickness (Bennett *et al.* 2014), which correlates with mass and axial girth (Hall and McConnell, 2007). This may suggest that pups with greater *SOD* are more robust. Also, higher body mass and axial girth was associated with higher *CAT* and *GST* expression, respectively. Both CAT and GST are elevated in various tissues (for example, liver, kidney, heart and adipose tissue) of obese humans, rats and mice (i.e. animals with increased body masses and subsequently increased girths). Therefore the results presented here may be confirming these findings in a mammal with a significantly high percentage body fat. An alternative explanation is that pups with higher body mass and axial girth can better support higher cellular defences, as they have additional resources to do so.

Mass gain rate was not related to HSP or RE gene expression. Pups with higher cellular defences did not gain weight at a slower rate, suggesting that increased defences did not affect weight gain and pups were apparently able to allocate resources effectively to sustain both the stress markers increased during suckling (which potentially offset the stress that induced them to sustain weight gain) and the processes associated with rapid weight gain and fat deposition.

Increased rate of mass loss during the post weaning fast was associated with raised cellular defences, suggesting increased energy allocation to maintenance *Hsp90* and *Hsc70*, both ATP dependent chaperones, were positively related to rate of mass loss. This was also the case for the REs *CAT*, *SOD* and *Nox4*. This suggests that either the expression of these cellular defences incurs significant metabolic costs, or that animals with higher metabolic rates have increased defences to cope with higher RS generation. *CAT* expression was reduced as the fast progressed, which may alleviate the trade-off associated with its expression, especially as the function of *CAT* (the decomposition of hydrogen peroxide) could be taken over by *GPx*, which was not associated with an increased rate of mass loss.

4.4.3 Importance of additional factors in explaining gene expression

Plasma glucose did not have an effect on the expression of any of the cellular stress markers measured here. However the lowest values reported do not seem biologically real for healthy grey seal pups (Bennett *et al.*, 2017), especially as the individuals for whom the low values are reported did not exhibit especially low masses or appear to be in poor condition throughout suckling or the post weaning fast.

Importantly, it appears that fixed effects (i.e. nutritional state; suckling or fasting) do not explain a large portion of the variance in gene expression data, whereas individual does. Differences between individuals need to be considered for example, pollutant load, transferred from mothers during suckling (Debier *et al.*, 2003a; 2003b). Pups face an increased allostatic load during suckling due to pollutant exposure. Persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs), are transferred to pups via their mother's milk (Debier *et al.*, 2003a; 2003b). In marine mammals PCBs have been linked to developmental abnormalities and are known to act as endocrine disruptors, supress the immune system and induce oxidative stress which leads to increased expression of REs and HSPs. A high PCB load acquired during suckling may reduce fitness, particularly as this is a critical period of intensive growth and development.

Other individual differences that may be driving variance in gene expression data include: pathogen and/or parasite load, colony density and distribution of individuals and other physiological stressors (for example, hypoxia, which may be induced as a result of a large volume of adipose tissue).

4.4.4 Comparison with other studies in seals

In contrast to the current results, Bennett *et al.* (2014) found that suckling grey seal pups had higher levels of cellular defences, including HSP70 (protein abundance; blubber and liver) and CAT (enzyme activity; liver only), compared with fasting pups . There was also no change in SOD activity in blubber between suckling and fasting, which contrasts with the current results (showing increasing SOD throughout sucking and the post-weaning fast). No change in oxidative damage in liver and blubber was found between suckling and fasting. However, Bennett *et al.*

(2014) performed a cross-sectional study that did not distinguish different stages of suckling or fasting and relied on a small sample size ($n = 5$, suckling; $n = 11$, fasting), without the power of repeated measures. There were also differences in the measurements taken and a time delay between sample collection and storage at -80°C.

In Northern elephant seal pups, Vázquez-Medina *et al.* (2010) report increases in GPx, CuZnSOD, MnSOD and CAT protein content of muscle, also accompanied by increased enzyme activity. Though some of Vázquez-Medina *et al.*'s (2010) data mirror that found here, CAT decreased as development progressed and nutritional state transitioned to the post-weaning fast. However, the data generally agree with the increases of some REs (GPx, SOD, Nox4) observed during fasting in muscle and liver of other species of pinnipeds (Vázquez-Medina *et al.*, 2010; Vázquez-Medina *et al.*, 2011a; 2011b; 2011c) and the elevation of cellular stress defences seen in other fasting animals, including rodents, fish and humans (Ehrenfried *et al.*, 1996; Gasbarrini *et al.*, 1998; Cara *et al.*, 2005; Santos *et al.*, 2009). However there are other contrasting results, as these studies do not report an increase in oxidative damage to lipids (or proteins) during fasting in pinnipeds (Vázquez-Medina *et al.*, 2010; Bennett *et al.*, 2014), which was seen here as the fast progressed. MDA concentration increased in blubber late in the post weaning fast. This may be due to the fact that different measures of oxidative damage (e.g. TBARS and protein carbonyls) were measured in different tissues (e.g. serum and muscle, rather than blubber). Blubber in grey seals pups may be particularly susceptible to oxidative attack due to individual POP burden, the dramatic increase in tissue volume during suckling and the requirement for that tissue to then sustain

metabolic demand by catabolism as the pup transitions from one nutritional state to another.

These previous pinniped studies measured protein abundance or content and enzyme activity, which may help explain the differences in findings. Post translational modifications (PTM) of proteins could also account for variation and provides an explanation for why the patterns in the protein-level and gene-level studies are different (Cloutier and Coulombe, 2013). PTMs of CAT or GST in grey seals may explain differences in findings at the protein and gene level and differences between species. It is also important to consider different isoforms of the genes or proteins that result in subtle changes in function and/or expression.

This study identifies the post weaning fast as a nutritional state with higher cellular stress defences in blubber of grey seal pups. This life-history state may be vulnerable to additional stressors. However, this needs extra work to determine whether fasting pups can and do increase cellular defences when exposed to additional sources of stress. The data also concur with the results reported for adult females in Chapter 3, which suggests higher defences against oxidative stress in fasting compared to feeding individuals. The individual pattern of change also aligns with findings from females that show a strong individual component to variation in the expression of cellular defences.

This study forms the basis for additional work as we still need to determine 1) how the gene and protein level are related to each other; 2) the individual-related factors that drive expression (e.g. pollutant load); and 3) the consequences for tissue function of relatively lower or higher levels of cellular defences. Until such questions have been answered these markers cannot be used as indices of stress. This study

also shows the need to understand the natural drivers of cellular defences in order to interpret them as useful stress markers. However, our data show that increased cellular defences are associated with greater energetic costs in both suckling and fasting pups. Fasting pups may be most vulnerable to additional stressors because their defences are already elevated and may have limited scope to increase further, particularly given their restricted resources to meet additional energetic demands.

Chapter 5.

Heat shock protein and redox enzyme gene expression dynamics in grey seal (*Halichoerus grypus*) leukocytes in relation to nutritional state, morphometrics and blood glucose: abundance of glutathione-S-transferase and NADPH oxidase 4 mRNA is increased in fasting pups.

Abstract

Immune function is vital for health and is influenced by nutritional state. Altered investment in immune function has survival and fitness consequences. Organisms face trade-offs between immunity and other physiological functions when resources are limited, and these trade-offs may be most apparent during energetically demanding periods, such as reproduction or development. Grey seals (*Halichoerus grypus*) experience large changes in body fat content and prolonged fasting as part of their normal life-history. However, suckling grey seal pups appear to be more susceptible to infection than adults and pups after weaning. Natural variation in expression of HSPs and REs in immune cells, which mediate immune function and protect against proteotoxic and oxidative stress, have not been investigated in the context of development or lactation in grey seals. Here, I used qRT-PCR to examine relative gene expression of *Hsp70*, *Hsp90*, *SOD*, *GST* and *Nox4* in breeding adult females and *SOD*, *GST* and *Nox4* in pups to establish 1. If females show a reduction in investment in leukocyte cellular defences or RE during lactation; 2. Whether pups show developmental or nutritional changes in leukocyte cellular defences or RE and 3. Whether leukocyte cellular defences or RE are influenced by body condition or circulating metabolites. I performed total and differential WBC counts to determine whether changes in cell number or composition occurred in mothers or pups. Finally, I used MDA as an index of leukocyte oxidative stress. There was no change in HSP or RE expression during the lactation-fast in adult females. However, axial girth had a positive effect on *SOD*, suggesting that females in better condition can invest more in key cellular defences or in the ability of immune cells to perform respiratory burst. Leukocyte number or composition did not change and there was no change in cellular stress, indicated by MDA concentration,

during lactation. *Nox4* was positively related to MDA concentration. As in Chapter 3, it would be useful to compare these findings with pre-breeding females. Pups increased *GST* mRNA abundance 1.26-1.41 fold and *Nox4* 4.95 fold during the post weaning fast and there was no effect of mass, axial girth or sex on mRNA abundance on any of the genes of interest in this age group. *GST* is involved in cell cycle control and increases in expression may result from cell maturation. Alternatively, since POPs enter circulation from blubber during fasting, the role of *GST* in detoxification may be important in fasting pups. Altered cell defences are not able to help explain increased infection rate in suckling pups.

5.1 Introduction

The immune system is vital for health and underpins survival and fitness (Sheldon and Verhulst, 1996; Owens and Wilson, 1999). It provides essential protection for animals by cellular and molecular mechanisms that identify and protect native cells and substances, while simultaneously removing or inactivating foreign organisms and their products (Abbas and Janeway, 2000).

Immune responses are either innate or acquired (Andersen *et al.*, 2006). Cells involved in innate or 'natural' immune responses provide general defence against different pathogens. Previous exposure is not required to activate these rapid responses. Innate immunity includes activation (i.e. increased cell proliferation and clustering) of: phagocytes, such as neutrophils and macrophages, which mediate inflammation and engulf and kill pathogens via cytotoxic mechanisms, that include the production of RS (Mayadas *et al.*, 2014); eosinophils, which are involved in allergic inflammation and defence against parasitic helminths (Weltman, 2000; Muniz

et al., 2012); and monocytes, which play important roles in inflammation and tissue repair (Boyette *et al.*, 2017), are also part of innate immunity.

Acquired or 'adaptive' immunity is mediated by three different types of lymphocytes, T-helper cells, T-cytotoxic cells and B-cells, which exhibit remarkable specificity in terms of antigen-specific responses (Lanzavecchia, 1985; Altman *et al.*, 1996; McHeyzer-Williams and McHeyzer-Williams, 2005; Moody and Haynes, 2008; Tangye and Tarlinton, 2009). Lymphocytes have cell-surface receptor sites that recognise and bind to antigen present on an invading pathogen. The receptors are highly specific and thus respond to only one specific antigen. Mature T- lymphocytes are identified by the expression of cell-surface glycoproteins CD4 or CD8 (Swain, 1983; Gallagher *et al.*, 1989; Koretzky, 2010). CD4+ T-helper cells produce cytokines that direct and amplify other elements of the immune response (Luckheeram *et al.*, 2012); CD8+ T-cytotoxic cells monitor all cell types, and lyse infected and compromised cells (Andersen *et al.*, 2006). They can kill both cancerous and virally infected cells (Zweerink *et al.*, 1977; Sobao *et al.*, 2001; Andersen *et al.*, 2006; Martínez-Lostao *et al.*, 2015). CD8+ cells recognise melanoma-associated antigen presented by major histocompatibility complex (MHC) class I – derived molecules that bind to peptide fragments resulting from intracellular protein degradation, allowing T-cytotoxic cells to monitor antigenic changes within transforming/transformed cells (i.e. the conversion of normal cells to cancer cells; Castelli *et al.*, 2000). B cells produce antibodies, which neutralise bacterial toxins, inactivate viruses and stimulate antibody-antigen complex activated serum proteins and other white blood cells to attack pathogens (LeBien and Tedder, 2008). There is also evidence for monocytes playing roles in acquired immunity as antigen presenting cells (Jakubzick *et al.*, 2017).

Immune function is influenced by nutritional state (Chandra, 1996; Martí *et al.*, 2001; Schaible and Kaufmann, 2007). Negative effects of obesity, or excess adiposity, on immune function have been reported in humans, mice and rats (Flier *et al.*, 1998). Obesity is associated with increased levels of inflammation, including increased cytokine and chemokine production, and enhanced macrophage infiltration of adipose tissue (Weisberg *et al.*, 2003). Obese individuals show higher rates of infection and impaired wound healing with elevated neutrophil, monocyte and lymphocyte counts (Nieman *et al.*, 1999; Martí *et al.*, 2001). Furthermore, obese humans show reduced antibody production after vaccination (Weber *et al.*, 1986; Sheridan *et al.*, 2011).

Underweight or malnourished individuals, including humans, rats, mice and birds, can also experience diminished immune function and increased susceptibility to disease (Shears, 1991; Polack *et al.*, 1993; Calder and Jackson, 2000; Katona and Katona-Apte, 2008; Hegemann *et al.*, 2012). This includes: impaired phagocyte function, for example, bactericidal activity of peripheral blood phagocytes (both polymorphonuclear cells and monocytes) is compromised in humans, leading to reduced killing ability of common pathogens such as *E. coli* and *Staphylococcus aureus* (Douglas and Schopfer, 1974; Forte *et al.*, 1999). The phagocytic function of macrophages is also impaired by the reduced production of superoxide anion in malnourished mice (Redmond *et al.*, 1991). Antibody responses are impaired in malnourished individuals (Chandra, 1996; 1997). A reduced nasopharyngeal secretory immunoglobulin (Ig) A response to live measles and polio virus vaccinations in humans (Chandra, 1975) and decreased secretory Ig A concentrations in nasal secretions, saliva, duodenal fluid and tears have been reported in humans and mice (Sirisinha, 1974; Reddy *et al.*, 1976; Green and

Heyworth, 1980). The secretory antibody system (in particular Ig A) is vital to mucosal immunity (Sirisinha, 1974). Secretory Ig A production is also compromised in malnourished weanling mice (Ha and Woodward, 1997). Deficiencies in cell-mediated immunity are also a result of being underweight or malnourished (Chandra, 1996; 1997). There is strong evidence that suggests progression and final outcome of infections such as pneumonia, tuberculosis, measles and bacterial and viral diarrhoea, are affected adversely by malnourishment in humans (Chandra, 1996; Ikeogu *et al.*, 1997; Mondal *et al.*, 2009; Olofin *et al.*, 2013). Under-weight status is also associated with higher risk of recurrence and death in humans with breast or ovarian cancer (Moon *et al.*, 2009; Kim *et al.*, 2014). Low body weight may result in insufficient energy stores to meet the metabolic demands of immune responses to pathogens (Matarese and La Cava, 2004; Ritz and Gardner, 2006). Additionally, infections may alter nutritional status by inducing anorexia (i.e. loss of appetite leading to reduced food intake) which can contribute to a malnourished state (Hart, 1988). Therefore, in young animals infections can impair growth and development (Stoll *et al.*, 2004).

The effect of fasting and fast duration on immune function is less clear. Fasts of between 2-7 days suppress the immune systems of rodents and cats (Lord *et al.*, 1998; Freitag *et al.*, 2000; Xu and Wang, 2010). However, in healthy, normal weight humans, fasts of up to 10 days do not decrease immune function, in terms of humoral and cell-mediated immunity (Holm and Palmbald, 1976; Palmblad *et al.*, 1977; Neuvonen and Salo, 1984). Cheng *et al.* (2014) reported that, even after WBCs are dramatically reduced or damaged by chemotoxicity in mice, prolonged fasts of two days can reverse immunosuppression. Normal WBC numbers were restored and stem-cell-based regeneration of the hematopoietic system was

promoted by reduced circulating insulin-like growth factor 1 (IGF-1) and protein

kinase A (PKA) regulated self-renewal pathways. This suggests that fasting, at least

in the short -term in non-fast adapted species, can have beneficial effects on

immune function when the system is challenged by cytotoxic stressors. Common

eiders (*Somateria mollissima*) are a fast-adapted species that undergo an

incubation-fast, which is an energetically limited period that lasts between 24-26

days. Acquired immunity (T-cell-mediated response and the humoral immune

response) is suppressed (Bourgeon *et al.*, 2006a; 2006b; 2007), though the

components of the innate immune response (phagocytic function i.e. ability of

phagocytes to destroy pathogens by production of nitric oxide (NO)) are unchanged

during this time (Bourgeon *et al.*, 2006a; 2006b; 2007). In these examples, fasting

modulates immune function and is not always detrimental.

Wing *et al.* (1983) demonstrated that obese human subjects undertaking a

long-term fast have increased monocyte bactericidal activity, natural killer cell

(subpopulation of lymphocytes that exhibit spontaneous cytotoxicity against virus-

infected cells, amongst others) cytolytic activity and serum concentrations of

antibodies (IgG, IgA and IgM). Peripheral blood leukocyte counts (e.g. neutrophils T

and B-cells) did not change, whereas mitogen-induced transformation of peripheral

blood lymphocytes was decreased. When compared to the healthy human-subject

studies above, this highlights the pathological consequences of obesity and the

importance of nutritional state (i.e. immune function is compromised by obesity but

can be rescued by fasting) and its implications for immunity.

Increased adiposity (obesity) and fasting are physiologically stressful for most

animals. Additional negative impacts on immune function may result in reduced

fitness and/or survival. Obese or fasting animals may be more susceptible to

additional stressors or challenges such as toxicological or oxidative stress, which have also been shown to negatively affect immunity. Immunity may also share finite resources with other physiological functions such as reproduction or developmental processes, which involves trade-offs and may impact on health and survival during key life history stages. Numerous elements of the immune system require energy, including protein production (e.g. inflammatory cytokines), tissue repair, respiratory burst and generation of additional immune cells during B-cell production after antigen presentation (Buttgereit *et al.*, 2000).

As described previously, HSPs and REs provide vital protection for macromolecules against proteotoxic and oxidative stress. HSPs, as numerous and highly conserved molecular chaperones, have a variety of functions due to their ability to interact and form complexes with other molecules. HSPs play a variety of important roles in innate and acquired immune responses (Colaco *et al.*, 2013) and have been identified as key immunogens (i.e. molecules that stimulate immune responses) in the response against pathogens (Suzue *et al.*, 1997; Srivastava, 2002b; Wallin *et al.*, 2002; Osterloh and Breloer, 2008). HSP70 can stimulate human monocytes and macrophages to secrete cytokines, including IL-6, IL-10, IL-12 and TNF-α (Ohashi *et al.*, 2000; Vabulas *et al.*, 2001). HSPs also play a role in the maturation of dendritic cells (DCs; specialised antigen presenting cells derived from monocyte precursors that activate and induce differentiation of naïve T-lymphocytes; Simones and Moser, 2010). HSPs bind to immature DCs and upregulate of a number of receptors including CD40, CD83, CD86 and CD91 which are markers of DC maturation (Basu *et al,*. 2000; 2001; Kuppner *et al.*, 2001; Calderwood *et al.*, 2007b; Binder, 2009; Colaco *et al.*, 2013). Gp96 (a member of the HSP90 family, expressed exclusively in the endoplasmic reticulum) is involved in the

chaperoning, and in turn proper expression, of toll-like receptors (TLRs) which crucially sense and recognise microorganisms in antigen presenting cells (Liu *et al.*, 2010). Staron *et al.* (2010) showed mice with reduced gp96 expression are severely susceptible to bacterial infection due to compromised T- and B- cell development. This suggests gp96 can control immune responses to pathogens by controlling TLR binding (Binder, 2014). Binder (2014) considered HSPs, along with MHC, to be one of the major peptide binding families in immunology. HSP70 and HSP90 play vital roles in antigen presentation and cross-presentation. They bind and chaperone antigenic peptides to MHC class I molecules on the cell surface, where they can be presented to lymphocytes (Srivastava *et al.*, 1994; Tsan and Gao, 2009; Binder, 2014).

HSPs are also associated with numerous pathologies in which increased levels of chaperones occur in immune cells. In humans, sepsis significantly increased protein expression of HSP27, HSP70 and HSP90 in activated polymorphonuclear leukocytes (PMNLs) compared to healthy individuals (Hashiguchi *et al.*, 2001). The same study also reported increases in oxidative activity, specifically in PMNLs, along with enhanced HSP70 expression response to sepsis and inhibition of apoptosis. This suggests that HSPs may not only regulate PMNL function in some disease states, but HSP expression in immune cells could be used as an indicator of immune system activation.

REs also play an important role in immunity, protecting cells from RS generated physiologically (i.e. phagocytes such as neutrophils and macrophages) to destroy pathogens (these can be toxic to cells surrounding the activated phagocyte and the phagocyte itself), whilst preventing oxidative stress, which is associated with a variety of pathologies, such as hypertension, sepsis, cancer, atherosclerosis and

diabetes (Mühl *et al.*, 2011; Cheng *et al.*, 2013). RS can impair T-cell responses and decrease T-cell proliferation as a result of increased H_2O_2 production during chronic inflammatory conditions, such as rheumatoid arthritis (Otsuji *et al.*, 1996; Cemerski *et al.*, 2002). Oxidative stress can further impair immune cell function by inducing hypo-responsiveness in T-cells (Cemerski *et al.*, 2003).

RS affect protein structure, and cells exposed to moderate levels of H_2O_2 increase proteolysis rate (Grune *et al.*, 1995; 1996). Hypo-responsive T-cells are correlated with RS-induced structural protein alterations. Molecular chaperones, such as HSPs, may help protect protein structure against oxidative damage and maintain cell function during immune responses. Once activated, other immune cells, such as lymphocytes, are highly susceptible to oxidative-stress-induced cell death (Degasperi *et al.*, 2008). Therefore, when elements of the immune system are active, additional oxidative stressors (e.g. RS producing chemicals) that further challenge immune cells may lead to cell death and immune system dysfunction.

REs themselves play important intracellular roles in immune cell function and protection from RS. In humans and rats, mutations in genes encoding subunits of Nox enzymes result in 'chronic granulomatous disease' (CGD). Mutations associated with CGD impact phagocyte Nox's ability to generate antimicrobial RS (i.e. phagocytes are unable to destroy some pathogens) leading to increased susceptibility to acute bacterial and fungal infections (Heyworth *et al.*, 2003). In the past, CGD was originally associated with GPx deficiency; for example, neutrophils of selenium deficient rats that were also GPx deficient presented defective microbicial activity and were more susceptible to chronic infection (Serfass and Ganther, 1975). Although no longer considered to be a driver of CGD phenotype (Newburger *et al.*, 1994), in some animal models (rats and humans) *in vivo* GPx deficiency still affects

phagocyte function (McCallister *et al.*, 1980; Schulman *et al.*, 1980; Baker and Cohen, 1983; 1984; Cheng *et al.*, 2013) highlighting the importance of GPx activity in immunity.

CAT and SOD have been also implicated in the protection of leukocytes from phagocytic activity (Salin and McCord, 1975) and variation in these REs has been observed during a number of pathological states (Mühl *et al.*, 2011). Both Mn-SOD and Cu, Zn-SOD activities were reduced in neutrophils and lymphocytes of diabetic humans compared to healthy controls, suggesting that diabetics may be more vulnerable to infection and immune system dysfunction (Uchimura *et al.*, 1999). Lower SOD activity in polymorphonuclear leukocytes (PMNs) compared to healthy control subjects has been associated with inflammatory conditions such as acne, which is induced by *Propionibacterium acnes* bacterium (Kurutas *et al.*, 2005).

It has been proposed that high levels of circulating glucose (i.e. during a diabetic state) can increase the virulence of certain pathogens and the ability of microorganisms to adhere to diabetic cells (Geerlings and Hoepelman, 1999). As glucose is also an important metabolic fuel for immune cells and can be pro-oxidative, it may have a special involvement in alterations to immune function (e.g. inflammation) in obese and diabetic humans (Wolowczuk *et al.*, 2008).

Grey seals experience dramatic changes in nutritional state during development and lactation, both of which are physiologically challenging life-history states associated with high metabolic costs (costs are lower in pups compared to females; Reilly, 1991; Iverson *et al.*, 1993). Breeding adult females fast during the ~18-21 day lactation period (Fedak and Anderson, 1982), lose approximately 40% of their initial mass (Pomeroy *et al.*, 1999) and 61%-84% of their fat reverses (Fedak

and Anderson, 1982; Baker *et al.*, 1995). During development pups triple in body mass while suckling, gaining up to 2.5 kg day^{-1} and rapidly accumulate fat in the form of subcutaneous blubber. After weaning, they then enter a post weaning fast of 10 days to >4 weeks. Fasting grey seals and suckling pups generally have high levels of circulating glucose relative to terrestrial mammals of similar sizes (Schweigert, 1993; Bennett *et al.*, 2013; Houser *et al.*, 2012; 2013; Schermerhorn, 2013).

Pre-weaned grey seal pups are more susceptible to infection than adults (Baker, 1984; Carter *et al.*, 1990; King *et al.*, 1994). Indeed, Baker (1984) and Carter *et al.* (1990) considered pups to be immunodeficient compared to adults, with increased occurrences of opportunistic infections seen in pups. Adult seals were also identified as carriers of some bacterial pathogens (the commonest being *Streptococci* and *Corynebacterium* of various species) that went on to infect pups on UK breeding colonies (Baker, 1984). Levels of antibodies (crucial markers of immune function), in particular immunoglobulin G (IgG), were also low in pups compared to adults (Carter *et al.*, 1990). However, concentrations of IgG were also low in lactating females compared to adult males and sub-adults, potentially as a result of immunosuppression associated with pregnancy (King *et al.*, 1994). Hall *et al.* (1998) determined total and differential WBC counts in grey seal pups of various age categories from the same population of animals considered here. There was no difference in total WBC counts between suckling and fasting pups and percentage differential counts were relatively constant across the nutritional states. Neutrophils made up the largest proportion of the leukocytes population (approximately 60%), followed by lymphocytes (approximately 30%), with monocytes (approximately 5%) and eosinophils (approximately 2%) making up the smallest proportions of WBCs.

There is limited information regarding WBC counts in adult female grey seals. Lehnert *et al.* (2014) reported total WBC counts in five captive adult grey seals (with a median weight of 123kg; range 104 – 154kg) in May and October of the same year. Total WBC counts ranged from 6.7 – 9.8 x 10^3 cells per mm^3 and 6.4 – 7.3 x 10^3 cells per mm^3 in May and October, respectively. Differential cell counts were not taken. Cook and McNamara (1988) determined total and differential WBC counts in one immunologically-compromised nine year old free-ranging female grey seal where a diagnosis of dystocia (obstructed labour) of a macerated foetus was made. Treatment involved manual extraction of the foetus followed by immediate and 72 hourly uterine flushes with sterile saline and 600mg gentamicin sulphate, and dinoprost tromethamine and trimethoprim-sulfaclozine (broad-spectrum antibiotic; 24%; 34mg per kg body weight) administered intramuscularly once daily for 10 days. After first treatment: total WBC count, 4950 cells; differential count, 445 monocytes, 842 lymphocytes, 1336 segmented neutrophils and 2327 banded neutrophils. 72 hours after first treatment: total WBC count 14,700 cells; differential count, 2499 monocytes, 1470 lymphocytes, 10,731 neutrophils. Despite treatment the female died 11 days post observation of dystocia and removal of the macerated foetus. Cause of death was determined to be an overwhelming infection leading to necrotising vaginitis extending into peripelvic tissues. The major pathogen was identified as *Clostridium perfringens*.

In pinnipeds, little has been done to investigate natural variation in gene expression of HSPs and REs in leukocytes, in relation to development and nutritional state, which may modulate their immune function and influence disease susceptibility. Previous studies have considered *Hsp70* gene expression in whole blood. However, the examination of a broader suite of HSPs was not undertaken and

REs were not considered. Fonfara *et al.* (2008) examined *Hsp70* mRNA expression in white blood cells, along with a suite of cytokines, in rehabilitated harbour seal pups (*Phoca vitulina*). Expression was compared between admission to the rescue centre and before release without consistent patterns. *Hsp70* expression was positively related to the condition of the animals when they were admitted to the rescue centre for rehabilitation, suggesting animals in better condition have more robust cellular defences in their WBCs. Cytokine expression was reduced prior to release. Lehnert *et al.* (2014) followed a similar experimental design to Fonfara *et al.* (2008), and examined *Hsp70* gene expression in whole blood of rehabilitated grey seal pups and rescue centre-resident adults and pups born in captivity. *Hsp70* expression was higher at admission than before release in pups, and captive adults had higher *Hsp70* expression than captive-born pups. Understanding variation in expression of HSPs and REs in wild phocids during natural physiological stressors will provide baseline data and offer a greater insight and ability to interpret data on response to additional stressors and pathogens.

Here, I aim to 1) determine the relative gene expression of HSPs and REs in circulating blood leukocyte populations of breeding adult females and in suckling and fasting pups; 2) ascertain whether body mass and axial girth affect gene expression of HSPs and REs, because body condition may influence immune function in healthy wild animals; 3) ascertain whether circulating glucose can explain variation in the expression of HSPs and REs, because high glucose levels can negatively impact immune cells and increase susceptibility to infection, which may stem from altered expression of HSPs and REs; 4) ascertain whether blubber MDA concentration, used as a marker of oxidative stress and tissue damage, can explain variation in the expression of HSPs and REs 5) determine whether animals with higher investment

in HSPs and REs have lower rates of mass gain and higher rates of mass loss 6)
perform total and differential WBC counts to identify white blood cell populations in
breeding females and pups, and ascertain if any changes in HSP or RE expression
are a result of altered WBC population. Changes in WBC counts during different
nutritional states and development may detect if/when the immune system is
activated, providing information regarding immune status.

5.2 Materials and Methods

5.2.1 Field and laboratory techniques

Plasma drawn into ethylenediaminetetraacetic acid (EDTA) coated
vacutainers from the epidural sinus were obtained from adult female grey seals
during early and late lactation, and pups during early and late suckling and early and
late in the post-weaning fast in 2013, using methods described in detail previously
(Chapter 2; General Methods; sections 2.1.2.4 and 2.1.2.5). Whole blood was taken
for blood smears and differential cell counts (see section 5.2.1.2 below), before
vacutainers were centrifuged. After centrifugation at 2000g, buffy coats were
removed and red blood cells (RBCs) disrupted using a potassium-based lysis buffer
(for more details see Chapter 2; General Methods; section 2.1.2.7.2). WBCs were
washed in fresh lysis buffer, pelleted in a microcentrifuge at 2000g and flash frozen
in liquid nitrogen. RNA was extracted from WBC pellets and 500ng was reverse
transcribed (using a gDNA elimination step) as described in Chapter 2; General
Methods; sections 2.2.4, 2.2.5. WBC RNA samples were run on a 2100 Bioanalyzer
System using a Agilent RNA 6000 Nano Kit and a Eukaryote Total RNA Nano assay
(Agilent Technologies, California, U.S.A). The Bioanalyzer is a gold standard of

nucleic acid analysis (including integrity and quantification) and provides

electropherogram and gel image outputs to determine RNA integrity.

qRT-PCR experiments were performed using gene specific primers (female

samples: *Hsp70*, *Hsc70*, *Hsp90*, *Hsp40*, *Hsp27* and *GPx*, *CAT*, *SOD*, *GST*, *Nox4*;

pup samples: *GPx*, *CAT*, *SOD*, *GST*, *Nox4* only) including two reference genes,

previously selected using *NormFinder* and *BestKeeper* softwares (see Chapter 2;

General Methods; sections 2.2.2 and 2.2.8). Primer efficiencies (i.e. efficiency of

amplification) were calculated using log serial dilutions of pooled cDNA template

(Chapter 2; General Methods; sections 2.2.7; equation 1). Raw C_T values generated

by qRT-PCR reactions were inspected using QuantStudio™ 12K Flex Software

version 1.1.1 (Applied Biosystems). Additional quality control checks were performed

on all experimental data, including examination of melt curves for single peaks,

baselines for similarity for all genes across all plates and threshold values (Chapter

2; General Methods; section 2.2.8). For each gene the average threshold was

calculated and each threshold was then set to this value to allow comparability

across all plates (i.e. each threshold for each gene was the same across all plates,

where the samples were directly compared as part of the same experiment).

To allow for comparison between conditions (i.e. EARLY and LATE lactation)

the reference genes were the same in each condition. Lactating female ($n = 16$; two

animals, 72830 and 72448/9, were discounted due to poor RNA integrity) and pup

samples ($n = 18$) were run with the reference genes *L8* and *UXT*.

5.2.1.1 Differential WBC counts

Blood film smears were performed as described in Kerr (1989). Briefly, a drop

of whole blood was applied near the end of a clean, polished microscope slide and,

using another slide as a spreader, the drop of blood was manipulated until it adhered along the edge of the spreader in a straight line. The leading edge of the blood was spread down the slide in a continuous movement to produce a thin monolayer of cells. Slides were air dried for 24 hours and then stained with Leishman's stain (0.15g Leishman's Stain (eosin-methylene blue; Sigma-Aldrich, Dorset, UK) dissolved in 100mL methanol (HPLC grade, Fisher Scientific, Loughborough, UK) at 56°C) as follows: slides in a glass slide holder were submerged in neat Leishman's stain for two minutes, then transferred to a 50:50 Leishman's stain: water solution for 10 minutes. Slides were rinsed thoroughly in distilled water for approximately one minute, drained, blotted and air dried before differential counts were performed.

The slide was placed under oil immersion using a light microscope and 200 cells were classified into four categories based on appearance and staining characteristics: neutrophils, eosinophils, monocytes and lymphocytes. Double counting was prevented by moving the slide in a systematic way: from left to right, down to the lower edge of the smear and across for two fields of view, then up to the upper edge of the smear. This was repeated until 200 cells had been counted. Seven female EARLY and LATE lactation and seven pup EARLY and LATE suckling and EARLY and LATE POST-WEANING fast differential counts were performed.

The number of each cell type per mL of blood was then calculated from overall WBC number (equation 2):

$$\text{Number of cell type} = \frac{\text{cell type count}}{200} \times \text{WBC number (2)}$$

5.2.1.2 Malondialdehyde (MDA) concentrations

Lipid peroxidation (LP) was determined in lactating females and suckling and fasting pups by measuring MDA concentrations in blubber tissue. The colorimetric product formed during the reaction of MDA with thiobarbituric acid (TBA) was measured, as described in Chapter 2; General Methods; section 2.2.10. A standard curve was constructed from the absorbance values of known standards and the resulting equation was used to calculate MDA concentrations (nmol/µL) of unknown samples.

5.2.1.3 Circulating glucose concentrations

Glucose was measured in breeding female plasma using glucose oxidase and the subsequent reaction of hydrogen peroxide with phenol and 4-aminophenazone. The red-violet quinoneimine dye formed acts as an indicator. Standards and samples were read in duplicate on the RX Monza Clinical Chemistry Analyser (Randox Glucose GOD-PAP GL 364; Randox Biosciences, County Antrim, UK) as described in Chapter 2; General Methods; section 2.2.9.2. Glucose was measured in pup deproteinised plasma using glucose oxidase (Bennett *et al.*, 2017) as described in General Methods, Section 2.2.9.1. Visibly haemolysed samples were not analysed.

5.2.2 Statistical Analysis

For detailed information regarding statistical analysis, refer to Chapter 3, section 3.2.3 and Chapter 4, section 4.2.3. Statistical analyses were performed in R software (R Version 2.15.2; Ihaka and Gentleman, 1996; R Core Development Team, 2012). Linear mixed effects (LME) models were used to investigate changes in Delta C_T for each GOI from EARLY to LATE lactation/suckling and EARLY to

LATE in the POST-WEANING fast. EARLY lactation/suckling was used as the control condition to calculate delta C_T, relative gene expression and when fitting the intercept in statistical models. The same model was run with the different timepoints captured in the intercept (LATE suckling and EARLY to LATE in the POST-WEANING fast) to explore differences within the post-weaning fast and between LATE suckling and the post-weaning fast time-points. Models were fitted by maximum likelihood using the lme function, part of the nlme library in R. Individual was included as the random effect. Fixed effects for females included state of lactation, body mass, daily rate of mass loss, axial girth, circulating glucose and MDA concentration. For pups, fixed effects included: developmental state (either feeding or fasting), body mass, daily rate of mass gain/loss, axial girth, sex, circulating glucose and MDA concentration. Forward stepwise regression was used to find the most suitable model. Residuals were plotted against fitted values and assessed for structure or pattern. Marginal and conditional R^2 values were calculated for each of the model fits using the sem.model.fits function, part of the piecewiseSEM library, in R (Nakagawa and Schielzeth, 2013; Johnson, 2014).

A separate model was fitted for each gene because no *a priori* assumptions were made that the genes would respond in a similar way to each other. I used a sequential Bonferroni method, ranking *p*-values by significance, to adjust for multiple comparisons. Paired t-tests were used to examine differences in total and differential WBC counts for breeding females; LMEs were used to explore differences in total and differential WBC counts between suckling and fasting pups.

5.3 Results

5.3.1 Breeding females: HSP and RE gene expression

There was no change in relative expression of HSPs or REs during the lactation-fast. The increase in log *SOD* expression approached significance (Table 5.1). Individual improved the model fit and explained a large portion of the variance for HSPs and REs (Table 5.1; 5.2).

Table 5.1. Model output for linear mixed effect models (LMEs) that explain variability in normalised gene expression (ΔC_T) of HSPs (heat shock proteins), where LATE lactation is compared to EARLY lactation (intercept); measured in lactating wild grey seal females in 2013. Significant difference where $p < 0.05$.

Gene	Marginal R^2	Conditional R^2	*n* observations	*n* individuals	*T*	*p*
Log *Hsp70*	3.93×10^{-6}	0.65	32	16	-0.018	0.99
Log *Hsp90*	0.00026	0.66	32	16	0.15	0.88

Table 5.2. Model output for linear mixed effect models (LMEs) that explain variability in normalised gene expression (ΔC_T) of redox enzymes (superoxide dismutase, *SOD*; glutathione-S-transferase, *GST*; and NADPH oxidase 4, *Nox 4*), where LATE lactation is compared to EARLY lactation (intercept); measured in lactating wild grey seal females in 2013. Significant difference where $p < 0.05$.

Gene	Marginal R^2	Conditional R^2	*n* observations	*n* individuals	*T*	*p*
Log *SOD*	0.048	0.65	32	16	1.99	**0.065**
GST	0.0013	0.35	32	16	0.24	0.81
Log *Nox4*	0.0040	0.32	32	16	0.42	0.68

The models that best described variation in log *Hsp70* and log *Hsp90* relative expression included circulating glucose. However, the positive relationship between log *Hsp70* expression and circulating glucose and the negative relationship between log *Hsp90* expression and circulating glucose were not significant (LMEs: log *Hsp70*, marginal R^2 = 0.032, Conditional R^2 = 0.75, *n* (observations) = 26, *n* (animals) = 13, df = 12, *T* = 1.52, *p* = 0.16; log *Hsp90*, marginal R^2 = 0.0040, Conditional R^2 = 0.71, *n* (observations) = 26, *n* (animals) = 13, df = 12, *T* = 0.50, *p* = 0.62). The inclusion of nutritional state (EARLY or LATE lactation), body mass, axial girth and blubber MDA concentration did not improve model fit (Table 5.3).

The model that best described variation in log *SOD* relative expression included nutritional state (EARLY or LATE lactation) and an additive effect of axial girth. Larger axial girth at both time-points (though females EARLY in the lactation-fast have significantly higher axial girths than LATE lactation) resulted in higher *SOD* relative gene expression (Table 5.3). The model that best described variation in *GST* mRNA abundance included circulating glucose, though the positive relationship was not significant (LME: marginal R^2 = 0.041, Conditional R^2 = 0.46, *n* (observations) = 26, *n* (animals) = 13, df = 12, *T* = -1.22, *p* = 0.25). Log *Nox4* mRNA abundance variation was best described by including blubber MDA concentration. There was a significant positive relationship between log *Nox4* mRNA abundance and blubber MDA concentration (LME: marginal R^2 = 0.12, Conditional R^2 = 0.79, *n* (observations) = 24, *n* (animals) = 12, df = 11, *T* = 3.21, *p* = 0.0083; Table 5.3). Body mass or axial girth did not improve *GST* or log *Nox4* models' fit to the data (Table 5.3).

Table 5.3. Additional explanatory variables included as covariates in LMEs to explain variability in the normalised gene expression of the HSPs and REs. Each HSP and RE superoxide dismutase, *SOD*; glutathione-S-transferase, *GST*; and NADPH oxidase 4, *Nox 4*) was modelled separately. Models in which body mass, axial girth, circulating glucose or blubber MDA concentration was included were then tested against the simpler model form with no covariates using the anova function. Significant difference where $p < 0.05$.

Gene	Model	Marginal R^2	Conditional R^2	L ratio	p
Log *Hsp70*	Circulating glucose (mM) + Axial girth (cm)	0.043	0.79	2.60	0.11
	Circulating glucose (mM) + Body mass (kg)	0.035	0.77	0.28	0.59
	Circulating glucose (mM) + MDA concentration (nmol/μL)	0.098	0.73	0.52	0.47
Log *Hsp90*	Circulating glucose (mM) + Axial girth (cm)	0.0087	0.71	0.31	0.58
	Circulating glucose (mM) + Body mass (kg)	0.0040	0.71	0.0018	0.97
	Circulating glucose (mM) + MDA concentration (nmol/μL)	0.15	0.53	1.97	0.16
Log *SOD*	**Time-point + Axial girth (cm)**	**0.23**	**0.71**	**5.61**	**0.018**
	Time-point * Axial girth	0.22	0.74	1.33	0.25
	Time-point + Axial girth (cm) + Body mass (kg)	0.22	0.70	0.48	0.49
	Time-point + Axial girth (cm) + Circulating glucose (mM)	0.34	0.69	0.034	0.85
	Time-point + Axial girth (cm) + MDA concentration (nmol/μL)	0.25	0.81	0.29	0.59

GST	Circulating glucose (mM) + Axial girth (cm)	0.048	0.46	0.13	0.72
	Circulating glucose (mM) + Body mass (kg)	0.056	0.46	0.32	0.57
	Circulating glucose (mM) + MDA concentration (nmol/µL)	0.13	0.51	1.24	0.27
Log *Nox4*	MDA concentration (nmol/µL) + Body mass (kg)	0.12	0.80	0.15	0.70
	MDA concentration (nmol/µL) + Axial girth (cm)	0.12	0.80	0.26	0.61
	MDA concentration (nmol/µL) + Circulating glucose (mM)	0.24	0.78	0.79	0.37

5.3.2 Pups: RE gene expression

Log *GST* gene expression increased during the post weaning fast (Figure 5.1). Relative to EARLY suckling, there was a 1.26 fold increase EARLY in the POST- WEANING fast and a 1.41 fold increase LATE in the post weaning fast in *GST* expression. There was no change in log *GST* relative expression during suckling (Table 5.4) or the post weaning fast (LME: $T = 1.62$; $p = 0.11$). Nutritional state explained 20% of the variation in *GST* and an additional 45% was explained by individual.

Log *Nox4* expression increased 4.95 fold LATE in the POST-WEANING fast (Figure 5.1), but marginal and conditional R^2 values were low. The increase in log *Nox4* expression during suckling and EARLY in the POST-WEANING fast approached significance (Table 5.4). Increased log *SOD* relative expression during suckling and LATE in the POST-WEANING fast approached significance (Table 5.4). There was no change in log *Nox4* or log *SOD* relative expression during fasting

(LMEs: log *Nox4*; *T* = 1.37; *p* = 0.18; log *SOD*; *T* = 1.39; *p* = 0.17). Individual explained a larger portion of the variance in expression of each gene.

The model that best described variation in log *SOD*, log *GST* and log *Nox4* relative expression included nutritional state (EARLY or LATE suckling or EARLY or LATE in the POST-WEANING fast). Body mass, axial girth, sex, circulating glucose or blubber MDA concentration did not improve the models' fit to the data (Table 5.5). However, the relationship between log SOD relative expression and sex (higher expression in females compared to males) and the positive relationship between log GST relative expression and axial girth approached significance (Table 5.5).

Table 5.4. Model output for linear mixed effect models (LMEs) that explain variability in log normalised gene expression of redox enzymes (*SOD*; glutathione-S-transferase, *GST*; and NADPH oxidase 4, *Nox 4*), in which LATE suckling (L.S), EARLY POST-WEANING fast (EPWF) and LATE POST-WEANING fast (LPWF) are compared to EARLY suckling (intercept); measured in suckling and fasting wild grey seal pups in 2013. Bold font indicates a significant difference where *p* < 0.05. *n* (observations) = 69, *n* (individuals) = 18.

Gene	Marginal R^2	Conditional R^2	L.S *T*	L.S *P*	EPWF *T*	EPWF *P*	LPWF *T*	LPWF *p*
Log *SOD*	0.029	0.68	1.91	**0.062**	0.62	0.54	1.98	**0.053**
Log *GST*	0.20	0.65	0.35	0.73	3.60	**0.0007**	5.01	**<0.0001**
Log *Nox4*	0.13	0.22	1.90	**0.064**	1.85	**0.071**	3.18	**0.0026**
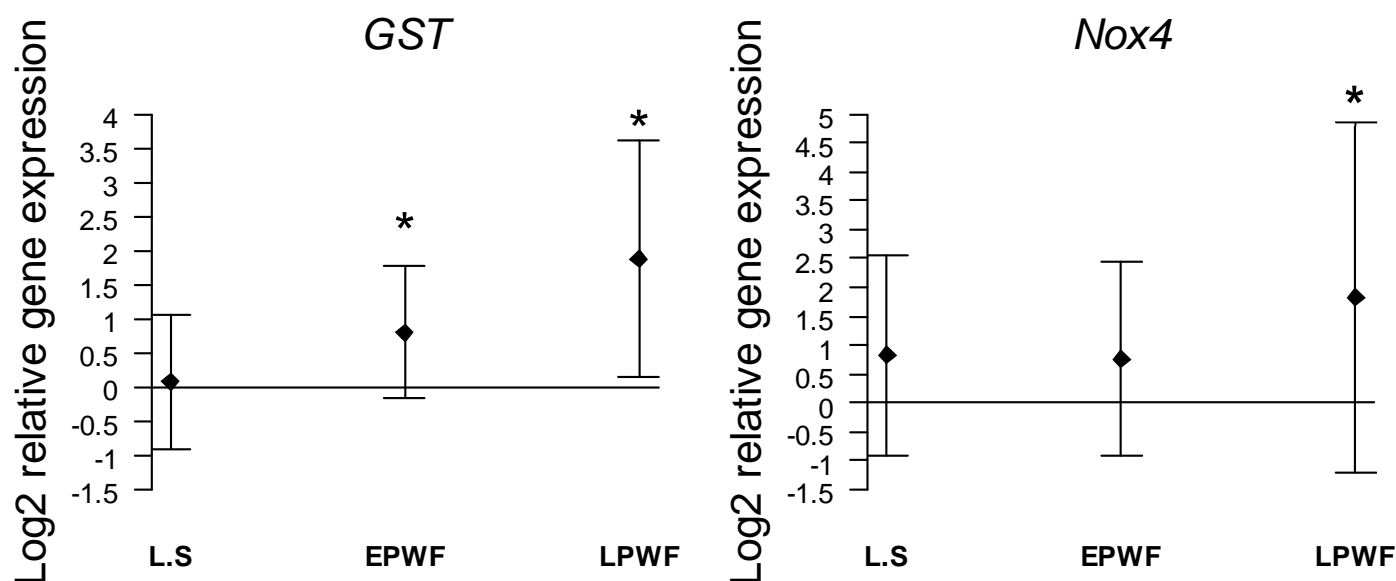
Figure 5.1. Mean (± SD) log2 relative gene expression for each redox enzyme (RE) gene measured, where LATE suckling (L.S), EARLY POST-WEANING fast (EPWF) and LATE POST-WEANING fast (LPWF) are relative to EARLY suckling. Glutathione-S-transferase, *GST*; NADPH oxidase 4, *Nox4*. The symbol "*" indicates a significant difference ($p < 0.05$) in expression between EARLY suckling and the EARLY and LATE post weaning fast.

Table 5.5. Additional explanatory variables included as covariates in LMEs to explain variability in the normalised gene expression of REs. Each RE was modelled separately. Models in which body mass, axial girth, sex, circulating glucose or blubber MDA concentration was included were then tested against the simpler model with no covariates using the anova function. Bold font indicates $p < 0.05$.

Gene	Model	Marginal R^2	Conditional R^2	L ratio	p
Log *SOD*	Time-point + Axial girth (cm)	0.037	0.67	0.52	0.47
	Time-point + Body mass (kg)	0.055	0.68	3.67	0.30
	Time-point + Sex	0.15	0.69	3.03	0.082

	Time-point + Circulating glucose (mM)	0.062	0.69	0.78	0.38
	Time-point + MDA concentration (nmol/µL)	0.012	0.68	0.27	0.60
Log *GST*	Time-point + Axial girth (cm)	0.23	0.70	3.66	0.056
	Time-point + Body mass (kg)	0.22	0.68	1.39	0.24
	Time-point + Sex	0.21	0.65	0.30	0.58
	Time-point + Circulating glucose (mM)	0.22	0.64	1.94	0.16
	Time-point + MDA concentration (nmol/µL)	0.22	0.68	1.36	0.24
Log *Nox4*	Time-point + Axial girth (cm)	0.13	0.22	0.16	0.69
	Time-point + Body mass (kg)	0.13	0.22	0.090	0.76
	Time-point + Sex	0.15	0.22	1.32	0.25
	Time-point + Circulating glucose (mM)	0.12	0.25	1.00	0.32
	Time-point + MDA concentration (nmol/µL)	0.15	0.19	0.056	0.81

5.3.3 Differential WBC counts

5.3.3.1 Breeding females

Range and mean ± SD (*n*) total and differential white blood cell counts are displayed in Table 5.6. There was no difference in mean total WBC count between early and late lactation (paired t-test; df = 6; T = -1.89; p = 0.11). Neutrophils were the most numerous cell type at both early and late lactation, making up between 69 – 74% of differential counts. During lactation, there was no change in neutrophil (paired t-test; df = 6; T = -1.87; p = 0.11), lymphocyte (paired t-test; df = 6; T = -1.15; p = 0.29), monocyte (paired t-test; df = 6; T = -1.12; p = 0.31) or eosinophil numbers (paired t-test; df = 6; T = 0.086; p = 0.93). Percentage differential counts were relatively constant across lactation, with the exception of eosinophils. Composition was: 13% lymphocytes and 6% monocytes. Eosinophil percentage differential counts ranged between 11% at early lactation to 6% at late lactation. This may have been due to a small sample size (*n* = 7).

5.3.3.2 Pups

Range and mean ± SD (*n*) total and differential white blood cell counts are displayed in Table 5.11. There was no difference in total WBC count between suckling and fasting (Table 5.6). There was no difference between early and late post weaning (Table 5.6). The total WBC counts were variable, potentially due to the small sample size (*n* = 7 (E.S, L.S, EPWF); *n* = 5 (LPWF)).

Table 5.6. Model output for linear mixed effects models (LMEs) that explain variability in total WBC counts ($x10^6$ L^{-1}) in which the intercept is varied to compare all nutritional states to one another. E.S, EARLY suckling; L.S, LATE suckling; EPWF, EARLY POST-WEANING fast; LPWF, LATE POST-WEANING fast; counts measured in suckling and fasting wild grey seal pups in 2013. n (observations) = 26, n (individuals) = 7.

Total WBC count ($x10^6$ L^{-1})	Marginal R^2	Conditional R^2	df	T	P
E.S vs L.S	0.20	0.20	16	1.27	0.22
EPWF vs LPWF	0.20	0.20	16	0.29	0.78
E.S vs EPWF	0.20	0.20	16	1.91	0.074
L.S vs EPWF	0.20	0.20	16	0.64	0.53
E.S vs LPWF	0.20	0.20	16	2.03	0.060
L.S vs LPWF	0.20	0.20	16	0.88	0.39

Neutrophils were the most numerous cell type at all time-points, making up approximately 64% of differential counts. There was no change in neutrophil numbers between suckling and fasting and no change across the post weaning fast (Table 5.7).

Table 5.7. Model output for linear mixed effects models (LMEs) that explain variability in neutrophil counts ($x10^6$ L^{-1}) in which the intercept is varied to compare all nutritional states to one another. E.S, EARLY suckling; L.S, LATE suckling; EPWF, EARLY POST-WEANING fast; LPWF, LATE POST-WEANING fast; counts measured in suckling and fasting wild grey seal pups in 2013. n (observations) = 26, n (individuals) = 7.

Neutrophil count ($x10^6$ L^{-1})	Marginal R^2	Conditional R^2	df	T	P
E.S vs L.S	0.17	0.17	16	1.24	0.23
EPWF vs LPWF	0.17	0.17	16	0.55	0.59
E.S vs EPWF	0.17	0.17	16	1.52	0.15
L.S vs EPWF	0.17	0.17	16	0.29	0.78
E.S vs LPWF	0.17	0.17	16	1.94	0.071
L.S vs LPWF	0.17	0.17	16	0.81	0.43

Table 5.8. Model output for linear mixed effects models (LMEs) that explain variability in eosinophil counts ($\times 10^6$ L^{-1}) in which the intercept is varied to compare all nutritional states to one another. E.S, EARLY suckling; L.S, LATE suckling; EPWF, EARLY POST-WEANING fast; LPWF, LATE POST-WEANING fast; counts measured in suckling and fasting wild grey seal pups in 2013. n (observations) = 26, n (individuals) = 7.

Eosinophil count ($\times 10^6$ L^{-1})	Marginal R^2	Conditional R^2	df	T	*P*
E.S vs L.S	0.10	0.10	16	-0.49	0.63
EPWF vs LPWF	0.10	0.10	16	-0.21	0.83
E.S vs EPWF	0.10	0.10	16	0.94	0.36
L.S vs EPWF	0.10	0.10	16	1.43	0.17
E.S vs LPWF	0.10	0.10	16	0.65	0.53
L.S vs LPWF	0.10	0.10	16	1.09	0.29

There was no difference in eosinophil numbers between suckling and fasting. With the exception of E.S, eosinophil percentage differential counts were reasonably constant across suckling and fasting, at approximately 5%; E.S was approximately 10% (Table 5.8).

Table 5.9. Model output for linear mixed effects models (LMEs) that explain variability in monocyte counts ($x10^6$ L^{-1}) in which the intercept is varied to compare all nutritional states to one another. E.S, EARLY suckling; L.S, LATE suckling; EPWF, EARLY POST-WEANING fast; LPWF, LATE POST-WEANING fast; counts measured in suckling and fasting wild grey seal pups in 2013. *n* (observations) = 26, *n* (individuals) = 7.

Monocyte count ($x10^6$ L^{-1})	Marginal R^2	Conditional R^2	df	T	*P*
E.S vs L.S	0.36	0.36	16	3.02	**0.0081**
EPWF vs LPWF	0.36	0.36	16	0.40	0.70
E.S vs EPWF	0.36	0.36	16	2.48	**0.025**
L.S vs EPWF	0.36	0.36	16	-0.54	0.59
E.S vs LPWF	0.36	0.36	16	2.66	**0.020**
L.S vs LPWF	0.36	0.36	16	-0.098	0.92

Monocyte numbers were lower at E.S than all other time-points. There was no change in monocyte numbers during the post weaning fast and no change in the transition from L.S to the post weaning fast. Monocytes were more variable during suckling, from 6-11%, though approximately 9% during fasting (Table 5.9).

Table 5.10. Model output for linear mixed effects models (LMEs) that explain variability in lymphocytes counts ($x10^6$ L^{-1}) in which the intercept is varied to compare all nutritional states to one another. E.S, EARLY suckling; L.S, LATE suckling; EPWF, EARLY POST-WEANING fast; LPWF, LATE POST-WEANING fast; counts measured in suckling and fasting wild grey seal pups in 2013. *n* (observations) = 26, *n* (individuals) = 7.

Lymphocyte count ($x10^6$ L^{-1})	Marginal R^2	Conditional R^2	df	T	*p*
E.S vs L.S	0.23	0.23	16	0.74	0.47
EPWF vs LPWF	0.23	0.23	16	-0.63	0.54
E.S vs EPWF	0.23	0.23	16	2.36	**0.032**
L.S vs EPWF	0.23	0.23	16	1.62	0.13
E.S vs LPWF	0.23	0.23	16	1.52	0.15
L.S vs LPWF	0.23	0.23	16	0.84	0.41

Lymphocyte numbers remained constant throughout suckling and fasting, with the exception of lower cell numbers at E.S compared to the EPWF. Lymphocytes were approximately 18% of all leucocytes during suckling and 20-26% during fasting (Table 5.10).

Table 5.11. Range and mean ± SD (*n*) total and differential white blood cell counts in lactating-fasting adult females (EARLY lactation (E.L), LATE lactation (L.L)) and suckling and post weaned (fasting) wild grey seal pups (EARLY suckling (E.S), LATE suckling (L.S), EARLY POST-WEANING fast (EPWF) and LATE POST-WEANING fast (LPWF)) in 2013.

	Cell type									
	Total WBC number		Neutrophils		Eosinophils		Monocytes		Lymphocytes	
	Range x10^6 L^{-1}	Mean ± SD (*n*) x10^6 L^{-1}	Range x10^6 L^{-1}	Mean ± SD (*n*) x10^6 L^{-1}	Range x10^6 L^{-1}	Mean ± SD (*n*) x10^6 L^{-1}	Range x10^6 L^{-1}	Mean ± SD (*n*) x10^6 L^{-1}	Rang e x10^6 L^{-1}	Mean ± SD (*n*) x10^6 L^{-1}
Females E.L	3.41 – 18.50	6.89 ± 5.09 (7)	2.80 – 14.90	6.30 ± 4.26 (7)	0.39 – 2.87	1.04 ± 0.86 (7)	0.085 – 2.22	0.57 ± 0.74 (7)	0.41 – 2.50	1.16 ± 0.70 (7)
Females L.L	6.05 – 25.70	12.73 ± 7.03 (7)	4.05 – 18.12	11.13 ± 5.16 (7)	0.43 – 1.96	1.01 ± 0.59 (7)	0.45 – 1.88	1.01 ± 0.52 (7)	0.74 – 5.01	1.91 ± 1.51 (7)
Pups E.S	2.53 – 22.20	6.45 ± 7.52 (7)	1.94 – 15.76	6.34 ± 5.46 (7)	0.18 – 1.67	0.73 ± 0.52 (7)	0.14 – 1.89	6.00 ± 0.62 (7)	0.25 – 3.90	1.92 ± 1.61 (7)
Pups L.S	5.17 - 21.70	12.75 ± 5.59 (7)	3.29 – 15.40	9.87 ± 4.26 (7)	0.39 – 0.89	0.60 ± 0.19 (7)	0.52 – 3.15	1.67 ± 0.84 (7)	1.00 – 4.63	2.79 ± 1.50 (7)
Pups EPWF	9.68 – 32.70	14.74 ± 9.02 (7)	6.24 – 21.90	10.68 ± 6.35 (7)	0.29 – 1.96	0.97 ± 0.58 (7)	0.87 – 2.30	1.47 ± 0.50 (7)	1.95 – 10.63	4.69 ± 3.04 (7)
Pups LPWF	6.16 – 24.50	16.48 ± 6.36 (7)	4.84 – 19.86	13.65 ± 5.34 (7)	0.40 – 2.35	1.15 ± 0.77 (7)	0.59 – 2.71	1.81 ± 0.72 (7)	1.33 – 6.63	4.15 ± 2.20 (7)

5.3.4 Grey seal WBC RNA integrity and qRT-PCR primer failure

Details of RNA integrity analysis, which revealed the presence of a distinct third band are provided in Appendix 3. Cloning of this band to attempt to identify it is also described in the appendix. Information regarding poor primer performance of *GPx*, *CAT*, *Hsc70*, *Hsp40* and *Hsp27*, hence why only data for select HSPs and REs are presented above are given in Appendix 3.

5.4 Discussion

The present study shows the profiles of some HSPs and REs in leukocytes of breeding female grey seals and pups, during the lactation-fast, suckling and the post-weaning fast.

5.4.1 HSP and RE gene expression in breeding female leukocytes

There was no change in expression of any HSPs (*Hsp70*, *Hsp90*) or REs (*SOD*, *GST*, *Nox4*) for females during the lactation-fast period. Total and differential WBC counts (i.e. cell types making up the WBC population) also remained constant. Individual explained most of the variance in gene expression, whilst fixed effects were of small importance.

There is no evidence here that females showed a reduction in investment in leukocyte cellular defences or REs during the lactation-fast. However, axial girth had a positive effect on *SOD*, suggesting that females in better condition can invest more in key cellular defences or in the ability of immune cells to perform respiratory burst. *SOD* provides protection from superoxide anion (for details see Chapter 1, section 1.6) generated by Noxs in mammalian phagocytes to destroy microorganisms. Superoxide anion generated in this way may also cause damage to surrounding

tissues or induce apoptosis in other immune cells (Dahlgren and Karlsson, 1999). Therefore, if females can invest in adequate protection of phagocytes via increased expression of *SOD*, they may be better able to perform respiratory burst, which is also an ATP-dependent process. Activation of the immune machinery is energetically costly due to the metabolic requirements of immune cells and other indirect consequences associated with immune up-regulation (Lochmiller and Deerenberg, 2000), for example anorexia during infection and inflammation and tissue degradation (Bonneaud *et al.*, 2003). Trade-offs between reproduction and immune responses may be detrimental to breeding female grey seals that are already energetically limited. However, we have no evidence that females were compromised here. In Chapter 3, I highlighted the importance of comparing life-history states; increases in oxidative markers would not have been identified using lactating-breeding females alone. Therefore, I would recommend carrying out the same protocol with pre-breeding female leukocytes as without the contrast we cannot make further conclusions regarding the sacrificial status of blubber or make comparisons with previous chapters.

There was no change in expression of HSPs during the lactation-fast. HSPs may be maintained throughout the lactation-fasting period to protect against intracellular proteotoxic stress from either natural or anthropogenic sources. During this time toxins, such as PCBs, enter circulation as they are liberated from the fat layer (Debier *et al.*, 2003a; Bourez *et al.*, 2012), which is reduced by catabolism to maintain metabolic fuel availability during lactation. As these toxins may compromise cellular function by damaging macromolecules, such as proteins, lipids and DNA, maintained HSP (and RE) expression throughout the lactation-fast would provide consistent protection for cells. In whole blood of three Antarctic seal species

(Weddell seals, *Leptonychotes weddellii*, Ross seals, *Ommatophoca rossii*, and Crabeater seals, *Lobodon carcinophaga*), pollutant load (mercury, Hg, measured in fur) was a potential driver of gene expression of *Hsp70* (though Hg had a negative relationship with Hsp70 expression) and immune system function markers (the cytokines *IL-2* and *IL- 10*; Lehnert *et al.,* 2017). As an alternative interpretation, females may avoid additional energetic costs of mounting cellular stress responses during fasting and therefore keep cellular defence gene expression low and stable.

Both the production and binding action of HSPs are ATP dependent (Mallouk *et al.*, 1999; Tomanek, 2010), which could add considerably to the energy demands of the cell, which already has a high energetic requirement from its role in immune function. It is essential to save energy at a time when there is a dramatically increased energy demand in the face of resource limitation, during which females lose approximately 40% of initial mass (Pomeroy *et al.*, 1999) and 61-84% of their fat reserves (Fedak and Anderson, 1982). The absence of elevated HSPs in this context may be as a result of grey seals being a fasting adapted species.

No change in total and differential WBC counts may indicate females are not experiencing challenges to the immune system. Increased numbers of leukocytes may suggest that animals are adequately fighting infection or could be indicative of latent infection. This work does not provide data regarding immune function (such as respiratory burst or phagocytosis) or data that allows us to make inferences regarding immune system activation (activation of immune cells) or immune status (for example, are animals immunologically compromised). Therefore it is more difficult to relate no-change in cellular defences to immunity and make comments about distinguishing between eustress and distress.

As demonstrated in Chapter 3, comparison of gene expression with pre-breeding females indicated increased mRNA abundance of some REs and HSPs in blubber tissue of breeding individuals. Therefore, it would be informative to compare HSP and RE gene expression reported here in leukocytes with that of pre-breeding females to determine importance of these genes in different tissues and life-history states. This would help us see if breeding females are keeping levels of REs and HSPs low or high. Such comparisons would allow the data presented here to be interpreted in a more meaningful way.

5.4.2 Increased mRNA abundance of GST and Nox4 during fasting in pups

Pups exhibited increased *GST* and *Nox4* expression during the post weaning fast and there was no change in total WBC counts between suckling and fasting, though differential WBC counts varied with lower numbers of lymphocytes at E. S compared to EPWF, and lower monocyte numbers at E.S compared to all other time-points. Interestingly, body mass, axial girth, circulating glucose or pup sex did not affect gene expression.

There was an increase in *GST* in pups during fasting. This may be attributed to the role of GSTs in conjugation reactions that detoxify harmful electrophilic compounds. During lactation, mothers transfer pollutants, such as PCBs, to their pups, via their high fat milk (Debier *et al.*, 2003a; 2003b). Pups sequester these into the fat droplets of their rapidly growing subcutaneous adipose tissue layer, which is required to sustain metabolism during the post weaning fast; if pups are beginning to catabolised their fat reserves, they may be mobilising PCBs (which may then be present in the circulation) and increasing the requirement for a detoxification enzyme

such as GST. GST has been shown to catalyse conjugation reactions involving PCBs in a number of tissues in rats and mice.

Alternatively, various classes of GSTs have been shown to play regulatory roles in the mitogen-activated protein (MAP) kinase pathway that participates in cellular survival and death signals (Laborde, 2010). Increased expression in fasting pups may be related to immune cell maturation.

GSTs from parasites including *Schistosoma mansoni* have been used as candidate antigens in vaccines to protect against these parasites and associated diseases, for example schistosomiasis (Riveau *et al.*, 1998). GSTs in such vaccines generate strong immune responses that limit the fecundity of these parasitic worms, even reducing parasite burden (Riveau *et al.*, 1998).

Lee *et al.* (2010) identified *Nox4* as a novel source of intracellular RS in human monocytes and mature macrophages. *Nox4* mediates oxidised low-density lipoprotein cell death in macrophages, which is linked to the pathology of atherosclerosis (Lee *et al.*, 2010).

Neonates are quickly colonised by surrounding microbiota after birth and it is believed this process stimulates post-natal immune development. The rapid acquisition of immunity in neonate mammals can be influenced by a number of factors, including environmental conditions and maternal microbial input (de Agüero *et al.*, 2016). Neonates are classed as having immature immune systems as, compared to adults, they have limited adaptive immunity, deficiencies in T- and B-cell activation and limited antibody production (Georgountzou and Papadopoulos, 2017)

In humans, the early weeks of life represent a period of large fluctuations in cell populations, cell functional capacity (including signalling and recognition

pathways). This is closely linked to surrounding environment, which can lead to varying geno- and phenotypes. Georgountzou and Papadopoulos (2017) described the developmental changes associated with individual components of innate immunity and found individual molecules, cells, or recognition or signalling pathways, within different compartments/anatomical sites (e.g. skin, neutrophils), demonstrate variable maturation patterns over the course of the human post-natal period. Neutrophils may increase massively in newborn humans, to levels more abundant than an adult, however the cells are functionally defective and do not offer the same level of protection (Carr, 2000). During post-natal development, young mammals undergo rapid and undulating changes to their immunity, including associated cells (Georgountzou and Papadopoulos, 2017). Therefore, it is possible that this induces cell-states that are not necessarily stressful but would impact cellular defences and potentially increase or decrease mRNA expression accordingly (i.e. rapid cell turnover and protein synthesis could lead to increased requirement for HSPs; greater quantities of ROS may be produced as phagocytes develop and are utilised as part of an immune response). Assessing trade-offs also becomes more important to ascertain whether pups are able to meet developmental and immune-related energetic costs. Much is changing as a result of development. Simply identifying if HSPs or REs are useful cellular defences in a developing immune system would be beneficial. To do this I would need to take measurements of immune cell activation and functional capacity to try and link cellular defences with immunity in grey seals. Although clearly identifying eustress and distress was not possible in this tissue type, mRNA abundance of REs in pups should therefore be interpreted with caution and consider the influences of major changes in immune genotype and phenotype.

Here, I have described mRNA abundance of cellular defences in leukocytes in both lactating-fasting females and suckling and fasting pups. Nothing explained the variation in gene expression, with the exception of SOD where larger axial girth of lactating-fasting females explained higher mRNA abundance. In keeping with other chapters, this supports SOD as a maker of body condition in grey seals and is a unifying concept between blubber and leukocytes. There is no obvious state of eustress or distress identified in leukocytes, but, for females at least, that may be due to a missing comparison with another life-history state. For pups this could be masked by development of immunity. Our cellular defence data does not help explain increased infection rate in suckling pups however that might be explained by investigating the development of adaptive immune responses, which are deficient early in life (Georgountzou and Papadopoulos, 2017), as WBC counts reported here do show any changes throughout suckling and fasting.

Chapter 6

HSP gene expression in blubber explants exposed to hydrocortisone (HC) and polychlorinated biphenyls (PCBs): optimising culture conditions for experimental exposures

Abstract

Experimental approaches are important to establish and characterise causal effects of altered environmental conditions and stress exposure on animal function, health and survival. Marine mammals are often protected species and considered important sentinels of ecosystem health, but are not easily accessible or tractable for whole-animal level experiments. *In vitro* experiments on cells or tissue are therefore important approaches that need to be developed for these species. Adipose tissue is essential for fitness and survival of marine mammals but *in vitro* methodologies to understand its physiological responses are not always well established for wildlife species, and adipose tissue/cells can be difficult to maintain. Optimising suitable culture conditions is essential to ensure adipose explants are maintained in a viable state for experimental exposures to be performed on healthy and representative tissue. Here, we cultured blubber explants from grey seals in a range of media over 24-36 h. We used RNA integrity as a proxy for viability and qPCR to investigate the relative gene expression of a range of HSPs to investigate cellular stress. Of the conditions tested, M199 media supplemented with 5% FBS and fatty acid supplement produced the best quality tissue. The time-course suggested that explants survived under those conditions for up to 33 hours. We then used explant culture to investigate absolute gene expression of *Hsp70* and *Hsc70* in response to intrinsic persistent organic pollutant (POP) load and experimental treatments of HC, PCBs and a combination of both HC and PCBs in inner and outer blubber section of suckling and fasting grey seal pups from the Isle of May in 2016. There was higher mRNA abundance of both HSPs in outer compared to inner blubber sections, which may be as a result of loss of lanugo and increased blood flow to the surface of the skin as the seal pups begin to moult late in the suckling period. Alternatively, outer

blubber at 37°C may have experienced greater thermal stress, since it may be maintained *in vivo* closer to lower ambient temperatures. Intrinsic dioxin-like PCB levels, accumulated during suckling, explained 31% of variation in gene expression of *Hsp70,* but not *Hsc70*. PCB and HC/PCB treatments were also associated with increased *Hsp70* transcript number. This chapter highlights the importance of adopting experimental approaches to supplement observational data in an effort to explore variables that induce cellular stress responses. Furthermore, I have identified the role of whole-animal stress and contaminants in inducing cellular stress and showed that *Hsp70*, not *Hsc70*, may be a useful marker to identify POP exposure.

6.1 Introduction

Knowledge of physiological responses to environmental challenges underpins our understanding of the consequences of natural and anthropogenic stressors at cellular, organismal and population levels. If we are to identify and understand causal effects of altered environmental conditions on animal function, health and survival, we need to use experimental approaches. However, experimental approaches are ethically and logistically challenging to implement in wild species at the organismal level (Bennett *et al.,* 2017). Experimental methods *in vitro*, such as tissue culture, allow us to infer causal relationships between physiological function and exposure to conditions that cannot be easily investigated *in vivo*, and allow us to tease apart individual and synergistic effects of altered conditions, such as effects of specific hormones or contaminants. However, *in vitro* methodologies are not always well established for wildlife species.

In wildlife, *in vitro* approaches using various cell types have been used, most commonly to determine the effects of persistent organic pollutants (POPs) on cell function. Hammond *et al.* (2005) maintained grey (*Halichoerus grypus*) and harbour seal (*Phoca vitulina*) peripheral blood polymorphonuclear cells (PMNCs) and mononuclear cells (PBMCs) in culture for up to 24 hours to determine the effects of polychlorinated biphenyls (PCBs) on immune cell function (i.e. degree of phagocytosis and change in respiratory burst). In another short-term experiment, Routti *et al.* (2016) maintained polar bear (*Ursus maritimus*) adipose tissue-derived stem cells for up to 14 days, to investigate the effect of environmental pollutants on cell differentiation. As a further example, Murk *et al.* (1994) examined the ability of harbour seal, harbour porpoise (*Phocoena phocoena*), common tern (*Sterna hirundo*; all environmentally exposed to PCBs) and eider duck (*Somateria mollissima*), rainbow trout (*Salmo gairdneri*) and flounder (*Platichthys flesus*; experimentally exposed to PCBs) liver microsomes to metabolise ^{14}C-labelled PCBs. In all species ethoxyresorufin-O-deethylase (EROD), a biomarker of PCB exposure, activity was correlated with PCB metabolism. The ratios of the different hydroxy metabolites formed differed between species. Furthermore, slices of tissue, rather than isolated cells or organelles, can also be used for experimental exposures *in vitro*. Tissue slices are thin to maintain normoxia at the cell level. Fossi *et al.* (2010) maintained slices of fin whale (*Balaenoptera physalus*) skin in culture for 24 hours during exposures to various concentrations of organochlorines.

Cells from wildlife (non-model organisms) can be difficult to obtain and maintain. Routti *et al.* (2016) and Murk *et al.* (1994) obtained samples opportunistically. For example, polar bear adipose tissue was collected after a bear was shot in self-defence, and harbour porpoise and harbour seal liver samples were

collected from stranded individuals. Collecting samples from stranded individuals can make planning experiments very challenging and keeps sample sizes small. Also, it is generally difficult to use stranded specimens because tissues are likely to be in poor condition or cells are already dead. Cells of non-model organisms can also behave in an unexpected manner. Louis *et al.* (2015) reported that progenitor cells of northern elephant seals (*Mirounga angustirostris*) did not differentiate into adipocytes or accumulate fat according to standard protocols established in humans, rats and mice.

Adipose tissue is a remarkably complex secretory and metabolic organ (Trayhurn and Beattie, 2001; Kershaw and Flier, 2004; Trayhurn and Wood, 2005; Berry *et al.*, 2013). In mammals, there are two distinct types of adipose tissue that differ in histological appearance, anatomical distribution, function and molecular signature (Cousin *et al.*, 1992; May *et al.*, 2017). Brown adipose tissue (BAT) also generates heat, dissipates energy (Cannon and Nedergaard, 2003; Kajimura *et al.*, 2010) and is characterised by the presence of numerous mitochondria and high levels of expression of uncoupling protein 1 (UCP1; the protein that controls non-shivering thermogenesis; Lowell and Spiegelman, 2000; Ouellet *et al.*, 2012). Though most commonly associated with newborn infants (to help maintain normal body temperature), it has been established that BAT is also present in healthy adult humans (Virtanen *et al.*, 2009). White adipose tissue (WAT) is the predominant adipose tissue in mammals, and the tissue type considered in this study. It performs a number of vital functions that includes chemical energy storage in the form of triglycerides, which can be mobilised as an energy source (by hormone-sensitive lipase (HSL) action resulting in the release of fatty acids (FAs)) during resource limited periods, such as fasting (Sztalryd and Kraemer, 1994; Kraemer and Shen,

2002; Duncan *et al.*, 2007). In more recent times, adipose tissue has been identified

as a key endocrine organ, involved in both the secretion and metabolism of

hormones (adipokines, such as leptin and adiponectin; Zhang *et al.*, 1994; Scherer

et al., 1995; and steroid hormones such as cortisol; Stimson *et al.*, 2009; Hughes *et al.*, 2010) and cytokines (such as TNF-α and IL-6; Hotamisligil *et al.*, 1993), which

control inflammatory responses, glucose homeostasis, insulin sensitivity, appetite

and energy balance (Kershaw and Flier, 2004; Kwok *et al.*, 2016; Wang and

Scherer, 2016). In addition, WAT provides insulation against cold and protection

against physical trauma (Berry *et al.*, 2013). Adipose tissue is important for fitness

and survival in wildlife (Young, 1976; Barboza *et al.*, 2009). Since many wildlife

species undergo profound changes in body condition as a result of life history cycles,

such as moulting, migration, and reproduction, they are unlikely to have the same

body fat regulation mechanisms as humans and other model species (Speakman *et al.*, 2008; Houser *et al.*, 2013; Fowler *et al.*, 2018; Halsey, 2018). Understanding how

their WAT works and responds to environmental change and physiological stress

would help underpin our knowledge of their energetic requirements and our ability to

predict effects of anthropogenic stress. The ability to study fat tissue function and

responses to stress in wildlife species would therefore benefit from an *in vitro*

approach.

Culturing adipocytes (both primary cells and cell lines) *in vitro* is technically

challenging. Primary adipose systems (cells and tissue) have a number of

limitations, including failure to propagate in culture, interference of microscopy and

biochemical analyses as a result of large triacylglycerol stores, difficulty transfecting

with DNA, which is required for immortalisation or for reporter assays, and variation

due to the genetics and body condition of the animals from which they are isolated

(Wolins *et al.*, 2006; Ruiz-Ojeda *et al.*, 2016). Additionally, isolation of primary mature adipocytes can be technically difficult to execute because such cells are large and osmotically fragile, therefore very likely to lyse (Wolins *et al.*, 2006; Armani *et al.*, 2010; Ruiz-Ojeda *et al.*, 2016). As adipose tissue contains a number of different cell populations (including mature adipocytes, pre-adipocytes, multi-potent stem cells, fibroblasts, endothelial cells and immune cells, such as macrophages, neutrophils and lymphocytes; Armani *et al.*, 2010), extra care must be taken to ensure high purity mature adipocytes or pre-adipocytes (i.e. the desired cell type) are isolated, to avoid the co-isolation of contaminating cells (Shah *et al.*, 2016). Homogenous cell populations must be achieved to ensure uniform physiological and metabolic responses to treatments. Other cell types, in particular macrophages, can adhere to isolated adipocytes (Weisberg *et al.*, 2003) and can be difficult to remove, requiring additional disturbance of the culture environment (for example, a number of passages or centrifugation steps) that may damage fragile adipocytes (Thalman *et al.*, 2008). There may also be a significant loss of cells during purification, which is a major issue when tissue availability is limited (Fernyhough *et al.*, 2005). Mature adipocytes are fragile cells that have large lipid droplets in their cytoplasm (Walther *et al.*, 2009; Church *et al.*, 2012). When cultured, they do not adhere to the surface of the culture vessel and remain buoyant in the culture media, where they clump together, resulting in unequal and insufficient access to the medium (i.e. nutrients and treatments; Zhang *et al.*, 2000; Toda *et al.*, 2009). Therefore, it is difficult to observe morphological changes and cells typically lyse after approximately 72 hours of incubation (Zhang *et al.*, 2000), unless a suitable alternative attachment surface is provided (for example, 'ceiling culture' allows buoyant adipocytes to adhere to the top inner surface of a culture flask that is completely filled with medium; Sugihara *et*

al., 1986; Fernyhough *et al.*, 2004). These limitations make primary adipocyte culture particularly challenging, especially under field conditions in wild life, which is compounded by limited tissue volume. In addition, although cell culture tries to obtain a pure cell population, often cells in single culture do not behave in the same manner as when in co-culture with other cell types (Edmonson *et al.*, 2014). Therefore, it may be more physiologically relevant to retain mixed cell populations.

Adipose explants are small pieces of tissue that, under adequate *ex vivo* culture conditions, maintain structure and cellular integrity, making them more likely to mirror *in vivo* tissue responses (i.e. to experimental stimuli), whilst preserving metabolic characteristics and cell to cell communication (Fried *et al.*, 2001; Thalman *et al.*, 2008). Adipose explants are simpler and faster to prepare and lose fewer cells in comparison to more complex methods that involve separating and purifying individual cells for culture (i.e. individual mature adipocytes or stromal vascular fraction cells; Fried *et al.*, 2001; Thalman *et al.*, 2008). The explant method has been widely used to establish much of the basic understanding of adipose tissue function and is widely used in biomedical science to work with tissue from obese patients (Prins *et al.*, 1997; Fain *et al.*, 2003; Ramanjaneya *et al.*, 2010). Therefore, the explant method is ideal when faced with restricted tissue volume and challenging field conditions, as often occurs when working with wild animals. In addition, explant culture provides a unique opportunity to investigate tissue responses of large, wild animals (such as marine mammals) that are not easily accessible for whole-animal level experiments, or cannot be exposed to experimental conditions under investigation for ethical reasons. However, culturing adipose explants is a technique that has not been explored thoroughly using marine mammal blubber, and has only recently been applied to grey seals (Bennett *et al.*, 2017b).

Adipose tissue of marine mammals faces many specific challenges, including: large changes in volume; potential for hypoxia as a result of both substantial blubber depth and from repeated ischaemia during diving; and high contaminant load, all of which are physiologically stressful states. Adipose tissue is of great importance for marine mammals, providing a source of metabolic fuel, insulation, buoyancy and streamlining (Irving and Hart, 1957; Young, 1976; Koopman *et al.*, 2002; Strandberg *et al.*, 2008). Adipose tissue is essential for survival and fitness for different sexes and age classes (Pomeroy *et al.*, 1999; Hall *et al.*, 2002; Lidgard *et al.*, 2005). Grey seals have extensive fat stores, which show dramatic seasonal fluctuations; body fat content can be as low as 12% in juveniles after moulting, approximately 30% in pre-breeding adults, reaching up to 45% in weaned grey seal pups (Reilly, 1991; Beck *et al.*, 2003; Sparling *et al.*, 2006; Hall and McConnell, 2007). Adipose tissue is an essential metabolic fuel for adults during fasting when breeding and moulting and for pups during the post-weaning fast. For example, larger mothers raise larger pups and 'skip' a breeding year following a large investment (Pomeroy *et al.*, 1999). Male grey seals that are larger and fatter sire more offspring and/or fare better during aggressive encounters (Lidgard *et al.*, 2005). Deposition of large subcutaneous adipose tissue depots during suckling is essential for first year survival (Hall *et al.*, 2002).

Previously in this thesis (Chapter 3-5), I have shown that blubber experiences changes in cellular defences during periods of natural physiological stress, including fasting during periods of high energy demand or development. I wanted to know more about the drivers of such changes, including teasing apart the individual level effect identified already that could be a result of contaminant load.

POPs are highly lipophilic and accumulate in adipose tissue leading to biomagnification up marine food chains (Muir *et al.*, 1988; Ross, 2000; Ross *et al.*, 2000; Burreau *et al.*, 2006), which is particularly problematic for top predators, such as marine mammals, as they accumulate substantial depots of adipose tissue and are at the top of the food chain (Ross *et al.*, 2000). POPs, such as PCBs, are widely distributed in terrestrial and marine ecosystems and detectable far from their anthropogenic sources of origin (Nisbet and Sarofim, 1972; Wolska *et al.*, 2012). Due to the physicochemical properties of PCBs (i.e. the number and position of chlorine atoms in biphenyl rings), they do not degrade easily by physical, chemical or biological means and remain in the environment indefinitely, resulting in chronic toxicity to wildlife (Loganathan and Kannan, 1994). PCBs affect wildlife in a variety of different ways: they disrupt endocrine and immune function (Hammond *et al.*, 2005; Hall *et al.*, 2009); behave as carcinogens and induce DNA damage (Safe, 1994; Oakley *et al.*, 1996); impair reproduction (Haave *et al.*, 2003; Pavlova *et al.*, 2016) and development (Bergman *et al.*, 1986); are associated with neurotoxicity (Tilson and Kodavanti, 1998); whilst altering adipocyte function and energy homeostasis (Regnier and Sargis, 2014; Routti *et al.*, 2016; Robinson *et al.*, 2018). PCBs also induce proteotoxic and oxidative stress in cells (Lee and Opanashuk *et al.*, 2004).

Cellular oxidative or proteotoxic stress induces the heat shock response (HSR), which is characterised by an increase in expression of heat shock proteins (HSPs). HSPs have already been described elsewhere in this thesis (Chapter 1, section 1.2.1; Chapter 3, section 3.1) as essential molecular chaperones that maintain proteostasis and prevent apoptosis by, for example, repairing damaged proteins and assisting the proper folding of newly synthesised proteins (Feder and Hoffman, 1999; Hartl and Hartl-Meyer, 2002). In cell and tissue culture systems, HSP expression can

be used to provide information regarding the likelihood of cell survival during and after stress exposure. For example, Song *et al.* (2014) found higher HSP70 protein expression with decreased incidence of apoptosis and necrosis in prostate cancer cell lines subjected to hyperthermia, when comparing 2D and 3D culture environments. This suggests higher HSP70 enhanced cell survival. Measurement of HSP expression in blubber thus provides information regarding the ability of cells to mount an effective stress response to a range of challenges. Indeed, HSPs are widely used as markers of cellular-level stress and increase in response to various stressors including exposure to heavy metals and other pollutants, including POPs, such as polycyclic aromatic hydrocarbons (PAHs; de Pomerai, 1996; Aït-Aïssa *et al.*, 2000; Rössner Jr. *et al.*, 2003; Mahmood *et al.*, 2014) and HSP70 has been suggested as a sensitive marker of toxicant exposure (Gupta *et al.*, 2010). I have already shown (in Chapter 3, 4 and 5) that HSPs are not very responsive to developmental or life-history changes, suggesting they may be useful as indicators of exogenous sources of stress.

Transcription and translation of HSP genes and proteins can also be affected by stress hormones, such as glucocorticoids (GC) (Basu *et al.*, 2001; LeBlanc *et al.*, 2012). GCs are essential steroid hormones, synthesised from cholesterol, that play important roles in regulating energy balance and mediating responses to stress. Cortisol is the major GC secreted by the adrenal cortex and is regulated by the hypothalamic-pituitary-adrenal (HPA) axis, which mediates the neuroendocrine response to stress (Sapolsky *et al.*, 2000; Tsigos and Chrousos, 2002; Atkinson *et al.*, 2015). Cortisol increases rapidly and dramatically in response to acute stress and becomes elevated during chronic stress. Corticosteroid- binding globulin (CBG) transports cortisol in the circulation (Lewis *et al.,* 2005; Gagliardi *et al.*, 2010).

Cortisol has wide-ranging effects on various systems, including whole body energy storage and distribution, immunity and cardiorespiratory functions (Sapolsky *et al.*, 2000; Tsigos and Chrousos, 2002; Atkinson *et al.*, 2015). Environmental pollutants, such as PCBs, impair the cortisol stress response in fish (Hontela *et al.*, 1992). In adipose tissue, cortisol is involved in both fat storage (i.e. triglyceride deposition) and fat depletion (i.e. triglyceride catabolism and subsequent release into circulation). Blubber cortisol levels increase during moulting in harbour seals, suggesting it mediates or reflects a functional change in the tissue (Kershaw and Hall, 2016). Cortisol and other GCs influence adipocyte differentiation and adipokine and inflammatory cytokine secretion. However, there is currently little research linking the whole animal stress response, in the form of cortisol production, with the cellular stress response in adipose tissue.

Intracellular GC action directly involves HSPs, because HSP70 and HSP90 are bound to glucocorticoid receptor (GR) in the cytoplasm and act as chaperones as part of steroid-receptor complexes (Pratt, 1993; Beato and Klug, 2000). Once cortisol binds and activates steroid-receptor complexes, HSP70 and HSP90 dissociate from the complex into the cytoplasm. The steroid-receptor complex then migrates into the nucleus and binds to the 'glucocorticoid-response element' (a palindromic CGTACAnnnTGTACT enhancer sequence in the genome), stimulating or repressing GC-responsive gene transcription (Pratt, 1993; Beato and Sanchez-Pacheco, 1996; Beato and Klug, 2000). Cortisol may therefore influence gene and protein expression of HSPs. Basu *et al.* (2001) showed that cortisol suppressed the elevation in HSP70 induced by heat stress in liver and gill tissues of rainbow trout and tilapia (*Oreochromis mossambicus*).

HSP gene expression in response to environmental contaminants, such as PCBs, has been measured in some marine mammal species. *Hsp70* gene expression is higher in skin of Mediterranean fin whales (*Balaenoptera physalus*), which experience higher toxicological stress from POPs sequestered in blubber compared to fin whales from pristine environments (Fossi *et al.*, 2010). Panti *et al.* (2011) also reported increased expression of *Hsp70* in skin of striped dolphins (*Stenella coeruleoalba*) resident in a region of the Mediterranean with high recorded levels of POPs, relative to dolphins from a Mediterranean area with low recorded levels of POPs due to limited human activity. HSP70 has also been used as a stress biomarker in whole blood of wild-rehabilitated grey and harbour seals (Fonfara *et al.*, 2008; Weirup *et al.*, 2013; Lehnert *et al.*, 2014). *Hsp70* mRNA abundance was decreased at release (back into the North Sea) compared to admission, which may suggest exposure to POPs during lactation that fell during rehabilitation, and/or habituation to rehabilitation and reduced stress due to improved health and feeding (after abandonment, dehydration, handling and transportation to the rehabilitation centre; Fonfara *et al.*, 2008; Weirup *et al.*, 2013; Lehnert *et al.*, 2014). However, none of these studies considered GC levels and whether there was any relationship between cellular and whole-animal level responses to stress. In addition, few studies have investigated the drivers of HSP expression experimentally, with the exception of Fossi *et al.* (2010), described briefly earlier in this section, in which *Hsp70* mRNA expression was examined in skin and blubber slices of fin whales and increased in response to PCB treatment *in vitro*.

Experimental protocols *in vitro*, such as adipose explant culture, provide a unique opportunity to test the consequences of exposure to physiologically relevant levels of hormones, including GCs, and environmental contaminants, including

POPs, for the HSR in seal blubber. These data will help understand the drivers of differences in HSPs seen in the wild, which cannot be explained by other covariates such as natural fasting or development (see Chapters 3 and 4), and facilitate understanding and interpretation of HSPs as markers of stress exposure in seals.

This chapter therefore aims to: 1) determine how culture conditions and time in culture affect RNA integrity and the relative gene expression of a range of HSPs, as indices of cellular stress and viability; 2) investigate absolute gene expression of *Hsp70* and *Hsc70* in inner and outer blubber explants of suckling and fasting grey seal pups in response to the artificial GC, hydrocortisone (HC) and PCBs, to ascertain the ability to induce the HSR alone or in combination; 3) determine whether other factors including mass, girth, sex and intrinsic concentrations of PCB congeners already sequestered in blubber can explain variation in absolute gene expression data; 4) consider if is there a functional relationship between neuroendocrine and cellular stress responses in grey seals.

6.2 Materials and Methods

6.2.1 Optimising culture conditions and time courses

6.2.1.1 Field and laboratory techniques

To determine the most suitable culture conditions for blubber explants and the time-course over which such conditions may adequately maintain blubber explants, two 10mm blubber biopsies were obtained from moulting juvenile grey seals that had previously been captured on the Isle of May, Scotland in May 2016 and transported to the Sea Mammal Research Unit captive facility, St. Andrews, Scotland. Animals were maintained on a fish-based diet with access to water and haul-out space.

Biopsies were taken under general and local anaesthesia using methods described in detail previously (Chapter 2; General Methods; section 2.1.2.6; Bennett *et al.*, 2017b). 10mm biopsies were transferred to 37°C Krebs-Ringer solution ($0.070gL^{-1}$ K_2HPO_4; $7.89gL^{-1}$ NaCl; $0.12gL^{-1}$ $MgSO_4$; $0.11gL^{-1}$ $CaCl_2$; $4.77gL^{-1}$ HEPES; $0.99 gL^{-1}$ glucose; $0.373 gL^{-1}$ KCl; dissolved in deionised H_2O; pH 7.4; all chemicals obtained from Sigma-Aldrich Co., Dorset, UK) in 15 mL Falcon tubes, kept in a Thermos® flask containing water at 37°C, and immediately transported to the laboratory for processing, as described by Bennett *et al.* (2017b). Blubber was washed in fresh Krebs-Ringer and skin, hair, muscle, connective tissue and large blood clots removed. Whole biopsies were weighed and minced into 5 – 10mg pieces. 100mg portions were weighed onto the surface of a sterile Petri-dish and then placed in wells of pre-prepared culture plates.

Initial experimental culture conditions were as follows: M199 media supplemented with 10% 'unstripped' fetal bovine serum (FBS) (1); M199 media supplemented with 10% charcoal 'stripped' FBS and supplemented with FFAs (2); M199 media supplemented with 10% charcoal 'stripped' FBS only (3). Charcoal stripped FBS has had small molecules, such as endogenous cortisol and FFAs, removed so they cannot interfere with experimental treatments by driving background stimulation (i.e. HC) and allow the tissue to be in a basal state. However, stripping also removes FFAs, which cells may need for metabolism. Explants were cultured with and without the presence of 5% CO_2 for either 18 or 24 hours using each experimental media composition (1, 2 and 3). 'Open' explants had the culture plate lid removed ('opened') at 18 and then 24 hours for additional media to added or changed, simulating experimental conditions and to explore whether media changes would extend explant survival. Explants were removed from media

after the specified time, immediately flash frozen, then stored at -80°C before analysis.

In a subsequent time-course experiment, explants were cultured in M199 media supplemented with 10% 'stripped' FBS and 2% FFAs in the presence of 5% CO_2. Explants were harvested and immediately flash frozen after 24, 48 and 72 hours to initially determine how long explants remained viable under optimal conditions (suitable media, supplements and CO_2 concentration).

A detailed time-course was then performed in which explants were cultured in M199 media supplemented with 10% 'stripped' FBS and 2% FFAs in the presence of 5% CO_2 and then harvested every three hours from 24 hours onwards to 48 hours (at 24, 27, 30 and 33 hours etc. up to 48 hours) to determine the changes in HSPs and RNA integrity over the duration within which explants appear to remain viable on the basis of RNA integrity. Explants were immediately flash frozen when harvested.

RNA was extracted as described in Chapter 2; General Methods; section 2.2.4, using 1-bromo-3-chloropropane (Sigma-Aldrich Co., Dorset, UK) as a phase separation reagent. Total RNA was visualised on a 1% agarose gel (Chapter 2; General Methods; section 2.2.4). 960ng total RNA was reverse transcribed using the QuantiTect Reverse Transcription kit (QIAgen, Manchester, UK), following the manufacturer's instructions. qRT-PCR experiments were performed using gene specific primers (*Hsp70*, *Hsc70*, *Hsp90*, *Hsp40*, *Hsp27* and reference genes *CycA*, *S9* and *L8* that had previously been selected using *NormFinder* and *BestKeeper* softwares based on changes recorded in this experiment) on a StepOne™ Real-Time PCR System (Applied Biosystems).

6.2.1.2 Statistical analysis

There were insufficient or no replicates to perform statistical analyses on experimental culture condition time-course data, therefore only patterns are described.

6.2.2 Blubber explants exposed to HC and PCBs and Hsp70/Hsc70 gene expression

6.2.2.1 Field and laboratory techniques

One 6 mm and two 10 mm blubber biopsies were obtained from grey seal pups late in the suckling period (approximately day 12) and late in the post-weaning fast (approximately day 12) in 2016, using methods described in detail previously (Chapter 2; General Methods; sections 2.1.2.2, 2.1.2.3, 2.1.2.4). The 6 mm biopsies were wrapped in tin foil (marking inner and outer sections of the blubber core), placed in sterile 1.8mL Nunc™ cryogenic tubes (Thermo Scientific, Basingstoke, UK), then immediately placed on ice until storage at -20°C within one hour of sampling.

The 10mm biopsies were divided into inner and outer sections, transferred to 37°C Krebs-Ringer solution and transported immediately from the field to the laboratory for processing, as described above in section 6.2.1.1. Inner and outer biopsy sections were weighed separately and minced into 5 – 10mg pieces, before being divided into eight 100mg portions (i.e. eight inner and eight outer explants). Each 100mg explant was added to one of two pre-prepared (at least one hour before the addition of cells) 12 well culture plates: four wells containing pre-warmed complete media (M199 media (Gibco by Life Technologies) supplemented with 5% 'stripped' FBS (Sigma Aldrich), 1% FFAs (Sigma Aldrich) and 1% Antibiotic Antimycotic Solution (100x; Gibco by Life Technologies)) and four containing

complete PCB media (M199 media supplemented with 5% 'stripped' FBS, 1% FFAs

and 1% Antibiotic Antimycotic Solution with Aroclor Standard PCB mix (Sigma

Aldrich) at a concentration of 150ng mL^{-1}). Explants were left to acclimate at 37°C,

5% CO_2 for 18 hours to allow PCB-mediated effects on gene expression to occur.

Explants (Figure 6.1) were then exposed to control or +PCB media, or 500 nM HC or

HC+ PCB media for six hours to allow time for HC mediated gene expression

changes to manifest, before being harvested, immediately flash frozen in liquid

nitrogen and stored at -80 °C until analysis.
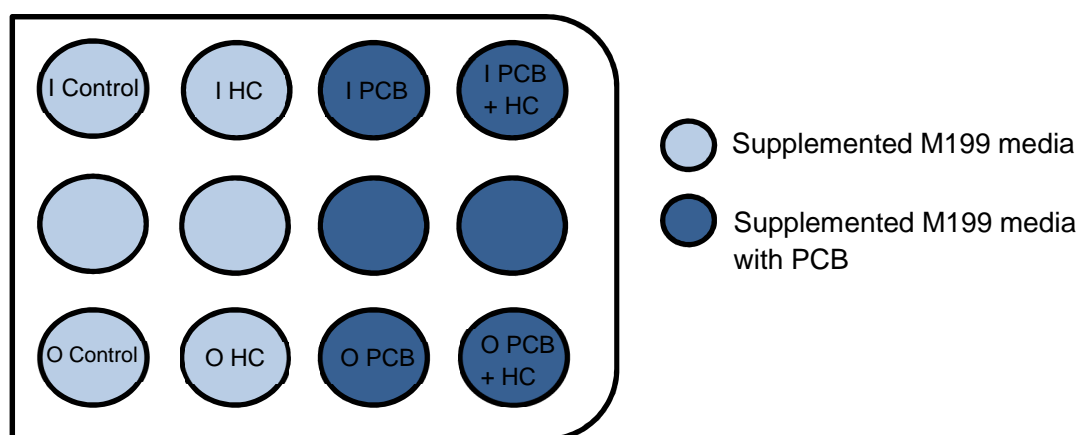
Figure 6.1. Schematic diagram depicting culture plate set-up. I = inner explants; O =

outer explants. Light blue colour indicates supplemented M199 media that was untreated

(I/O Control) or treated with 500nM HC (I/O HC). Dark blue colour indicates supplemented

M199 PCB media that was treated with PCB mixture only (I/O PCB) or additionally treated

with 500nM HC (I/O PCB + HC). Replicate outer core explants were plated on a second

pre-prepared plate with the same lay out as the bottom row. Unlabelled wells represent

inner core replicates.

6.2.2.2 Sample analysis

The 6 mm blubber biopsies were sent to the Centre of Analytical Research and Technology, Laboratory of Animal Ecology and Ecotoxicology, University of Liège, Liège, Belgium for analysis to determine concentrations of PCB congeners 28, 52, 101, 138, 153, 180, 105, 114, 118, 123, 156, 157, 167, 189. Briefly, lipids were extracted from whole blubber cores (weighing between 270 and 540 mg) by accelerated solvent extraction (ASE; Dionex 200, Sunnyvale, USA). Purified lipid extracts were analysed using high-resolution gas chromatography-mass spectrometry (GC-MS; ThermoQuest Trace GC, 2000; ThermoQuest, Milan, Italy).

RNA was extracted from the explants as described in Chapter 2; General Methods; section 2.2.4, using 1-bromo-3-chloropropane (Sigma-Aldrich Co., Dorset, UK) as a phase separation reagent. Total RNA was visualised on a 1% agarose gel (Chapter 2; General Methods; sections 2.2.4) to determine RNA integrity. 400ng total RNA was reverse transcribed using the qPCRBIO cDNA Synthesis Kit (PCR Biosystems Ltd, London, UK), according to the manufacturer's instructions.

6.2.2.3 Relative verses absolute quantification: blubber explants exposed to hydrocortisone and PCBs

Considerable variance in reference gene expression meant finding a stable gene with which to perform relative expression analyses was not possible with HC and PCB blubber explant samples. Though *NormFinder* software did suggest the 'most stable' reference gene from a selection of L8, S9, YWHAZ and CYCA, subsequent analyses by *BestKeeper* software showed high standard deviations and variances that made none of the candidate genes suitable for use, because none were stably expressed across all nutritional states (i.e. suckling or fasting),

treatments (i.e. control, HC, PCB and HC+PCB) and tissue sections (i.e. inner or outer blubber core). Due to this fluctuating expression of reference genes we decided to perform absolute quantification of gene expression using the standard curve method. The standard curve was constructed using known quantities (of the amplicon copy number) and then used to compare unknowns and extrapolate a transcript/copy number value. Standard curve amplicons were generated as follows: pooled cDNA was used in endpoint PCR (GeneAmp® Fast PCR Mastermix (2x); Applied Biosystems, Warrington, UK) performed on a C1000™ Thermal Cycler (Bio-Rad Laboratories Ltd., Hertfordshire, UK) using *Hsp70* and *Hsc70* specific primers. PCR products were run alongside a HyperLadder™ 50bp ladder (Bioline Reagents Ltd, London, UK) on a 3% agarose gel stained with SYBR® Safe DNA gel stain (Invitrogen, Life Technologies, Paisley, UK) and visualised on a G:BOX Chemi XX6 gel imaging system (Syngene, Cambridge, UK). Bands were then excised and amplicons purified using a QIAEX II Gel Extraction Kit (QIAgen, Manchester, UK), following the manufacturer's instructions. Purified amplicons were quantitated using Qubit 3.0 Fluorometer (Invitrogen, Life Technologies, Paisley, UK). Amplicons were stored at -20°C until use in qRT-PCR experiments. To ensure double stranded (opposed to single stranded) DNA was present another 3% agarose gel (as above) was run to observe band migration. Copy number/number of molecules was then calculated using the following equation (3; where ng = amount of purified amplicon quantitated using the Qubit Fluorometer; 6.022×10^{23} = Avogadro's constant, the number of molecules in one mole of a substance; length = amplicon size, either *Hsp70* or *Hsc70*, in base pairs, bp; 1×10^9 = ng/g; 650 = Daltons, average mass of one dsDNA bp):

$$\text{Number of copies (molecules)} = \frac{((\text{ng} \times (6.022 \times 10^{23})))}{(\text{length} \times (1 \times 10^{9}) \times 650)}$$

qRT-PCR experiments were performed using gene specific primers (culture conditions and time-course samples: *Hsp70*, *Hsc70*, *Hsp90*, *Hsp40*, *Hsp27*; HC and PCB blubber explant samples: *Hsp70*, *Hsc70* only) on a StepOne™ Real-Time PCR System (Applied Biosystems) and a MxPro - Mx3000P QPCR System (Agilent Technologies, Inc., Stockport, Cheshire, UK).

6.2.2.4 Statistical analysis

For detailed information regarding statistical analysis, refer to Chapter 3, section 3.2.3 and Chapter 4, section 4.2.3. The raw transcript copy number and PCB concentration data were log transformed for use in linear mixed effects models (LMEs). Blubber biopsy section (i.e. inner or outer), nutritional state (i.e. suckling or fasting), treatment (i.e. HC or PCB exposed tissue), mass, girth, sex and tissue PCB concentration (ng g^{-1} lipid; sum of all PCBs, sum of dioxin and non-dioxin-like PCBs, individual PCB congeners 28, 52, 101, 138, 153, 180, 105, 114, 118, 123, 156, 157, 167, 189) were included as fixed effects to explain variation in absolute gene expression (copy number). Individual was included as the random effect. Pearson's correlations were performed to investigate the relationships between individual dioxin- and non-dioxin-like PCBs. A paired t-test was used to investigate differences in body mass and axial girth between suckling and fasting pups.

6.3 Results

6.3.1 Time-course and culture conditions: HSP gene expression in response to experimental culture conditions without CO_2

Explants cultured without the presence of CO_2 showed increased expression of *Hsp70* at 18 and 24 hours under all culture conditions (described below in sections 6.3.1.2, 6.3.1.3 and 6.3.1.4), compared to fresh flash frozen control samples. Expression of *Hsc70*, *Hsp90* and *Hsp40* was decreased under all culture conditions relative to fresh flash frozen control samples. There was no clear pattern of *Hsp27* expression, which either increased or decreased depending on culture conditions, which are described below in sections 6.3.1.2, 6.3.1.3 and 6.3.1.4. All those HSP gene expression values obtained from similar culture environments were different to the fresh flash frozen controls, but not different from each other.

6.3.1.1 'Un-stripped' FBS

Explants cultured in M199 media supplemented with 'un-stripped' FBS, without the presence of CO_2, showed increased expression of *Hsp70* at 18 (~3.65 fold) and 24 hours (~1.45 fold) and under 'open' conditions (~3.35 fold), relative to control samples. *Hsc70* (~2.43, ~3.86 and 1.83 fold), *Hsp90* (~1.91, ~3.26 and ~1.61 fold) and *Hsp40* (~1.54, ~2.93 and ~1.43 fold) were decreased at both time-points and under 'open' conditions; the reduction in all three genes was greater after 24 hours and increased under 'open' conditions. *Hsp27* was increased under 'open' conditions (~1.31 fold), though was slightly decreased after 18 hours (~0.30 fold) and further after 24 hours (~0.38 fold), compared to control samples.

6.3.1.2 'Stripped' FBS

Explants cultured in M199 media supplemented with 'stripped' FBS and without CO_2, showed increased expression of *Hsp70* at 18 (~ 4.62 fold) and 24 (~ 2.14 fold) hours and under 'open' conditions (~ 1.37 fold), relative to control samples. *Hsp27* followed the same pattern (~1.05 fold; ~0.72 fold; ~0.081 fold), though with less pronounced changes in expression. *Hsc70*, *Hsp90* and *Hsp40* followed a similar pattern to one another; all decreased relative to control samples at 18 hours (~ 0.98 fold, ~ 0.43 fold and ~ 0.36 fold, respectively) with a further decrease at 24 hours (~ 2.11 fold, ~ 2.71 fold and ~ 2.04 fold, respectively), though increased slightly under 'open' conditions (~ 1.44 fold and ~ 2.23 fold, respectively) compared to 24 hours, with the exception of *Hsp40* which decreased further by ~ 2.77 fold under 'open' conditions.

6.3.1.3 'Stripped' FBS and FFAs

Explants cultured in M199 media supplemented with 'stripped' FBS and FFAs also increased expression of *Hsp70* at both 18 (~ 5.33 fold) and 24 (~ 1.43 fold) hours and under 'open' conditions (~ 1.83 fold), relative to control samples. *Hsp27* followed a similar pattern (increased by ~ 1.63 fold at 18 hours and ~ 0.62 under 'open' conditions), though decreased expression by ~ 0.37 fold after 24 hours. *Hsc70*, *Hsp90* and *Hsp40* again followed a similar pattern to one another; all decreased relative to control samples at 18 hours (~ 0.34 fold, ~ 0.54 fold and ~ 0.30 fold, respectively) with a further decrease at 24 hours (~ 2.98 fold, ~ 3.40 fold and ~ 2.95 fold, respectively), though increased slightly under 'open' conditions compared to 24 hours (by ~ 2.65 and ~ 2.76 fold, respectively), with the exception of *Hsp40* which decreased further by ~ 3.29 fold under 'open' conditions.

6.3.2 24 – 72 hour time-course: HSP gene expression under optimised culture conditions in the presence of CO_2

To initially investigate how long blubber explants may be viable under the most suitable culture conditions tested above (M199 media supplemented with stripped fetal bovine serum (FBS) and free fatty acids (FFA) in the presence of 5% CO_2) tissue explants were cultured for up to 72 hours. These were chosen as the most suitable culture conditions, based on the relative merits of the culture medium components: FBS is essential, maintaining cells in contact with vital proteins and signalling molecules; 'stripped' FBS has had small molecules, such as endogenous cortisol, removed so they cannot interfere with experimental treatments; FBS is re-supplemented with FFAs to maintain adipocyte function. This provides cells with the richest conditions of those examined. Expression of all HSPs decreased after 48 hours, relative to control samples. *Hsp70* was increased at 24 hours (~3.26 fold) relative to control samples, though expression decreased ~1.83 fold from 24 to 48 hours. *Hsc70* (~2.11 fold, 24 hours; ~2.50 fold, 48 hours)*, Hsp90* (~2.49 fold, 24 hours; ~2.34 fold, 48 hours) and *Hsp40* (~1.22 fold, 24 hours; ~2.71 fold, 48 hours) were decreased at both 24 and 48 hours relative to control samples. *Hsc70* (~ 0.39 fold) and *Hsp40* (~ 1.49 fold) expression decreased further at 48 hours. *Hsp27* expression was increased after 24 hours (~ 1.22 fold relative to control samples) and decreased at 48 hours both compared to 24 hours (~1.60 fold) and relative to control samples (~0.38 fold). RNA had totally degraded after 72 hours under the above culture conditions, therefore HSP gene expression was not measured at this time-point.

6.3.3 24 – 33 hour time-course: HSP gene expression under optimised culture conditions

There was increased RNA degradation with time, visible on the 1% agarose gel (Figure 6.2); though the time-course ran from 24 to 48 hours RNA had totally degraded after 33 hours, therefore expression of HSPs was not examined after that time because the RNA was not usable.

Under suitable culture conditions (M199 media supplemented with 10% 'stripped' FBS, 1% FFAs and 1% pen/strep, with 5% CO_2 to allow correct partial pressure of CO_2 and pH) expression of all HSPs decreased after 27 hours, relative to fresh flash frozen control samples. There was a 6.55 fold increase in expression of *Hsp70* after 24 hours which stayed constant up to 27 hours (~6.45 fold). Compared to 24 and 27 hours, expression of *Hsp70* decreased at 30 hours (~0.99 fold) with further reduced expression at 33 hours (~1.65 fold). Relative to fresh flash frozen control samples, expression of *Hsp70* was increased at both 30 hours (~5.51 fold) and 33 hours (~4.85; Figure 6.2). *Hsc70* expression increased during the first 24 hours (~1.19 fold) and remained constant up to 27 hours (~1.21 fold higher than control samples) before decreasing at 30 hours (~0.60 fold reduction between 27 and 30 hours). *Hsc70* expression appeared to increase slightly after 33 hours (~1.08 fold higher than control samples). *Hsp90* expression decreased relative to control samples after 27 hours (~0.55 fold at 30 hours and ~0.35 fold after 33 hours). *Hsp90* expression was slightly elevated at 24 (~0.36 fold) and 27 hours (~0.34 fold). *Hsp40* decreased as the time-course progressed, though was higher relative to control samples at all time-points (~0.21 fold at 33 hours to 1.53 fold at 24 hours). *Hsp27* followed the same pattern as *Hsp70*, increasing at 24 hours (~3.14 fold) and

remaining constant up to 27 hours (~3.06 fold), before decreasing 0.67 fold at 30

hours and a further 0.60 fold at 33 hours (Figure 6.2).
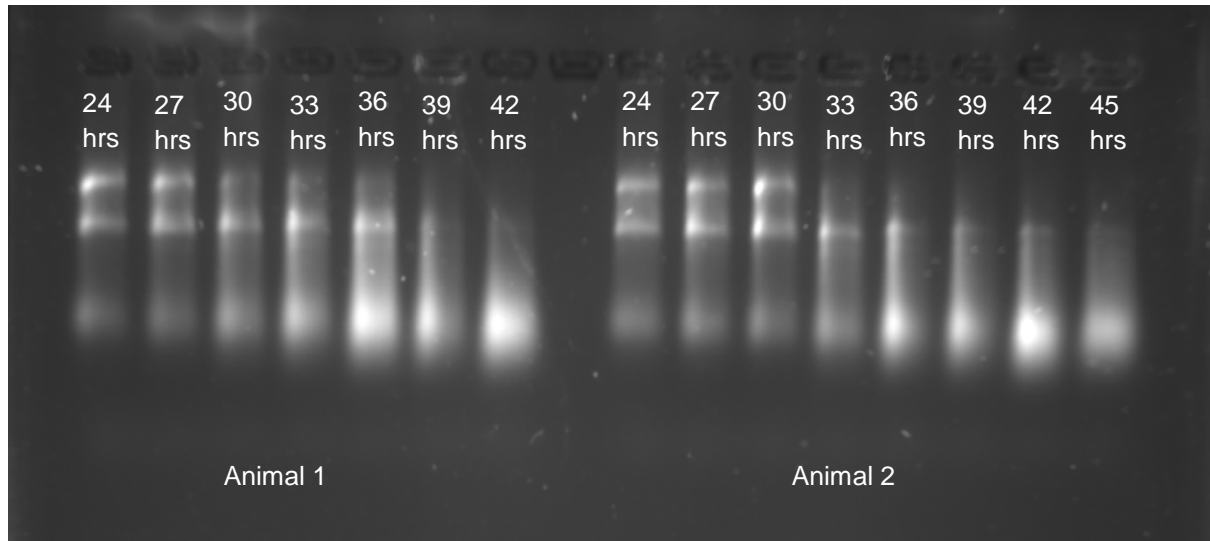
Figure 6.2. 960ng total RNA from time-points 24-45 hours were run on a 1% agarose gel.

Total RNA from blubber explants of juvenile moulting grey seals maintained in optimal

culture conditions for 24 up to 45 hours. Two different animals (1 and 2) are presented.

Gel and image courtesy of Dr. James Turton.

6.3.4 Blubber explants exposed to HC and PCBs: pup morphometrics

There was a significant decrease in body mass (paired t-test, df = 8, T = 2.90, p = 0.019) and axial girth (paired t-test, df = 8, T = 2.30, p = 0.050) between suckling and fasting pups (Table 6.1).

Table 6.1. Mean (± SD (*n*)) and range of body mass (kg) and axial girth (cm) at both late suckling and the late post-weaning fast. Mean (± SD (*n*)) and range of birth mass (kg), mass change from birth to weaning (kg), rate of mass gain (kg day^{-1}), rate of mass loss (kg day^{-1}) and maternal mass transfer to pup (%) is also presented for 2016 wild grey seal pups from the Isle of May, Scotland. "M:F" indicates the proportion of pups that were male (M) or female (F).

	Range		Mean ± SD (*n; M:F*)	
	Suckling (age 15-17 days)	Fasting (age 30 – 37 days)	Suckling (age 15-17 days)	Fasting (age 30 - 37 days)
Mass (kg)	34.2 – 44.8	34.2 – 44.8	42.64 ± 4.10 (10; 7:3)	39.82 ± 3.16 (9; 7:2)
Axial girth (cm)	89 - 103	88 - 99	95.2 ± 4.44 (10; 7:3)	92.11 ± 3.41 (9; 7:2)
Birth mass (kg)	10.0 – 16.78		13.46 ± 2.23 (10; 7:3)	
Weaning mass (kg)	35.16 – 49.18		44.23 ± 4.19 (10; 7:3)	
Mass change (birth to weaning; kg)	17.85 – 39.15		28.84 ± 5.98 (10; 7:3)	
Rate of mass gain (kg day^{-1})	1.63 – 2.70		2.03 ± 0.33 (10; 7:3)	
Rate of mass loss (kg day^{-1})	0.45 – 0.62		0.55 ± 0.057 (9; 7:2)	
Maternal mass transfer to pup (total pup mass change, birth-weaning, as a % of total maternal mass change, post-partum – weaning)	34.66 – 64.58		45.09 ± 9.49 (10; 7:3)	

6.3.5 Blubber explants exposed to HC and PCBs and Hsp70/Hsc70 *gene expression*

We investigated how absolute gene expression of *Hsp70* and *Hsc70* changes in inner and outer blubber explants of suckling and fasting pups after exposure to HC and a standard Aroclor PCB mixture and in response to intrinsic POP load.

6.3.5.1 Hsp70

The model that best described variation in log *Hsp70* transcript number included an interaction between blubber biopsy section and nutritional state and an additive effect of treatment and log sum of dioxin-like PCBs (LME: marginal R^2_{LME} = 0.31, conditional R^2_{LME} = 0.44, *n* (observations) = 129, *n* (animals) = 10). Models that included an additive effect of individual PCB congeners 52, 101, 118 and 167, tested against the additive effect of treatment alone, were a marginally better fit and explained the same or 1-2% more variance when compared to the additive effect treatment and log sum of dioxin-like PCBs (Table 6.2). However, these individual congeners are positively correlated with one another (Table 6.3) meaning it is difficult to ascertain which congener(s) are truly responsible for the effect on *Hsp70* gene expression; therefore the best model fit was taken as that described above, with an additive effect of log sum of dioxin-like PCBs.

Outer blubber explants had a significantly higher number of log *Hsp70* transcripts (~1.90 fold) compared to inner explants (LME: df = 118, T = 5.02, *p* = <0.001, marginal R^2_{LME} = 0.15, conditional R^2_{LME} = 0.26, *n* (observations) = 129, *n* (animals) = 10; Figure 6.3). Fasting pup explants had a significantly higher number of log *Hsp70* transcripts (~1.25 fold) compared to suckling pup explants (LME: df = 118, T = 2.66, *p* = 0.0088, marginal R^2_{LME} = 0.050, conditional R^2_{LME} = 0.15, *n* (observations) = 129, *n* (animals) = 10; Figure 6.3). PCB (LME: df = 116, T = 2.18, *p*

= 0.032, marginal R^2 $_{LME}$ = 0.042, conditional R^2 $_{LME}$ = 0.13, n (observations) = 129, n

(animals) = 10) and HC-PCB explants (LME: df = 116, T = 2.02, p = 0.045) had

significantly higher log *Hsp70* transcripts than control explants (Figure 6.3).

Compared to control conditions there was no change in the number of log *Hsp70*

transcripts in response to HC treatment (LME: df = 116, T = 1.23, p = 0.2229; Figure

6.3).

Table 6.2. Model output for linear mixed effect models (LMEs) that explain variability in

Hsp70 transcript numbers in which the interaction between blubber biopsy section and

nutritional state and the additive effect of treatment and either log PCB 52, 101, 118, 167 or

log sum of dioxin-like PCBs were tested against the simpler model form containing the

interaction between blubber biopsy section and nutritional state and the additive effect of

treatment alone, using the anova function. Bold font indicates $p < 0.05$.

Covariate	Model output	
Log PCB 52	Marginal R^2	0.31
	Conditional R^2	0.44
	L ratio	5.64
	P	**0.018**
Log PCB 101	Marginal R^2	0.32
	Conditional R^2	0.44
	L ratio	5.87
	P	**0.015**
Log PCB 118	Marginal R^2	0.31
	Conditional R^2	0.44
	L ratio	4.72
	P	**0.030**
Log PCB 167	Marginal R^2	0.32
	Conditional R^2	0.46
	L ratio	4.39
	P	**0.036**
Log sum of dioxin-like PCBs	Marginal R^2	0.31
	Conditional R^2	0.44
	L ratio	4.51
	P	**0.034**

Table 6.3. Pearson's product-moment correlations between log PCB congeners 118, 167, 52 and 101 concentration in blubber tissue (ng/g lipid). Significant ($p < 0.05$) correlations are indicated by bold font.

	R	T	p
Log PCB 118 x log PCB 167	0.91	8.96	**<0.0001**
Log PCB118 x log PCB 52	0.93	9.97	**<0.0001**
Log PCB 118 x log PCB 101	0.96	13.10	**<0.0001**
Log PCB 167 x log PCB 52	0.76	4.61	**0.0003**
Log PCB 167 x log PCB101	0.80	5.26	**<0.0001**
Log PCB 101 x log PCB 52	0.93	10.51	**<0.0001**

All PCB congeners included individually (Table 6.2), body mass (ANOVA: L = 0.49, p = 0.49; marginal R^2_{LME} = 0.31, conditional R^2_{LME} = 0.43), axial girth (ANOVA: L = 0.43, p = 0.51; marginal R^2_{LME} = 0.32, conditional R^2_{LME} = 0.43), sex (ANOVA: L = 1.87, p = 0.17; marginal R^2_{LME} = 0.33, conditional R^2_{LME} = 0.43) or log non-dioxin-like PCB congeners (ANOVA: L = 0.40, p = 0.53; marginal R^2_{LME} = 0.31, conditional R^2_{LME} = 0.44) did not significantly improve model fit.
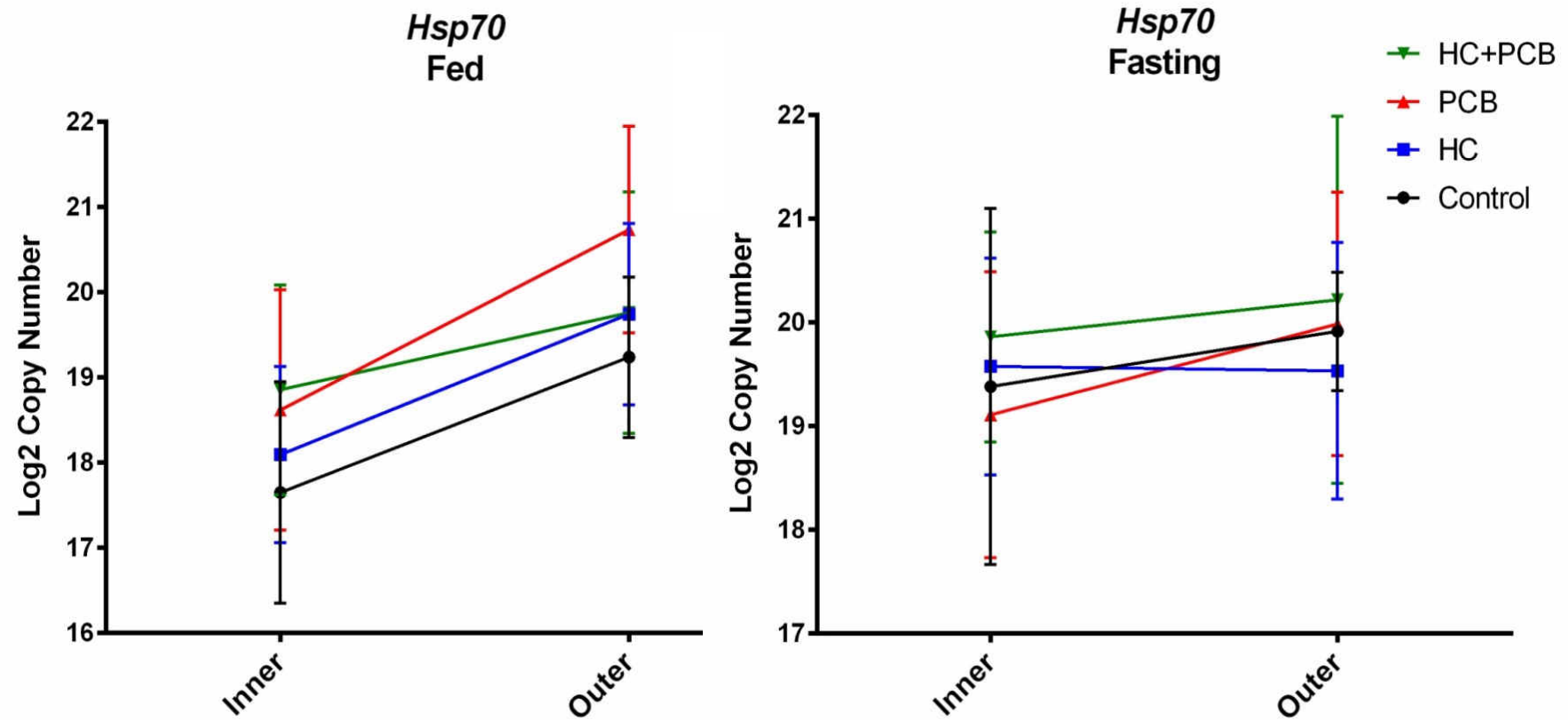
Figure 6.3. Mean (± SD) log2 *Hsp70* transcript copy number in inner and outer blubber explants exposed to hydrocortisone (HC), PCBs (standard Aroclor mix) or combination(PCB+HC) in a) suckling and b) fasting grey seal pups from the Isle of May, 2016.

6.3.5.2 Hsc70

The model that best described variation in log *Hsc70* transcript number included blubber biopsy section and an additive effect of sex (LME: marginal R^2_{LME} = 0.31, conditional R^2_{LME} = 0.45, *n* (observations) = 129, *n* (animals) = 10). Interestingly, sex significantly improved model fit (ANOVA: L = 4.37, *p* = 0.037; marginal R^2_{LME} = 0.31, conditional R^2_{LME} = 0.45) compared to the effect of blubber biopsy section alone. Overall females had higher (~1.79 fold) log *Hsc70* transcripts than males.

The addition of treatment (ANOVA: L = 3.60, *p* = 0.31; marginal R^2_{LME} = 0.22, conditional R^2_{LME} = 0.48) or nutritional state (ANOVA: L = 3.35, *p* = 0.067; marginal R^2_{LME} = 0.22, conditional R^2_{LME} = 0.47) as covariates to the above best-fit model did not improve model fit. There was no effect of either mass (ANOVA: L = 0.20, *p* = 0.65; marginal R^2_{LME} = 0.21, conditional R^2_{LME} = 0.46) or girth (ANOVA: L = 0.00014, *p* = 0.99; marginal R^2_{LME} = 0.21, conditional R^2_{LME} = 0.46) on expression of *Hsc70*.

Log sum of all PCBs did not improve model fit (ANOVA: L = 0.30, *p* = 0.58; marginal R^2_{LME} = 0.21, conditional R^2_{LME} = 0.47); neither sum log dioxin-like (ANOVA: L = 0.020, *p* = 0.88; marginal R^2_{LME} = 0.21, conditional R^2_{LME} = 0.47) or sum log non-dioxin-like (ANOVA: L = 0.40, *p* = 0.53; marginal R^2_{LME} = 0.21, conditional R^2_{LME} = 0.47) improved model fit. None of the dioxin and non-dioxin-like PCB congeners (PCB 28, 52, 101, 105, 114, 118, 123, 138, 153, 156, 157, 167, 180 and 189) included individually improved model fit and did not explain more variance than blubber explant section alone.

Outer blubber explants had a significantly higher number of log *Hsc70*

transcripts (~3.16 fold) compared to inner explants (LME: df = 118, T = 6.98, *p* =

<0.0001, marginal R^2 LME = 0.21, conditional R^2 LME = 0.46, *n* (observations) = 129, *n*

(animals) = 10; Figure 6.4). The change in transcript quantity of *Hsc70* between

suckling and fasting approached significance (LME: df = 118, T = -1.84, *p* = 0.067,

marginal R^2 LME = 0.022, conditional R^2 LME = 0.24; Figure 6.4). There was no change

in the number of log *Hsc70* transcripts in HC (LME: df = 116, T = 0.44, *p* = 0.66,

marginal R^2 LME = 0.015, conditional R^2 LME = 0.24; Figure 6.4), PCB (LME: df = 116,

T = 1.29, *p* = 0.20) or HC-PCB treated explants (LME: df = 116, T = 1.29, *p* = 0.20)

compared to control conditions.
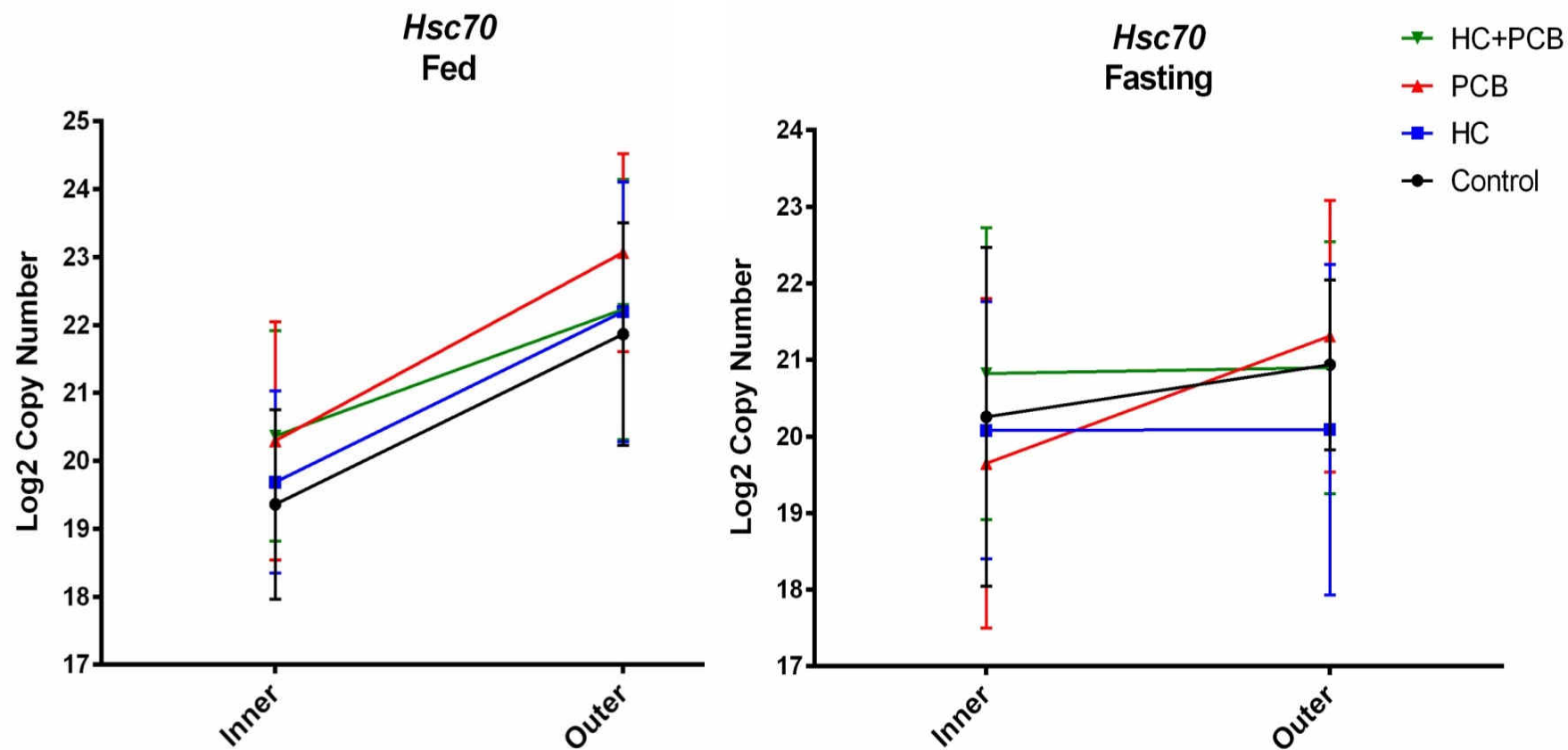
Figure 6.4. Mean (± SD) *Hsc70* transcript copy number in inner (I) and outer (O) blubber explants exposed to hydrocortisone (HC), PCBs (standard Aroclor mix) or combination (PCB+HC) in a) suckling and b) fasting grey seal pups from the Isle of May, 2016.

6.4 Discussion

6.4.1 Time-courses and culture conditions

Determining suitable culture conditions for blubber explants, is essential for method optimisation because it provides information regarding a suitable time-course over which experiments can be performed. The most suitable culture, of those explored here, conditions for blubber explants are as follows: M199 media supplemented with 10% 'stripped' FBS and FFAs in the presence of 5% CO_2. However, these conditions may not be perfect. Determining optimal conditions to maximise viability in culture and durations of experiments, needs to be explored further.

Here we used RNA integrity and HSPs as markers of cellular viability and stress, respectively. Typical viability markers are not appropriate for explants because we are using tissues and not cells (i.e. Trypan blue; neutral red; lactate dehydrogenase (LDH) release). HSPs could be considered as markers of apoptosis and necrosis (Sato *et al.*, 2008). The HSP gene expression data alone did not easily distinguish between the different conditions or directly inform which is most suitable to maintain cells. For example, *Hsp70* was induced prominently under all tested conditions (varying supplements and with/without the presence of CO_2) and was greatly increased after 18 hours. This increased expression after 18 hours may be attributed to cells adapting to their new *ex vivo* conditions. It is also likely that cells are exposed to damaged or disrupted neighbours, resulting in the release of apoptotic or necrotic extracellular signalling molecules (Renz *et al.*, 2001; Scaffidi *et al.*, 2002; Sato *et al.*, 2008), which may stimulate the induction of cellular defence mechanisms in undamaged cells anticipating potentially damaging conditions

(Bergsbaken *et al.*, 2009; Chovatiya and Medzhitov, 2014). Changing the culture media (i.e. the 'open' treatment) did not help, though it should have removed some of these signalling molecules. However, such molecules potentially had already initiated effects. Maybe more rinsing of the tissue before plating would remove more of these signals and cell debris. Explants may also be experiencing an altered oxygen environment. Incubation in cell culture conditions likely represents hyperoxia for most cells and tissues because they are exposed to atmospheric rather than tissue levels of oxygen. In contrast, cells at the centre of an explant piece may experience hypoxia as a result of reduced perfusion. It is not currently known what conditions are normoxic for grey seal blubber (K. Bennett, pers. comm.). Whatever the source of stress, our data suggest that all cells in explant culture, even though viable (according to RNA integrity and oxygen consumption measures (not reported here), are likely to be experiencing heightened stress conditions that do not reflect the *in vivo* environment. It may therefore be difficult to detect increased stress responses over the elevated background in explant culture, making the method less sensitive to detect low level stress. By 24 hours, as cells acclimatise and recover homeostasis, stress responses may be dampened (as cells potentially experience less physiologic stress). Conversely, cells may become compromised and unable to mount an adequate stress response after a longer time in inadequate culture conditions.

Under the culture conditions used here, expression of *Hsp70*, *Hsc70*, *Hsp90*, *Hsp40* and *Hsp27* decreased after 27 hours. I interpret this reduction as an indication that after this time cell function is compromised. Cells are potentially unable to mount an effective HSR (which protects against proteotoxicity and promotes survival) resulting in reduced HSP expression and, eventually, cell death.

This was further confirmed by visibly increased RNA degradation with time (Figure 6.2). Song *et al.* (2014) also found that decreased HSP expression was associated with increased incidence of apoptosis and necrosis in both 2D and 3D culture environments, optimised for prostate cancer cells subjected to hyperthermia. It would be useful to establish whether explants in culture are experiencing eustress or distress by including other markers, such as MDA, oxyblots or other oxidative or proteotoxic damage markers. Caspase activation could be assessed to investigate whether and when apoptosis is initiated, and DNA fragmentation could be used as an indicator of necrosis. We did not have enough tissue to perform these extra measurements.

Overall, from our data we suggest that blubber explants are cultured for no longer than 24 hours under the conditions used here to maintain cellular function and viability, which will also ensure data collected from experimental exposures are as physiologically meaningful as possible, with the caveat that explant culture increases HSP70 expression and therefore cellular stress. It would be useful to further optimise explant culture conditions to extend the lifespan of the explants and identify the timing of onset and nature of cellular stress and its consequences.

6.4.2 Blubber explants exposed to HC and PCBs and Hsp70/Hsc70 *gene expression*

We investigated the effect of HC and PCBs on *Hsp70* and *Hsc70* absolute gene expression in blubber explants maintained under the best culture conditions established above.

In contrast to data reported elsewhere in this thesis (Appendix 1), there was a higher mRNA expression of both *Hsp70* and *Hsc70* in the outer section of the blubber biopsy, compared to the inner. This was unexpected because cells in the

inner section of the blubber are thought to be more metabolically active with a higher protein turnover (Strandberg *et al.*, 2008), which would require greater chaperone function of HSPs. Also, it may be expected that expression of HSPs would be increased in the inner section of the blubber core because PCBs concentrate here as blubber volume decreases during fasting (Debier *et al.*, 2003a; 2003b). Increased expression in the outer section of the blubber may be due to the loss of lanugo and increased blood flow to the surface of the skin as the seal pups begin to moult late in the suckling period. Another possibility is that the outer section of the blubber may experience thermal shock in the incubator (maintained at 37°C). Outer blubber may not be accustomed to such a temperature since it is likely to be maintained *in vivo* closer to ambient temperatures (being important in insulation and some distance from vascularisation and closest to the ambient environment).

Furthermore, discrepancies between the experimental work and the developmental time-course data presented in Appendix 1, may be attributed to the fact the tissue here was maintained in culture, rather than being immediately flash-frozen in liquid nitrogen after removal from the animal, and we have shown (above) that culture itself increases *Hsp70* gene expression. Other variables that may have contributed to the difference between experimental and observational data include the manner in which the tissue was divided into inner and outer sections i.e. blubber cores taken for explant culture were divided in half directly before being processed whereas only the extremities (the inner and outer 'tips') were taken from the blubber biopsies flash-frozen in 2013. This may result in differing proportions of cell types, numbers and sizes between the directly sampled tissue and the explants. Adipose tissue consists of mature adipocytes, pre-adipocytes, vascular smooth muscle cells, fibroblasts, endothelial cells, resident monocytes and macrophages, lymphocytes

and multi-potent stromal cells (Lindroos *et al.*, 2011; Tallone *et al.*, 2011; Berry *et al.*, 2013). Further research to ascertain blubber tissue structure and stratification, including constituent cell types, in grey seals during different nutritional and life history states would help provide a more meaningful explanation regarding the drivers of differences in gene expression between sampled tissue and explants. Methods such as histology and immunohistochemistry to stain and count relevant cell types would allow direct comparison of the inner and outer sections of blubber biopsies.

The gene expression data from fresh flash frozen and explant experiments were generated by two different qPCR quantification methods; relative (2013 flash-frozen inner and outer blubber tissue) and absolute (inner and outer blubber explants) quantification. Both are suitable methods, equally sensitive to changes in transcript levels and should yield the same outcome (Lee *et al.*, 2006) However, the various methods of data presentation and differences in units (fold change vs absolute copy number) can make comparisons more difficult. Lee *et al.* (2006) reported identical and reproducible results from relative and absolute quantification methods to determine plasmid copy number in *E. coli* cells. Conversely the findings of Whelan *et al.* (2003) corroborate our findings, in which there were differences in gene expression patterns of cytokines in cultured human peripheral blood lymphocytes between the two quantification methods. I may also have been unable to ascertain a useable reference gene in the explant experiments due to differing amounts of tissue damage within and between explants that did not occur in fresh flash frozen tissue.

Hsc70 transcript numbers were significantly higher in blubber explants of female grey seal pups and the addition of sex significantly improved model fit.

Females may have a specific requirement for *Hsc70*, particularly in the outer blubber section because adipose tissue of female mammals has been shown to have higher protein content than males (Rodríguez-Cuenca *et al.*, 2002). Constitutively expressed HSP (as a molecular chaperone) may therefore be required at higher levels in females to ensure their greater protein content is properly maintained. Conversely, male rats exposed to a high fat diet had higher protein levels of glucose regulated protein (GRP75), a member of the HSP70 family, in brown adipose tissue compared to females (Choi *et al.*, 2011). We could investigate these possibilities in grey seals with a protein assay or measurements to ascertain rates of protein synthesis in male and female pups, but we did not have enough tissue to perform these extra measurements.

There was no change in *Hsp70* expression between suckling and fasting in the mid-section of the blubber biopsy of 2013 pups (Chapter 4, Sections 4.3.1), which agrees with the blubber explant data presented here. In Chapter 4 (Section 4.4.3) PCB concentration in blubber tissue was suggested as a possible explanation for un-explained variance in HSP gene expression LME models. Here, as in the observational Chapter 4 data, individual still accounts for a large portion of unexplained variance. However, the addition of dioxin-like PCBs improves model fit. Additionally, there was no effect of PCB or PCB and HC treatment on *Hsc70* transcript number, whereas both these treatments resulted in increased *Hsp70* transcript number. This suggests contaminants are capable of inducing cellular stress and that *Hsp70* is specifically a good biomarker for contaminant exposure in grey seal blubber. Contaminants may be driving HSP gene expression by inducing chemical stress. HSPs have been reported to be induced by various contaminants in a number of species, including PAHs, heavy metals and pesticides. In other marine

mammal species (fin whales and striped dolphins), increases in *Hsp70* gene expression in response to PCBs have been reported in skin and blubber (Fossi *et al.*, 2010; Panti *et al.*, 2011). However, Buckman *et al.* (2011) found no relationship between *Hsp70* gene expression and PCB concentrations (congeners quantified using the same biopsy) in blubber of killer whales (*Orcinus orca*). It is important to note Buckman *et al.* (2011) only considered gene expression in the outer section of the blubber biopsy, whereas here the contaminant data came from the full depth of the blubber and the explants were derived from both inner and outer regions. Our data suggest that *Hsp70* may be induced directly or mediated through aryl-hydrocarbon receptor, which is activated by dioxin-like but not non-dioxin-like PCBs. Importantly, these data show that seal pups early in life are experiencing the increased cost of elevated *Hsp70* production in blubber tissue, which may have energetic consequences at the whole animal level as well as localised alterations to tissue function. It would be interesting to see if animals that are underweight and have more limited resources have elevated *Hsp70* expression in response to POPs, and thus might help to explain the survival consequences of POP exposure for grey seal pups (Hall *et al.*, 2009). If intrinsic contaminant data were available for Chapter 3 and 4 individuals we could investigate if PCB concentration explained more variation in gene expression of cellular defence genes and improved model fit.

The blubber explant culture protocol offers a standardised laboratory approach to stress monitoring in seals. However, there is a need to improve how cell viability is assessed and show awareness that the explant system currently only allows short term exposures. Although Routti et al. (2016) managed to maintain polar bear adipose tissue-derived stem cells for up to 14 days, grey seal explants were not as robust as those from other animals. Adipose explants of humans, rats and mice

can be maintained in culture for some days (2 days – 1week reported; Gesta *et al.*, 2003; Aubin *et al.*, 2015). The underlying causes of this and a subsequent solution are needed, in order to refine the method and extend the utility of the approach for longer term use. Also, the explant method poses a significant challenge for field conditions. While it represents a success that sterile culture was maintained, the logistics keep the experiments small, contained and only possible at certain times of year. Access to boats and capture teams are required to operate outside of the breeding season. Transferring into a predominantly laboratory setting will allow in depth exploration of experimental conditions and rely less on repeated samples from wild animals.

There are currently many different study designs that employ varying approaches to address questions regarding stress in marine mammals. Though such studies may address similar questions, different results may be yielded as a result of differing study designs. For example, as discussed in section 6.1, *in vitro* approaches used in wildlife to ascertain the potential impacts of POPs use different tissue types (e.g. PMNCs and PBMCs, adipose-derived stem cells, liver microsomes and skin) and culture conditions (Murk *et al.*, 1994; Hammond *et al.*, 2005; Fossi *et al.*, 2010; Routti et al., 2016). In marine mammals, stress monitoring is often done using cortisol (Busch and Hayward, 2009).

The development of standardised culture protocols would reduce the need to make comparisons between different study designs and inferences regarding the effects of stressors, such as POPs. For grey seal adipose tissue, creating and maintaining an immortalised cell line would be an ideal way to do this. Cultured primary cells experience replicative senescence, whereby, after a certain number of cell population doublings (which varies by species, cell type, and culture conditions),

primary cells enter a state where they can no longer divide (Stewart, 2002). Immortalised cell lines, however, have an extended proliferative ability and can possess the same genotype and phenotype as the tissue from which they were taken. This ensures consistent material throughout a research project. This would reduce variation within and between different experimental groups. Importantly, the ethical benefits of such a cell line cannot be understated. There would not be the requirement to repeatedly sample live animals to create explants; the cell line could be cultured, cryopreserved and stored long-term (Stewart, 2002). This would provide an opportunity to further optimise culture conditions and have a standard cell line that could be used to examine the effects of other relevant stressors, including other pollutants such as heavy metals, heat shock and hypoxic conditions. There are some marine mammal cell lines; Carvan *et al.* (2006) isolated a bottlenose dolphin (*Tursiops truncatus*) kidney epithelial cell line and Yin *et al.* (2013) established a fibroblast cell line from skin of Indo-Pacific humpback dolphins (*Sousa chinensis*). No such cell lines exist for marine mammal adipose tissue. An *in vitro* three-dimensional (3D) blubber culture system to mimic natural adipose tissue would be an ultimate goal to standardise approaches investigating, not only responses to stressors, but also adipose function in marine mammals. Most established cell lines are monolayer or suspension cultured, however 3D culture systems could bridge the gap between two-dimensional (2D) culture methods (using immortalised cell lines or adipose explants) and whole-animal experiments. Such systems could provide a useful tool for ascertaining further biological characteristics of adipose tissue and more accurately predict responses at the whole-animal level to experimental exposures (Kokai *et al.*, 2015; Lv et al., 2017). Currently, 3D culture systems continue to be optimised (Louis *et al.*, 2019). There are numerous methods that can be used to

achieve 3D culture: hydrogel systems (including collagen hydrogels); silk protein scaffolds; collagen based scaffolds, which hold adipocytes and allow them to differentiate in what is considered to be a more physiologically accurate representation on *in vitro* conditions (Kokai *et al.*, 2015; Ravi *et al.*, 2015).

The system could even include a microfluidic system to simulate circulating plasma (Park *et al.*, 2014; Kokai *et al.*, 2015) and the effects of vasculature on adipocyte function. Once this system had been established, it would then need validating in wild animals, which has already been shown to be challenging.

Standardising across marine mammals is incredibly challenging because of issues such as inaccessibility and logistics associated with working in the wild. Therefore, moving towards laboratory based methods removes these issues and may allow for more effective monitoring of health and toxicant exposure. In top predators, this may also allow for ecosystem monitoring, as it is assumed that an ecosystem must be functioning adequately if top predators can be supported (Ross, 2000). Furthermore, it could provide a global picture of toxicant exposure in numerous species. A standard suite of genes, including HSP70, could be developed as biomarkers that are routinely measured in marine mammal tissues to indicate POP exposure. Assessing the biological effects of contaminants on individuals or cultured cells and tissues may be a proxy for species and population level responses (Ross, 2000). Other studies have attempted to describe gene-level biomarkers. Noël *et al.* (2017) found blubber mRNA levels of estrogen receptor alpha, thyroid hormone receptor alpha and glucocorticoid receptor to be increased with increasing concentrations of PCBs in harbour seals (*Phoca vitulina*). Brown et al. (2014) also identified PCB-related increases of thyroid hormone receptor alpha, along with retinoic acid receptor α in harbour seals. Brown *et al.* (2014) and Noël *et al.* (2017)

highlight genes that have been identified in POP exposed versus non exposed populations. These would be ideal to test in our explant system to see if such genes are induced experimentally. An issue that needs to be considered the gene induction time; if it is a long time course then it is unlikely that we would be able to see gene responses. Measuring mRNA expression of molecular indicators of POP exposure, such as the aryl hydrocarbon receptor (AhR) and xenobiotic detoxification enzymes, including cytochrome P450 enzymes (Cpy1a1, Cyp1a2, and Cpy1b1) and uridine 5' diphosphoglucuronosyltransferase (Ugt1a1; Stejskalova *et al.*, 2011), may provide additions the list of potential biomarkers. These genes, directly involved in mediating the actions of POPs, could provide early warning signals of toxicant exposure and potential for adverse health effects.

Pioneering the use of blubber explant culture provides a unique opportunity to experimentally test adipose responses to physiologically and environmentally relevant substances in a large marine mammal that depends on adipose tissue for survival, and for which whole-animal experiments of this sort are not feasible, yet is of conservation and management importance. This chapter highlights the value in adopting experimental approaches to supplement observational data in an effort to explore variables that induce cellular stress responses. It highlights the need for optimisation of tissue culture protocols and appropriate endpoints to assess viability and health in explants rather than cells. Furthermore, it identifies the role of contaminants in inducing cellular stress over and above the stress response induced by culture alone, and shows that *Hsp70*, not *Hsc70*, helps identify POP exposure in fat tissue in marine mammals.

Chapter 7

General Discussion

7. General Discussion

Links between cellular and physiological stress responses in marine mammals, and their impact on tissue damage and fitness are poorly understood. Although stress responses may be activated under specific conditions, we do need to recognise that the induction alone does not always represent a state of distress. If we are to understand the role of stress as a driver of individual health, population dynamics and life history trajectories we need to be able to distinguish between eustress and distress at the whole-animal and cell levels. When animals are in a poor state of health or when cells are dying distress is easy to detect, but identifying problems before they arise is a better way to achieve conservation success and set management priorities. There is also the need to distinguish natural stress and allostatic mechanisms from those caused by anthropogenic activity. This thesis aimed to investigate the causes of cellular stress responses in tissues essential to fitness and survival of grey seal adult females and pups, which are routinely exposed to natural and anthropogenic stressors, and consequences for cell damage and trade-offs in energy investment at the whole animal level. Grey seals experience numerous physiologically stressful states throughout development and adulthood that induce cellular defences in other species. This includes fasting during the energetically demanding lactation and developmental periods, and large seasonal fluctuations in fat stores with consequently significant variations in adipocyte size. Individual cellular stress responses to 'life-style' stressors may affect grey seals' ability to cope with additional stress, such as disease, disturbance and contaminants in prey. Identifying vulnerable life-history stages of grey seals, when animals experience specific additional stressors as 'distress', requires correct interpretation of markers of cellular stress. This is essential to inform management of seal

populations by predicting population level consequences of exposure to specific stressors. It is important to ascertain whether HSP and RE biomarkers can be used to detect cellular stress under natural and experimental conditions and are reliable and early indicators of distress. Such data can be used to inform pinniped physiologists, who may be interested in exploring how cellular defence data could be linked to other physiological processes in adipose tissue and leukocytes (this may help confirm that data presented are a result of true stress and not an artefact of another natural physiological process) and environmental managers. Being able to inform environmental managers about distressing natural physiological states (i.e. the lactation-fast) could inform wildlife management programmes. For example, during the breeding season when grey seal females are hauled out on land, environmental managers could reduce potential sources of *cumulative* external stressors to help ensure successful breeding i.e. reduce disturbance from human activities. Given that water access appears to be a key driver of grey seal behaviour that dictates breeding success (Twiss *et al.*, 2012a), where practical, managers could sensitively remodel natural land (outside of the breeding season) to include areas that could become natural pools. This approach would reduce the need for breeding females to commute to water if temperatures increase and there is a risk of thermal stress. Such information could provide further argument to governing bodies about the importance of protecting breeding and foraging areas (to provide food security).

The studies in this thesis investigated the nutritional and developmental changes in gene expression of key cellular defences, HSPs and REs, and to explore the role of intrinsic and extrinsic stressors in their induction at the gene expression level, in two vital tissues, blubber and leukocytes. MDA concentration (a marker of

oxidative damage) was also used to measure tissue-level oxidative damage in blubber. I focussed on the lactation-fast of breeding female grey seals, and the suckling period and post-weaning fast in pups, because these life history stages require large energetic expenditure when resources are limited, and therefore may involve greater physiological stress and trade-offs between investment in stress responses and other vital physiological processes. The same cellular defence and tissue-level damage markers were also measured in pre-breeding female grey seals to enable further comparison with a life-history state when regular energy intake eases resource limitation and energetic requirements are likely to be lower. Three chapters (Chapter 3, Chapter 4 and Chapter 5) used an observational approach, documenting natural changes in REs and HSPs and attempting to identify potential drivers of the observed changes by exploring associations with body size and condition, and glucose levels, which helps investigate impacts of resource limitation. The degree of HSR induction was linked to fitness consequences at the cellular (measures of tissue damage (MDA concentration)) and whole animal levels (performance (for example, rate of mass loss and gain) during the lactation-fast, suckling and the post-weaning fast) to determine the extent to which investment in increased cellular defences results in organismal trade-offs. For example, the high energetic cost of increased HSP expression (HSP function is ATP dependent; Garnier *et al.*, 2002) may result in increased rate of mass loss in fasting females or pups as limited energy derived from adipose is allocated to cellular defences, and this may have negative consequences for fitness or survival. Chapter 6 used an experimental approach to make causal inferences about the role of two potential stressors for grey seals, the artificial GC, HC, and PCBs, to induce the HSR (focusing on gene expression of *Hsp70* and *Hsc70*) in adipose cells. Chapter 6 used

the adipose explant method, which has only recently been applied to grey seals (Bennett et al., 2017b) and attempted to further optimise culture conditions. Chapter 1 reviews current knowledge regarding eustress and distress in tissues and cells of marine mammals, with a focus on grey seals. Chapter 2 provides detailed methods that are used here to answer questions regarding causes of cellular stress and consequences for tissue function and whole animal health, filling knowledge gaps identified by Chapter 1. Chapter 3 and Chapter 4 consider relative gene expression of cellular stress markers (HSPs and REs) in blubber of breeding and pre-breeding females and pups during suckling and the post-weaning fast, respectively, because blubber is a key tissue in energy balance. Chapter 5 examines the same gene expression in leukocytes of both lactating-fasting females and pups, because animals may experience a trade-off between stress responses and immune function when resources are limited. These chapters identify nutritional and developmental causes of expression of cellular defences, and consider relationships with markers of tissue damage (MDA concentration) and whole-animal fitness. Chapter 6 used an experimental approach to examine causal relationships between GC and PCBs, and HSP gene expression in blubber.

7.1 Summary of findings and contribution to knowledge

All studies undertaken in this thesis have provided novel information regarding cellular stress in grey seals, investigating tissue-level damage and whether there are trade-offs between investment in cellular defences and other measures of fitness in grey seals (i.e. mass loss rate during the lactation-fast and mass gain rate during suckling). Experimentally, I have then considered potential drivers of the HSR (HC and/or PCBs).

Chapter 3 investigated nutritional changes in MDA concentration and relative gene expression of HSPs and REs in blubber of breeding and pre-breeding females. Female seals experienced a 23.20 ± 4.71 (SD) % (39.56 ± 8.18 (SD) kg) average body mass loss over 10.75 days of the lactation-fast period (between EARLY and LATE lactation sampling), which may be expected to either increase cellular stress as a result of large adipocyte size changes or concentration of blubber contaminants, or to decrease them as a result of resource limitation. However, there was no change in MDA concentration or mRNA abundance of any HSPs and the majority of REs, suggesting there is no change in cellular stress or investment in cellular stress responses during lactation. However, during LATE lactation relative expression of *Nox4* was significantly decreased compared to EARLY in lactation. Rather than increased inflammation, which is the typical interpretation of increased *Nox4*, I suggest the change could instead reflect a reduction in cell turnover to reduce energy expenditure (Mouche *et al,*. 2007), or lower insulin sensitivity LATE in lactation (Mahadev *et al.*, 2004; Goldstein *et al.*, 2005b; Meng *et al.*, 2008; Schröder *et al.*, 2009). Most interestingly, when these data were compared with that of pre-breeding females, MDA concentration and relative gene expression of all REs were increased in breeding females. This suggests that female grey seals experience greater oxidative stress throughout the lactation-fast and provides support for the life-history-oxidative-stress theory (Al Jothery *et al.*, 2016). It would be interesting to know whether there is an abrupt transition (i.e. greater oxidative stress) when females begin to fast and/ or give birth or whether there is a gradual increase as females approach the breeding season. This could be tested using females that are hauled-out and fasting but not breeding or comparing lactation-fast data with the moulting-fast. However, *Hsp90* and *Hsp27* were increased in pre-breeding females,

which may suggest that these chaperone proteins are relied upon during a period of

increased lipid biosynthesis (Pechan, 1991; Liu and Chang, 2008; Kuan *et al.*, 2016),

as pre-breeding females grey seals are laying down substantial fat reserves in

preparation for the lactation-fast (Anderson and Fedak, 1987; Pomeroy *et al.*, 1999;

Beck *et al.*, 2003; Sparling *et al.*, 2006).

Furthermore, there was no evidence of reduced performance during lactation

as a result of greater oxidative stress as maternal mass loss rates, maternal mass

transfer and pup weaning mass were not affected by expression of RE genes or

MDA concentrations. Higher levels of RE genes or MDA did not result in increased

mass loss rates or reduced mass transfer and pup weaning mass. This suggests a

trade-off as more energy is allocated to pup-rearing than cellular defences, resulting

in oxidative damage to macromolecules. However, the increased cellular stress in

female blubber does not have whole-animal level negative consequences for fitness

measures, suggesting they are not experiencing distress. As the data are available,

it would be interesting to investigate if females with higher relative RE gene

expression or MDA concentrations were those that did not breed the following year.

Chapter 3 also highlighted the importance of considering multiple life-history states

which, here, allowed a more meaningful interpretation of gene expression and MDA

concentration data. However, there is no indication of higher distress when HSPs are

induced in this age class. Females with very elevated HSPs may be experiencing an

additional stressor not part of the normal physiological response to fasting and

lactation (i.e. we do not know what other stressors females were exposed to; it might

be useful to know more about hydration state and temperate, for example), but it is

important to know what baseline is for that life history stage, which needs to be

explored experimentally. This could be done by repeating the explant work,

pioneered in Chapter 6, but with adipose tissue sample from lactating-fasting females. Tracking individuals longer-term, outside of the breeding season represents a challenge, as even if tags could be financed, grey seal females moult almost immediately after breeding so coverage of their movements and behaviours would be limited.

Chapter 4 considered nutritional and developmental changes in MDA concentration and relative gene expression of HSPs and REs in blubber of suckling and fasting grey seal pups. Pups experienced rapid mass gain during suckling, at a rate of 1.81 ± 0.45 (SD) kg day^{-1} (n = 18) over an average period of 10.75 days (between EARLY and LATE suckling sampling), which may be expected to induce cellular stress responses from expanding adipocyte size and high fuel availability. There was no change in MDA concentration or gene expression of the majority of HSPs, during suckling. Interestingly *Hsp90* increased, along with REs *CAT*, *SOD* and *Nox4* mRNA abundance. Gene expression changes were not associated with daily mass gain rate, suggesting increased cellular defences in suckling pups did not occur at the expense of growth or fat deposition(potentially because during suckling pups are not resource limited).

During the post-weaning fast, pups lost mass at a rate of 0.49 ± 0.26 (SD) kg day^{-1} (n = 15) over an average period of 9.87 days (between EARLY POST-WEANING and LATE POST-WEANING fast sampling). There was a further increase in their relative expression of *SOD, Nox4* and *Hsp90* and increases in *GPx* in some individuals. MDA concentration increased LATE in the POST-WEANING fast, when *CAT* and *GST* abundance fell. These data suggest elevated defences during the later stages of fasting were insufficient to prevent lipid damage, and that *CAT* and *GST* may be particularly important for adequate blubber protection. During fasting,

higher mass loss rate was associated with greater abundance of *Hsp90*, *Hsc70*, *SOD*, *Nox4* and *CAT*, suggesting that increased defences require greater investment of limited resources, or that pups with higher energy expenditure require greater antioxidant capacity. Though this may suggest fasting is the most vulnerable time for pups, due to lower cellular defences, but on-colony observations (H. Armstrong) suggest that the POST-WEANING fast is not a period of high mortality for pups. This could mean that blubber gene expression is not as important as other tissues at predicting when stress becomes distress. Importantly, individual explained more of the variance in gene expression than did nutritional state. Factors that differ between individuals that were not examined here, and in other Chapters in which individual explained most of the variance, therefore need to be considered in understanding regulation of blubber cellular defences. For example, pollutant, pathogen or parasite load may be important mediators of cellular stress responses. Exploring genetic variability between individuals may provide additional answers. There may also be an environmental factor (a specific topographic feature?) or behaviour that has not been captured/recorded (e.g. levels of aggression, bites, proximity to water and commuting/movement behaviour). Developmental or nutritional changes in cellular defences may impact on grey seal pups' ability to cope with additional stressors.

Chapter 5 examined relative gene expression of HSPs and REs in leukocytes, immune cells essential to protecting animals against pathogens and maintaining whole-animal health. There was no change in leukocyte HSP or RE expression during the lactation-fast in adult females. However, axial girth had a positive effect on *SOD*, suggesting that females in better condition are able to invest more in key cellular defences or in the ability of immune cells to perform respiratory burst (Dahlgren and Karlsson, 1999). *Nox4* was positively related to MDA concentration,

which may be expected since it is a pro-inflammatory cytokine. Leukocyte number or composition did not change during lactation. This again suggests that HSPs are elevated above background, and may indicate an additional stressor, since baseline is stable in this age category. This would benefit experimental investigation. It would be useful to compare these findings with pre-breeding females, as in Chapter 3, though leukocyte samples were not available, since the equipment was not available in the field in those locations.

Pups increased leukocyte *GST* and *Nox4* mRNA abundance during the post weaning fast and there was no effect of mass, axial girth or sex on mRNA abundance on any of the genes of interest in this age group. *GST* is involved in cell cycle control and increases in expression may result from cell maturation (Laborde, 2010). Alternatively, since POPs enter circulation from blubber during fasting, the role of *GST* in detoxifying a wide range of toxicants may be important in fasting pups. Surprisingly, RNA integrity analysis revealed the presence of a distinct third band between the 28S and 18S rRNA bands in all samples that I was unable to explain, despite attempts to denature, clone and sequence the band. The additional band was not gDNA contamination and may instead be secondary structure of RNA that was difficult to remove and may have resulted in the poor performance of a number of the markers used in this thesis. This type of problem has not been identified in the literature, but is important if we are to use HSPs and REs as markers of stress, since failure to perform appropriate quality control could result in erroneous information and interpretation.

Different tissues and age classes respond differently to different stressors or modifiers, which makes interpretation of HSPs and REs more challenging if they are

taken in isolation. We need a better mechanistic understanding of what both suites of genes are doing in each cell type.

It is important to adopt experimental approaches to supplement observational data in an effort to explore variables that induce cellular stress responses versus those that are simply correlates. Chapter 6 used the adipose explant method in blubber from pups, which provided a unique opportunity to experimentally test adipose cell responses to physiologically and environmentally relevant substances, HC and PCBs, over background effects of intrinsic POPs, nutritional state and blubber depth. Suitable culture conditions were investigated before the main experiments were initiated, using RNA integrity and a range of HSPs. This preliminary work revealed that the explant approach has a narrow window of functionality that needs further exploration and optimisation, and that more replicates (as there was only one per condition) are essential to confirm our suggestions for suitable culture conditions. This system does also mean that we can only explore acute or intrinsic effects of experimental treatments, and this may not be appropriate for cumulative or slow acting stressors such as POPs.

The main experiment, using suitable culture conditions, found higher absolute gene expression of *Hsp70* and *Hsc70* in outer blubber explants compared to inner explants. This may be due to increased blood flow to the surface of the skin as pups begin to moult late in the suckling period. However, this isn't a very convincing explanation, because then we would only see it in suckling pups. Alternatively, outer blubber sections may experience thermal stress in the 37°C incubator, since it is likely to be maintained *in vivo* closer to ambient temperatures. It would be useful to know the normal temperatures of blubber tissue. Endogenous PCBs explained variation in gene expression of *Hsp70* but not *Hsc70*. PCB and HC/PCB treatments

were also associated with increased *Hsp70* transcript number. Furthermore, we have identified the role of contaminants in inducing cellular stress and showed that *Hsp70*, not *Hsc70*, helps identify POP exposure and could be used as a biomarker.

All Chapters make it clear that it is necessary to know what baseline is, therefore spot measures are useful but of limited value. We also need to know covarites and research needs to better identify what those covariates might be.

7.1.1 Expression of cellular defences: comparing adult females and pups

Hsp90 relative gene expression was higher in suckling pups and pre-breeding females, compared to either fasting pups, or fasting-lactating females, respectively. Both of the former life-history stages involve laying down significant volumes of adipose tissue in preparation for fasting. This suggests that *Hsp90* is important for lipid biosynthesis in grey seals (Pechan, 1991; Liu and Chang, 2008; Kuan *et al.*, 2016). Furthermore, *Hsp90* was also increased in pups during the post-weaning fast, relative to suckling, which also implicates this molecular chaperone in maintenance of adipose tissue and modulation of use of lipids.

In females and pups, both axial girth and body mass had a significant positive effect on *SOD* relative expression in blubber and leukocytes. This finding concurs with Bennett *et al.* (2014) who found SOD enzyme activity to be positively correlated with blubber thickness of suckling and fasting grey seal pups. This suggests that SOD could be used as a marker of body condition, which itself is a proxy for fitness and survival probability.

Oxidative stress markers (REs) are increased during fasting (compared to feeding) in both adults and pups. This suggests that fasting is a vulnerable state and

additional stressors could negatively affect fitness and survival during these times. Levels of oxidative stress may even play a role in terminating the fast (i.e. pushing females to wean their pups and return to sea to forage and driving pups to sea to forage for the first time), however there is no evidence of this in other species.

7.1.2 Expression of cellular defences: comparing blubber and leukocytes

In breeding female blubber and leukocytes there was no change in relative gene expression of HSPs or REs during the lactation-fast, with the exception of reduced *Nox4* during LATE lactation in blubber. These tissue types play different roles at the whole-animal level. However, there is communication between them and adipose tissue is considered an immunologically active organ (Huh *et al.*, 2014). As an endocrine organ, adipose tissue releases adipokines which have been implicated in the control of immune cell activity under conditions of limited nutrient availability (i.e. fasting; Wensveen *et al.*, 2015). Additionally, adipose tissue is made up of multiple cell-types, including immune cells such as macrophages and lymphocytes (Lindroos *et al.*, 2011; Tallone *et al.*, 2011; Berry *et al.*, 2013), which may impact the relative gene expression values reported (if we are considering a cell population with various cell-types).

Blubber tissue experiences increased oxidative damage (as evidenced by MDA concentration) during fasting in pups, therefore cellular defences that increased may do so to protect cells. In leukocytes, increased GST may be as a result of increased exposure to circulating PCBs, mobilised from blubber tissue. Differences in relative expression of cellular defences suggests specific functional roles for some genes in different tissues and different levels of exposure to stressors (i.e. oxidative stress). Although blubber and immune function are linked, the two tissue types

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respond in specific ways to natural stressors, such that they provide different information about cellular stress. Using these tissues as stress indicators therefore requires the question to be formulated in a tissue-specific way.

7.2 Limitations of the work and improvements

The studies in this thesis used limited tissue volume and morphometric data available from a free-ranging, large marine mammal, that requires expert handling and sampling, is usually inaccessible unless hauled out whilst breeding and moulting and is of management importance.

Each chapter focused on one analytical method: *q*RT-PCR. Though a well-established, reliable and cost-effective technique to examine levels of mRNA of genes of interest (Pfaffl, 2001; Bustin, 2002; Pfaffl *et al.*, 2002; 2004; Bustin *et al.*, 2009; D'haene *et al.*, 2010), it would be useful to corroborate gene-level data with protein-level data. Studies that have examined mRNA and protein expression have generally found poor correlation, which highlights the importance of combining the two measurements to make inferences regarding functional importance of genes and proteins. Additional measurements could include enzyme activity for the REs.

Throughout this thesis, individual explained more of the variance in HSP and RE gene expression than fixed effects, such as nutritional state. Factors that differ between individuals need further attention as they should be considered in understanding regulation of blubber and leukocytes cellular defences. There are other covariates that could have been used:

1. Female age: most ages are not known with certainty. Animals included in the studies presented in this thesis are a part of a long-term study

programme on grey seal breeding systems and physiology led by SMRU. Therefore all animals are marked (either branded or tagged). Some animals were marked as juveniles, meaning exact year of birth and consequently age is known, but the majority of females were marked as breeding adults, meaning their exact age is unknown. Grey seal females sexually mature at approximately five years old, but have their first pup closer to six years old (Hewer, 1964; Hammill and Gosselin, 1995). However, it would be very unwise to extrapolate age by simply adding six years to the year the female first joined the long-term study. This would assume that the individual was a first-time mother which is impossible to determine without long-term monitoring of an individual that had been marked before sexual maturity. This also may have implications for data interpretation as older, more experienced mothers tend rear more successful pups (i.e. heavier pups at weaning; Bowen *et al.*, 2006). It is also possible that females lose their tags so they may appear as being new to the study, but have actually been in the study previously with a different tag number. These issues can be overcome by comparing pelage patterns to the database to determine whether the female has previously been handled or seen on the colony, but this depends on good quality images and coverage of the area that the female comes ashore. Age can be determined by analysing annual rings in the cement deposited on the canine teeth. However, taking a tooth is relatively invasive and animals may become more susceptible to infection after an extraction. In some of the females in this study, teeth had been taken to verify age, but in most cases this data was not available. With the current dataset, the few known

ages (*n* = 4) are skewed in favour of older females (between 23 and 29 years old; Debier *et al.*, 2003a; 2003b).

2. Colony location: general location on the colony was recorded for every individual at each capture. I believe that for inclusion as a fixed effect in LMEs the exact definition of location, such as topographic features, would be essential to ensure meaningful interpretation. Previous research has suggested that female grey seals show preference for pupping habitat with sea-access points intermediate distances from their pupping site and near pools of water with low salinity (most likely as a source of drinking water and for behavioural thermoregulation). Competition for space will mean some animals will be unable to secure preferred pupping locations and have to commute to pools of water, which may be an important factor that distinguishes individuals (i.e. animals that have to commute further to pools may experience thermal stress, especially if environmental conditions are dry). Twiss *et al.* (2003) examined accurate fine-scale maps of seal locations, noting mother-pup pair densities and topographic features of pupping habitat (determining potential causes of pup mortality), which is possibly beyond the scope of this thesis. It would also be informative if colony location could be matched with behavioural observations to show how much females commute to and from pools.

3. Behavioural observations: these data are available, for some animals here, because a parallel study in 2013, piloted a data logger that recorded heart rate, and was partnered with detailed behavioural observations of individuals (N. B. L Brannan, MSc Thesis, 2017; Dr Sean Twiss, University of Durham, UK). Behavioural types (for example, proactive or reactive

coping styles or high or low heart rate variability) could be used to help explain variability in gene expression of cellular defences and provide a link between cellular and whole-animal level stress responses.

4. Intrinsic POPs in blubber from 2013 breeding and pre-breeding females and pups: Chapter 6 showed that *Hsp70* may be a useful indicator of POP exposure. Along with *Hsc70*, this was the only cellular defence gene examined in pup blubber in 2016 for which intrinsic POP concentrations were known. The effect of POPs on induction of other cellular defences, including REs, in both pups and adult females is unknown but could, on an individual-level, help explain variation in relative gene expression.

Working with a free-ranging animal in a heterogeneous environment is likely to introduce variability in data, for example, due to distinct genetic and behavioural differences between individuals and variation in sampling conditions. Therefore, we may also expect differences in the ability of individuals to respond to stress and the 'strength' of individual cellular stress responses. GC responses to stress can vary massively by individual (Cockrem *et al.*, 2013). Again, using GCs as an example, individual differences between coping strategies or stress-coping styles were correlated with high and low levels of cortisol (i.e. animals with very strong reactions to disturbance generally had high levels of cortisol, relative to animals that showed a limited reaction (Cockrem *et al.*, 2013)).

7.3 Informing conservation policy and practice

The experimental strategies developed by this thesis could be further explored for use in general wildlife management programmes. However, in marine mammals and other wildlife species, including birds, cellular level stress is not

routinely used to monitor potential stress experienced by individuals: instead, measurements of glucocorticoids (GCs; commonly corticosterone or cortisol) are widely used and have greater potential to inform species management (Busch and Haywood, 2009). Using this approach (GC measurements) as an example, it is clear that there are unifying concepts associated with standardising such approaches, that I would also suggest for cell-level data: ensuring standard methodologies when sampling animals and making stress measurement (i.e. using the same tissue type and method across laboratories to measure GCs, for example, an optimised, species suitable ELISA); establishing baseline measures (for example, high levels of GCs do not necessarily indicate stress as GC measurements do not change in a consistent and predictable manner in association with adverse conditions and animals can experience adrenal exhaustion; Busch and Haywood, 2009); understanding the physiological roles of the stress measure, both functional and mechanistic, outside of the stress response (for example, GCs play important roles in regulating energy balance). If such practices were coordinated across species, it may help make data more interpretable within the context of different species i.e. most cellular measures of stress in pinnipeds are measured in different tissues (muscle, liver, fat) and molecular levels (gene, protein, enzyme activity) but in the same life-history states. As there is no standard protocol, it is difficult to compare data (Chapter 4 and 6) and make inferences about how different species may respond to physiological stressors in similar ways, which could help identify baseline values. Even if standard protocols are prepared and adopted it is still very challenging to obtain data that is able to translate into practical conservation policy and practise. There is still much work required to match the suggestions above, especially at the cellular level.

7.4 Can we distinguish eustress from distress in grey seals?

The approaches used in this thesis to distinguish between eustress and distress were not detailed enough to answer the title question conclusively. However, by identifying vulnerable tissues and life history stages we could pre-empt distress. This might be useful as an early warning, but we need to better understand links with what causes (and is a marker) of compromise to health and well-being.

Though potential life-history states have been identified as experiencing elevated oxidative stress (lactating-fasting female, fasting pups, with heightened defences and MDA concentrations), it is harder to then make suggestions regarding whether the raised response goes on to be adaptive or provide protection from additional stressors, or whether higher oxidative stress matters at the whole animal level.

I believe more experimental work would be needed to test this. For example, in an explant system, cells could be exposed to a known stressor, such as an inducer of oxidative stress. Heightened defences could be confirmed and MDA measured as a proxy of oxidative damage. Then cells as part of the same system could be experimentally exposed to an additional stressor and the same measurements taken. If there is no increase in MDA concentrations and cellular defences are raised further we could interpret that as eustress. As we are also uncertain what baseline values of HSPs and REs are, it also makes it much more difficult to interpret results and attempt to identify eustress and distress. If pre-breeding individuals had not been included in Chapter 3, it would have appeared as if there was no change in RE relative gene expression and MDA concentrations and no real comment could be made regarding eustress or distress. Comparing data to another life-history state identifies the lactation-fast as a period of potential distress. However, as lactation

performance measures were not affected by gene expression, maybe this is eustress. Further work is required to ascertain if this might be the case (i.e. suggested experimental exposures, looking at RE activity or tissue level measures to see if there are other associated increases).

7.5 Future work

There are many avenues that could be explored to provide more in-depth answers to the questions posed by this thesis.

1. Additional experimental work: similar to the experimental design of Chapter 6, culturing primary leukocytes could be used to investigate immune cell function and cellular defences in response to POPs and stress hormones (Hammond *et al.*, 2005). Leukocytes are easy to culture and work has been done in this regard before, providing a solid basis for this proposed direction.

2. Considering other life history states: to add to the current developmental time-course over which HSP and RE gene expression has been reported, juvenile moulters could be considered as they are the most accessible consecutive life-history state and can be maintained at the SMRU captive facility (Chapter 6). We know very little about this life-history stage. Since we know that in adult harbour seals, cortisol levels increase throughout blubber and the cells experience increased blood flow and higher turnover of cells, we might expect an increase in HSPs and maybe REs to support replacement of fur.

3. Considering other stress and cell function markers: here the HSR was considered in detail, however other cellular stress mechanisms and their

components could be examined, such as the unfolded protein response (UPR) which is central to maintaining protein homeostasis in the endoplasmic reticulum (ER).

4. Protein-level work using the same cellular defence markers as those presented here at the gene level (discussed in more detail in section 7.2).

5. Determining with absolute certainty the identity of the distinct third band seen between the 28S and 18S rRNA bands on RNA integrity 1% agarose gels.

6. Combining cellular and tissue-level data with behavioural observations, which may be more informative for environmental managers.

7. Applying this work to other marine mammal species.

Distinguishing between eustress and distress in grey seals is not easy and one should be mindful of other physiological natural life-history processes that may impact upon mRNA expression of cellular defences. This thesis identifies a fasted state as one that is distressing for grey seals. Both females and pups experienced increased mRNA abundance of REs and MDA concentration, which potentially implicates oxidative stress in the termination of the lactation-fast and the post-weaning fast. Hsp70 was identified as a potential biomarker of POP/PCB exposure in grey seal pups, and the further optimisation of adipose explant culture techniques could provide a framework for the standardised testing of, not only POP exposure, but more general stressors.

Appendix 1: Accompanying Chapter 6

Determination of HSP gene expression in the inner and outer sections of freshly flash frozen blubber core

The blubber biopsies taken from both females and pups (as described in Chapter 2, General Methods, section 2.1.2.6) at various stages of lactation and development appear as a thin, cylindrical shaped sample of tissue, the blubber core (Appendix Figure 1), varying in length and circumference depending on when they are taken in the animal's life history. The blubber core can be divided into the inner core (IC; adjacent to the muscle layer), outer core (OC; located towards the surface of the skin or epidermis) and mid core (Debier *et al.*, 2003; Strandberg *et al.*, 2008). Blubber closer to the muscle, and a blood supply, is believed to be more metabolically active than blubber that is adjacent to the epidermis (Strandberg *et al.*, 2008). Fasting seals largely use fatty acid oxidation to meet the energetic costs of fasting (Fedak and Anderson, 1982; Anderson and Fedak, 1987; Reilly, 1991; Iverson *et al.*, 1993, 1995; Mellish *et al.*, 2000; Bowen *et al.*, 2001) and blubber closest to the blood supply is more likely to be catabolised first. In addition blubber exhibits stratification in fatty acid composition (Koopman *et al.*, 2007; Strandberg *et al.*, 2008) and contaminant load and type (Debier *et al.*, 2003). Therefore ascertaining whether there was a difference in the expression of genes of interest in the inner and outer blubber core was important.

Size and distribution of adipocytes, which vary in harbour porpoise blubber cores (Koopman *et al.*, 2002), may also be a factor that has the potential to influence gene expression.

This preliminary study aims to ascertain how changes in physiological state can influence gene expression of HSPs and REs in the inner and outer blubber core of breeding females and pups. Tissue collected from lactating-fasting adult females and suckling and fasting pups, from the Isle of May, 2013.

Materials and Methods

See Chapter 2, General Methods sections 2.1.2.1, 2.1.2.2, 2.1.2.3, 2.1.2.4, 2.1.2.6, 2.1.3.1, 2.1.3.2, 2.1.3.3, 2.2.2, 2.2.4, 2.2.5 for detailed methods regarding sample regime and collection, RNA extraction, cDNA synthesis and qRT-PCR conditions.

RNA was extracted from the inner and outer core of breeding adult female and pup blubber from 2013 ($n = 13$) at EARLY and LATE lactation and EARLY POST WEANING and LATE POST WEANING fast time-points from the samples for which inner and outer sections of the cores were visually distinguishable (Appendix Figure 1). Samples were chosen at random from a variety of different animals (both mothers and pups) and time points (EARLY/LATE lactation, EARLY/LATE POST WEANING fast), dependent upon whether they corresponded to the previously described criteria. Mass of the blubber core before tissue dissection was recorded.

Biopsies taken from mothers at EARLY lactation appear longer in length than biopsies taken at LATE lactation (pers. obs.). When collected in the field, the sample was always placed in the tube IC first, which allows the difference between the ends to be established. qRT-PCR was used to determine the expression of HSPs in the inner and outer core with YWHAZ and L8 used as reference genes as determined by methods described in Chapter 2, General Methods, section 2.2.2.
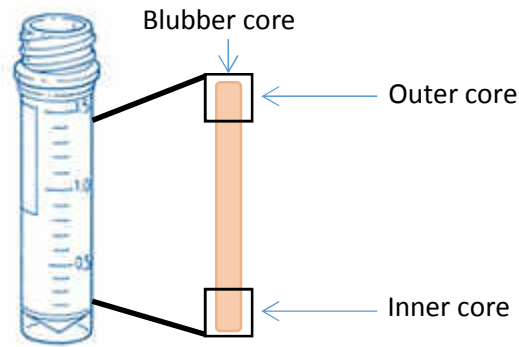
Appendix Figure 1. NOT TO SCALE Diagram to show the distinguishable position of the blubber core in the microcentrifuge tube, with the inner and outer section of the core clearly labelled.

Results

Data analysis using REST 2009 software suggests that there is no significant difference ($p > 0.05$; Appendix Figure 2) difference in the expression of HSPs between the inner and outer core.
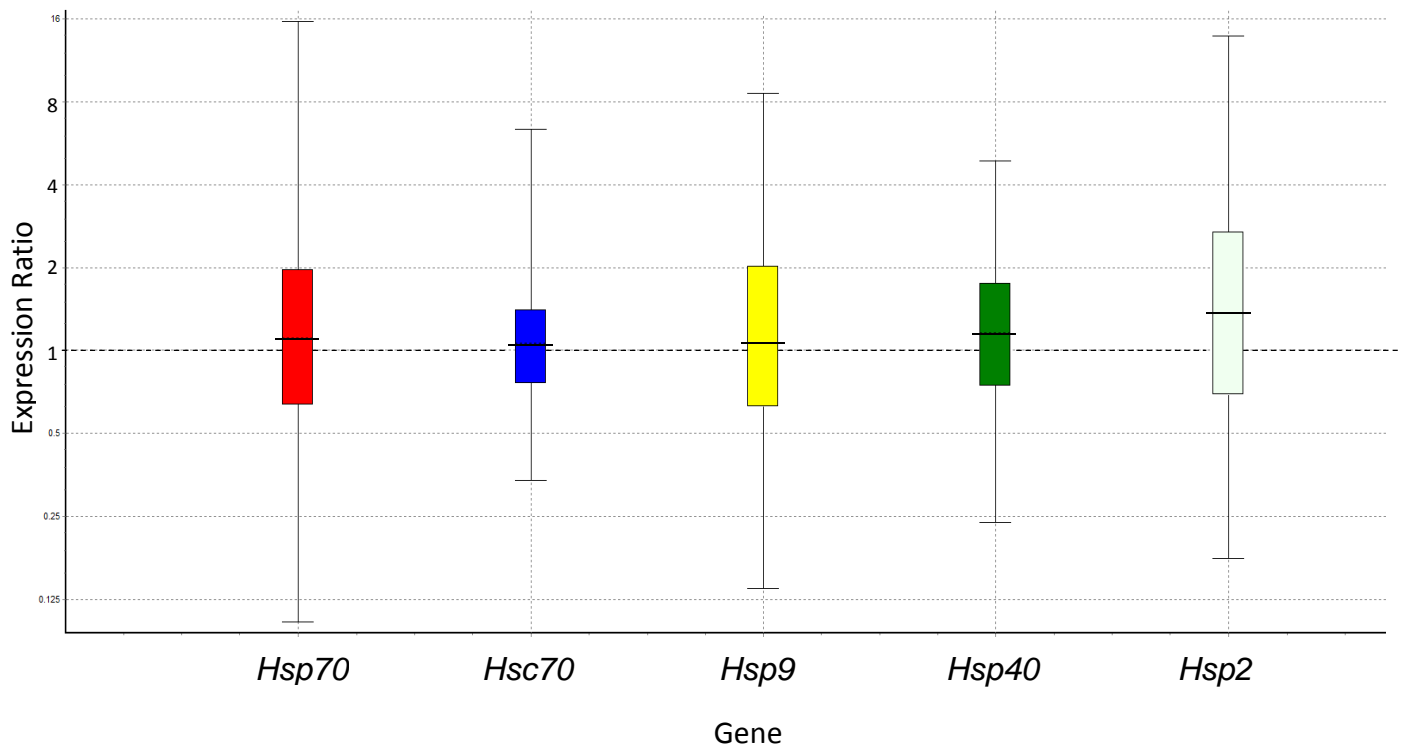
Appendix Figure 2. Relative mRNA abundance of *Hsp70, Hsc70, Hsp90, Hsp40, Hsp27* in 2013 adult female and pup grey seal blubber inner and outer core, at EARLY lactation (~day 5), LATE lactation (~day 15), EARLYPOST WEANING and LATE POST WEANING fast (REST 2009). Box shows interquartile range. Median expression of early lactation samples compared to late lactation samples for each gene is highlighted by the solid black line on each bar. Dotted black line across the graph indicates where median expression is not different in early and late lactation samples.

Appendix 2: Accompanying Chapter 4

Blubber heat shock protein and redox enzyme mRNA abundance and oxidative damage are higher during the post-weaning fast and associated with increased mass loss rates in grey seal pups.

Results

Tables A2.1, A2.2, A2.3 and A2.4 show outputs from LMEs performed in R, testing additional explanatory variables to explain variability in cellular defences (both HSPs and REs). Nutritional state ('Time-point') was the most important covariate and improved model fit in all cases. When 'Time-point' was removed and, for example, body mass included alone, model fit worsened (i.e. residuals showed a heteroscedastic pattern and AIC values increased). Therefore was 'Time-point' was included in all models.

Table A2.1. Additional explanatory variables included as covariates in LMEs to explain variability in log relative gene expression of the HSPs. Each HSP was modelled separately. Time-point represents nutritional state (i.e. EARLY or LATE suckling or EARLY or LATE POST-WEANING fast). Models in which body mass, axial girth, circulating glucose, sex or MDA concentration was included were then tested against the simpler model with no covariates using the anova function. Bold font indicates $p < 0.05$.

Gene	Model	Marginal R^2	Conditional R^2	L ratio	p
Log *Hsp70*	Time-point + Body mass (kg)	0.13	0.21	2.84	0.092
	Time-point + Axial girth (cm)	0.082	0.18	0.37	0.54
	Time-point + Sex	0.075	0.19	0.0071	0.93
	Time-point + Circulating glucose (mM)	0.072	0.22	0.0050	0.94

	Time-point + MDA concentration (nmol/µL)	0.0082	0.18	0.37	0.54
Log *Hsc70*	Time-point + Body mass (kg)	0.060	0.36	0.27	0.60
	Time-point + Axial girth (cm)	0.054	0.36	0.023	0.88
	Time-point + Sex	0.055	0.36	0.040	0.84
	Time-point + Circulating glucose (mM)	0.057	0.35	0.14	0.71
	Time-point + MDA concentration (nmol/µL)	0.062	0.50	0.53	0.47
Log *Hsp90*	Time-point + Body mass (kg)	0.17	0.40	0.53	0.47
	Time-point + Axial girth (cm)	0.15	0.41	0.061	0.81
	Time-point + Sex	0.16	0.41	0.20	0.65
	Time-point + Circulating glucose (mM)	0.17	0.39	0.050	0.82
	Time-point + MDA concentration (nmol/µL)	0.19	0.51	1.19	0.27
Log *Hsp40*	Time-point + Body mass (kg)	0.049	0.30	0.86	0.35
	Time-point + Axial girth (cm)	0.034	0.29	0.20	0.65
	Time-point + Sex	0.030	0.29	0.0011	0.97
	Time-point + Circulating glucose (mM)	0.035	0.29	0.20	0.66
	Time-point + MDA concentration (nmol/µL)	0.047	0.28	0.034	0.85
Log *Hsp27*	Time-point + Body mass (kg)	0.052	0.23	0.68	0.41
	Time-point + Axial girth (cm)	0.043	0.22	0.24	0.62
	Time-point + Sex	0.041	0.23	0.11	0.74
	Time-point + Circulating glucose (mM)	0.050	0.21	0.88	0.35
	Time-point + MDA concentration (nmol/µL)	0.12	0.23	1.90	0.17

Table A2.2. Further explanatory variables included as covariates in LMEs to explain variability in log relative gene expression/ relative gene expression of HSPs during suckling and fasting. Each HSP was modelled separately. Time-point represents nutritional state (i.e. EARLY or LATE suckling or EARLY or LATE POST-WEANING fast). Models in which body mass gain rate, maternal mass transfer to pup or mass loss rate were included were then tested against the simpler model with no additional covariates using the anova function. Bold font indicates $p < 0.05$.

Gene	Model	Marginal R^2	Conditional R^2	L ratio	p
Hsp70	Time-point + Mass gain rate (during suckling; kg day^{-1})	0.011	0.31	0.24	0.62
	Time-point + Maternal mass transfer to pup (during suckling; %)	0.021	0.31	0.47	0.49
Log *Hsp70*	Time-point + Mass loss rate (during fasting; kg day^{-1})	0.13	0.35	2.59	0.11
Hsc70	Time-point + Mass gain rate (during suckling; kg day^{-1})	0.068	0.58	1.27	0.26
	Time-point + Maternal mass transfer to pup (during suckling; %)	0.0030	0.58	0.015	0.90
Hsp90	Time-point + Mass gain rate (during suckling; kg day^{-1})	0.30	0.75	<0.0001	0.99
	Time-point + Maternal mass transfer to pup (during suckling; %)	0.30	0.75	0.00014	0.99
Hsp40	Time-point + Mass gain rate (during suckling; kg day^{-1})	0.039	0.47	0.43	0.51
	Time-point + Maternal mass transfer to pup (during suckling; %)	0.021	0.46	0.051	0.82
Log *Hsp40*	Time-point + Mass loss rate (during fasting; kg day^{-1})	0.022	0.12	0.64	0.42
Hsp27	Time-point + Mass gain rate (during suckling; kg day^{-1})	0.031	0.15	0.00012	0.99

	Time-point + Maternal mass transfer to pup (during suckling; %)	0.035	0.15	0.13	0.72
Log *Hsp27*	Time-point + Mass loss rate (during fasting; kg day^{-1})	0.025	0.26	0.46	0.50

Table A2.3. Additional explanatory variables included as covariates in LMEs to explain variability in the relative gene expression/ log relative gene expression of the REs. Each RE was modelled separately. Time-point represents nutritional state (i.e. EARLY or LATE suckling or EARLY or LATE POST-WEANING fast). Models in which body mass, axial girth, circulating glucose, sex or MDA concentration was included were then tested against the simpler model with no covariates using the anova function. Bold font indicates $p < 0.05$.

Gene	Model	Marginal R^2	Conditional R^2	L ratio	*p*
Log *GPx*	Time-point + Body mass (kg)	0.47	0.83	0.11	0.74
	Time-point + Axial girth (cm)	0.47	0.84	0.0063	0.94
	Time-point + Sex	0.54	0.83	2.92	**0.087**
	Time-point + Circulating glucose (mM)	0.47	0.84	0.90	0.34
	Time-point + MDA concentration (nmol/μL)	0.52	0.86	0.42	0.52
CAT	Time-point + Body mass (kg) + Axial girth (cm)	0.24	0.67	0.61	0.44
	Time-point + Body mass (kg) + Sex	0.27	0.64	2.31	0.13
	Time-point + Body mass (kg) + Circulating glucose (mM)	0.20	0.62	2.57	0.11
	Time-point + MDA concentration (nmol/μL)	0.29	0.60	0.0044	0.95

Log *SOD*	Time-point + Body mass (kg) + Axial girth (cm)	0.39	0.77	0.52	0.47
	Time-point + Body mass (kg) + Sex	0.41	0.77	0.80	0.37
	Time-point + Body mass (kg) + Circulating glucose (mM)	0.39	0.78	0.0041	0.95
	Time-point + Body mass (kg) + MDA concentration (nmol/µL)	0.54	0.79	<0.0001	0.99
GST	Time-point + Axial girth (cm) + Body mass (kg)	0.34	0.49	0.0013	0.97
	Time-point + Axial girth (cm) + Sex	0.35	0.49	0.38	0.54
	Time-point + Axial girth (cm) + Circulating glucose (mM)	0.33	0.47	0.53	0.46
	Time-point + Axial girth (cm) + MDA concentration (nmol/µL)	0.36	0.61	3.46	**0.063**
Log *Nox4*	Time-point + Body mass (kg)	0.098	0.38	0.13	0.71
	Time-point + Axial girth (cm)	0.096	0.37	0.045	0.83
	Time-point + Sex	0.097	0.36	0.034	0.85
	Time-point + Circulating glucose (mM)	0.12	0.44	1.21	0.27
	Time-point + MDA concentration (nmol/µL)	0.24	0.37	1.49	0.22

Table A2.4. Further explanatory variables included as covariates in LMEs to explain variability in the relative gene expression of REs during suckling and fasting. Each RE was modelled separately. Models in which body mass gain rate, maternal mass transfer to pup or mass loss rate was included were then tested against the simpler model with no covariates using the anova function. Bold font indicates $p < 0.05$.

Gene	Model	Marginal R^2	Conditional R^2	L ratio	p
Log *GPx*	Time-point + Mass gain rate (during suckling; kg day^{-1})	0.034	0.70	0.54	0.46
	Time-point + Maternal mass transfer to pup (during suckling; %)	0.098	0.65	1.68	0.19
	Time-point + Mass loss rate (during fasting; kg day^{-1})	0.14	0.84	0.15	0.69
CAT	Time-point + Body mass (during suckling; kg) + Mass gain rate (during suckling; kg day^{-1})	0.15	0.73	0.17	0.68
	Time-point + Mass gain rate (during suckling; kg day^{-1})	0.13	0.68	0.013	0.91
	Time-point + Body mass (during suckling; kg)	0.15	0.73	0.68	0.41
	Time-point + Maternal mass transfer to pup (during suckling; %)	0.24	0.58	1.21	0.27
	Time-point * Mass loss rate (during fasting; kg day^{-1}) + Body mass (during fasting; kg)	0.38	0.74	1.88	0.17
Log *SOD*	Time-point + Mass gain rate (during suckling; kg day^{-1})	0.29	0.79	0.35	0.56
	Time-point + Maternal mass transfer to pup (during suckling; %)	0.37	0.78	2.32	0.13
	Time-point + Mass loss rate (during fasting; kg day^{-1})	0.012	0.62	10.56	**0.0012**

GST	Time-point + Mass gain rate (during suckling; kg day^{-1})	0.14	0.41	1.76	0.18
	Time-point + Maternal mass transfer to pup (during suckling; %)	0.12	0.34	0.59	0.44
	Time-point + Mass loss rate (during fasting; kg day^{-1})	0.38	0.64	2.66	0.10
Nox4	Time-point + Mass gain rate (during suckling; kg day^{-1})	0.25	0.49	1.10	0.29
	Time-point + Maternal mass transfer to pup (during suckling; %)	0.30	0.30	0.11	0.74

Appendix 3: Accompanying Chapter 5

Heat shock protein and redox enzyme gene expression dynamics in grey seal (*Halichoerus grypus*) leukocytes in relation to nutritional state, morphometrics and blood glucose: abundance of glutathione-S-transferase and NADPH oxidase 4 mRNA is increased in fasting pups.

Grey seal WBC RNA integrity reveals three distinct bands

RNA integrity analysis revealed the presence of a distinct third band between the 28S and 18S ribosomal RNA (rRNA) bands in all samples that I was unable to explain, despite attempts to denature, clone and sequence the band. The additional band was shown not to be gDNA contamination. Further details are provided below and I speculate on additional explanations for the presence of the third band and offer some suggestions to investigate it further.

Materials and methods

Cloning

500ng WBC RNA (from various samples that had been tested on the Bioanalyzer and had RIN values of 6 and above; range 4.2 – 7.0, mean RIN 6.43, *n* = 12) was reverse transcribed and subsequent cDNA run out on a 1% agarose gel. Six separate bands were excised as precisely as possible from the gel and DNA fragments purified for cloning using PureLink ™ Quick Gel Extraction Kit (Invitrogen, Life Technologies Ltd, Paisley, UK). Cloning protocol was performed using the CloneJET™ PCR Cloning kit (#K1231; Thermo Scientific, Basingstoke, UK) as described in Chapter 2; General Methods; sections 2.2.7.3 – 2.2.7.3.7. Briefly, *E. coli* strain DH5α cells (Invitrogen, Thermo Fisher Scientific, Basingstoke, UK) were plated and exponential cell growth achieved before transformation. Purified DNA

fragments of 1μL volume (concentrations between 5 – 25 ng μL^{-1}) were blunted prior to ligation and were inserted into vectors using calcium chloride transformation. Subsequent transformants were used in colony PCR using pJET 1.2 Forward and Reverse sequencing primers (Thermo Scientific, Basingstoke, UK; Chapter 2; General Methods; section 2.2.7.3.7). PCR products (samples 1-6) were treated with ExoSAP (exonuclease I; Thermo Scientific, Thermo Fisher Scientific, Basingstoke, UK; and shrimp alkaline phosphatase; Fermentas Life Sciences, Thermo Fisher Scientific, Basingstoke, UK) before being sent for Sanger sequencing to Source BioScience Ltd. (Nottingham, UK). Sequence Scanner Software 2 (Applied Biosystems) was used to visualise sequences and call bases where appropriate. Sequences were then inputted into the nucleotide Basic Local Alignment Search Tool (BLAST) to compare and identify similar sequences.

Results

When RNA integrity was examined on a 1% agarose gel, all female and pup samples at all time points showed three distinct bands (Figure A3.1).
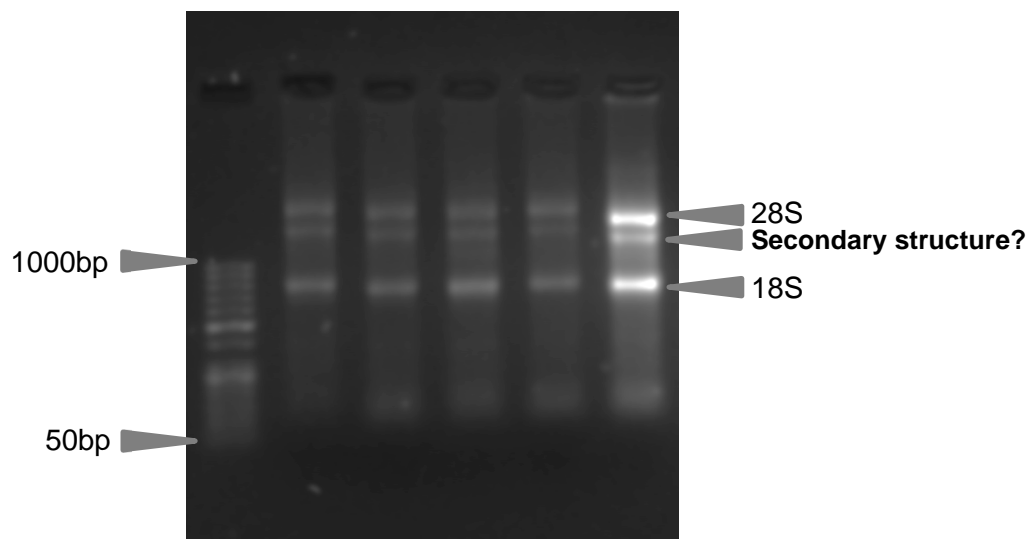
Figure A3.1 Examples of RNA from various female and pup WBC samples. In these examples, approximately 250ng total RNA were run alongside O'Generuler™ 50bp DNA ladder (Thermo Scientific) on a 1% agarose gel. An additional unexplained band is visible in between the 28S and 18S ribosomal units.

RNA was treated with a gDNA elimination buffer (taken from the reverse transcription protocol; QIAgen, Manchester, UK) to test whether the additional band was a result of gDNA contamination. Examination of a subsequent gel run showed the third band remained intact and visible after gDNA elimination treatment (Figure A3.2).
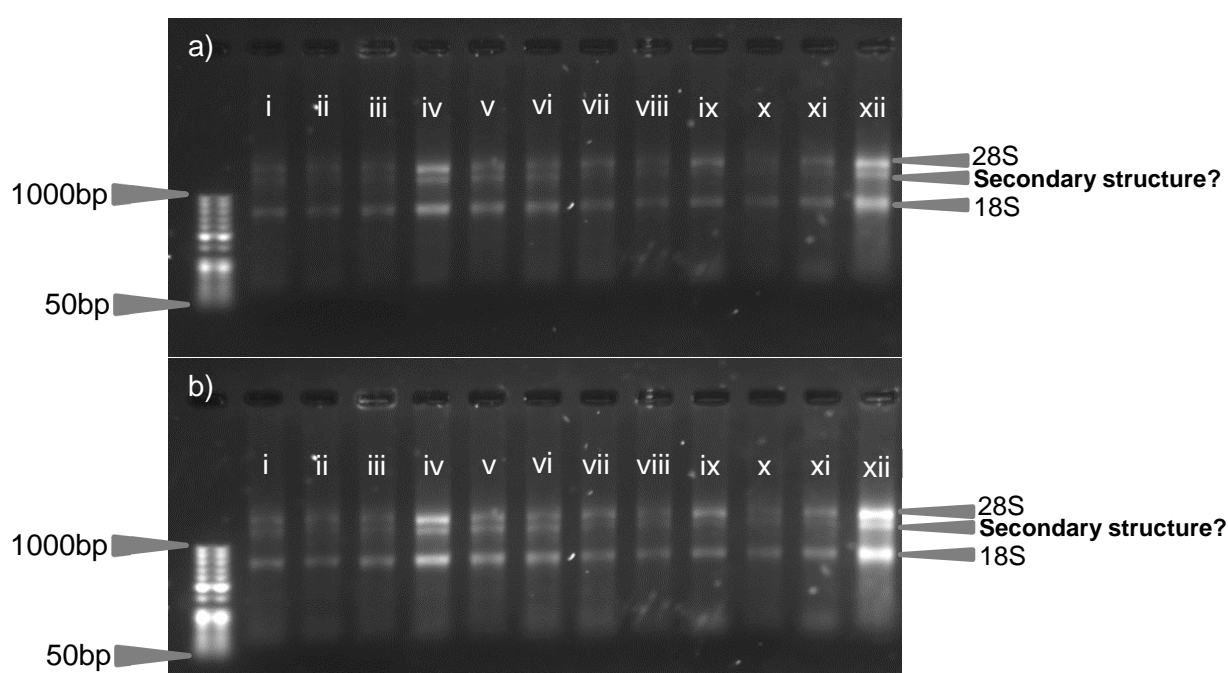
Figure A3.2. Examples of RNA from various female and pup WBC samples (all time-points represented). In these examples, approximately 250ng total RNA were run alongside O'Generuler™ 50bp DNA ladder (Thermo Scientific) on a 1% agarose gel. Images a) and b) are the same image at different exposures to improve the appearance of fainter lanes; a) 20 seconds; b) 30 second exposure. The additional unexplained band remains between the 28S and 18S ribosomal units. Lanes labelled i – xii are identified as follows: i, 4B late lactation (L.L); ii, 4B pup late suckling (L.S); iii, 4B pup early suckling (E.S); iv, 4B pup early post weaning fast (EPWF); v, 4B early lactation (E.L); vi, 7J E.L; vii, 7J L.L; viii, PFT E.L; ix, PFT L.L; x, PFT pup late post weaning fast (LPWF); xi, PFT pup EPWF; xii, PFT pup L.L.

To further explore the possible explanations for this third band, samples were then run on the Bioanalyzer Instrument (Agilent Technologies, California, U.S.A), which confirmed the third band, despite a heat denaturation step to eliminate secondary structures, via the electropherogram and gel image output (Figure A3.3).
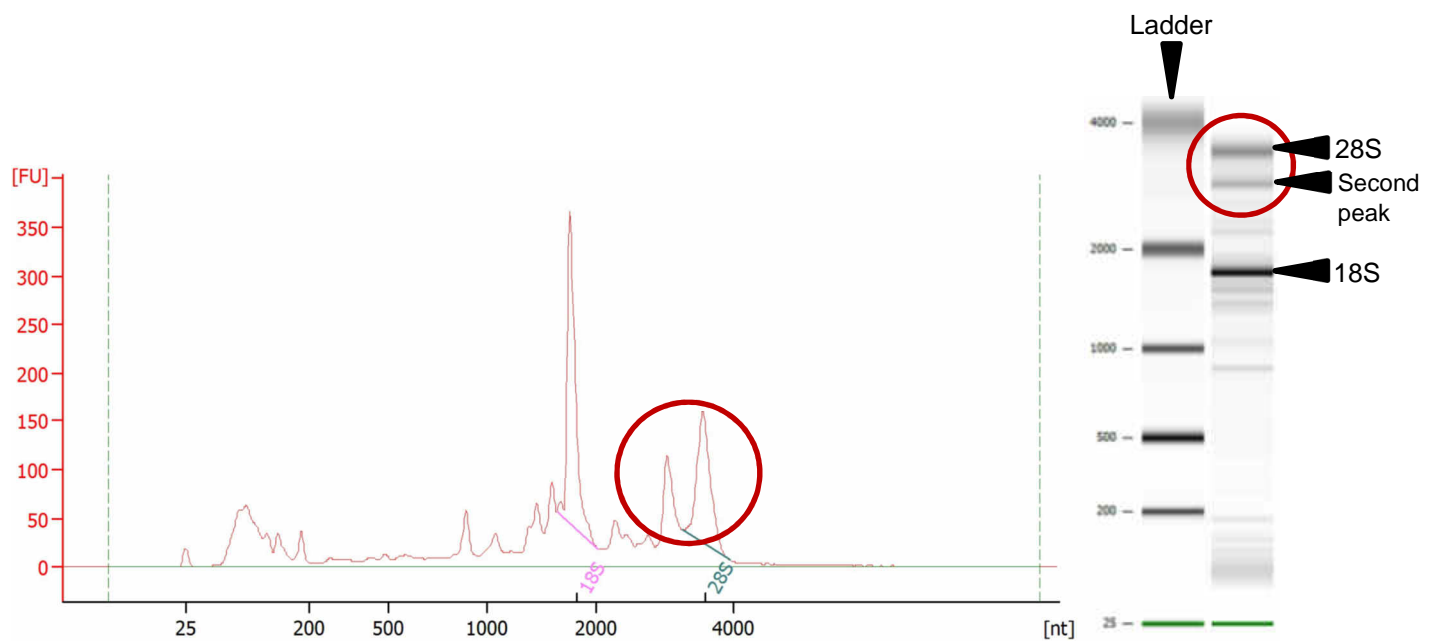
Figure A3.3. Electropherogram and gel image output from the Agilent Bioanalyzer 2100 system (assay class: Eukaryote Total RNA Nano, version 2.6). In this example, 2μL female (LATE lactation) RNA was run alongside the RNA 6000 NanoLadder (Agilent RNA 6000 Nano Kit; Agilent Technologies Inc.). These results are typical of all WBC samples (female and pup samples at all time-points): a peak of similar size next to the 28S peak; a band on the gel image next to the 28S band (which matches agarose gel runs); both are highlighted by red circles.

Sequences from cloning work confirmed, via BLAST, that the DNA from the PCR product had not inserted into the vector. BLAST results matched only the cloning vector sequence or completely unrelated sequences (including sesame seed (*Sesamum indicum*) genome matches). Poor transformation efficiency (i.e. few

transformants; mean 27.83 ± 14.08 (SD); range 8-46 colonies) had already suggested that this was most likely the case. Cloning experiments thus failed and provided no further information regarding the identity of the additional band.

Primers

Since the band was present in all samples and was not removed by gDNA elimination or denaturation, qPCR was performed. Gene specific primers that had previously been optimised for blubber (fat tissue) were used in WBC qRT-PCR experiments. However, during quality control checks of qRT-PCR experimental output, melt curves showed that not all primers amplified one specific amplicon and some efficiencies were not optimal (i.e. between 90-110%).

GPx, CAT and Hsp27: amplification and efficiency

Details of primer pairs, amplicons and efficiencies are displayed in Table A3.1. *GPx*, *CAT* and *Hsp27* were notably problematic; *CAT* (for both female and pup samples) and *Hsp27* (females only) had efficiencies that were 3.12x, 4.74x and 1.81x, respectively, higher than the maximum optimum (i.e. over 110%). *GPx* primers amplified two or three amplicons, as demonstrated by multiple Tm peaks (Figure A3.4), with amplification efficiencies 0.80x and 0.89x below the minimum optimum (i.e. under 90%). Though *Hsc70* showed specificity, amplification efficiency for this primer pair was also poor, 0.78x below the minimum optimum.

Table A3.1. Primer pair sequences, amplicon sizes and efficiencies of HSP and RE genes used in qRT-PCR experiments for both females and pups. Primers that amplify a single amplicon are identified as "Y" (Yes) and two or more amplicons "N" (N).

Gene	Primer Sequence 5'-3'		Amplicon size	Single amplicon? (Y/N)	Female efficiency (%)	Pup efficiency (%)
	Forward	Reverse				
L8	GGTGTGGCTATGAATCCTGT	ACGACGAGCAGCAATAAGAC	126	Y	92.21	94.10
UXT	CTCACAGAGCTCAGCGACAG	AGGTGTCTCCGGGAAATTCT	117	Y	84.50	108.83
Hsp70	AAGATCACCATCACCAACGA	AAATCACCTCCTGGCACTTG	238	Y	87.14	NA
Hsc70	AATCAAGTTGCGATGAACCC	CCTGCCAGCATCATTCAC	139	Y	69.86	NA
Hsp90	TGGAGCGTCTTCGGAAG	TCTGGAAGTTCCAAGCCCT	139	Y	96.85	NA
Hsp40	CCTGGAATGGTTCAGCAAAT	GCCATCTTTCATGCCTTTGT	159	Y	71.97	NA
Hsp27	AGCTGACGGTCAAGACGAAG	GGCAGCGTGTATTTTCGAGT	112	Y	199.09	NA
GPx	TTGTCAACGTGGCCAGCTA	TGAGGCTGGGTAGGATTTCC	158	N	71.79	80.46
CAT	GGTAATTGGGATCTTGTTGG	ATGGTCTGGGACTTCTGG	140	Y	342.88	521.35

SOD	CCTGGAGCCTCAC ATCAAC	TAGCTCTTCAGCCT GGGCT	127	Y	116.66	126.48
GST	CCTCAAGGAGAGA ACCCTGA	CTGGGCCATGTTAA CCACTT	123	Y	103.88	106.24
NOX4	AGCCTCCGCATCT GTTCTTA	CTTCTGGTTCTCCT GCTTGG	114	Y	101.60	85.77
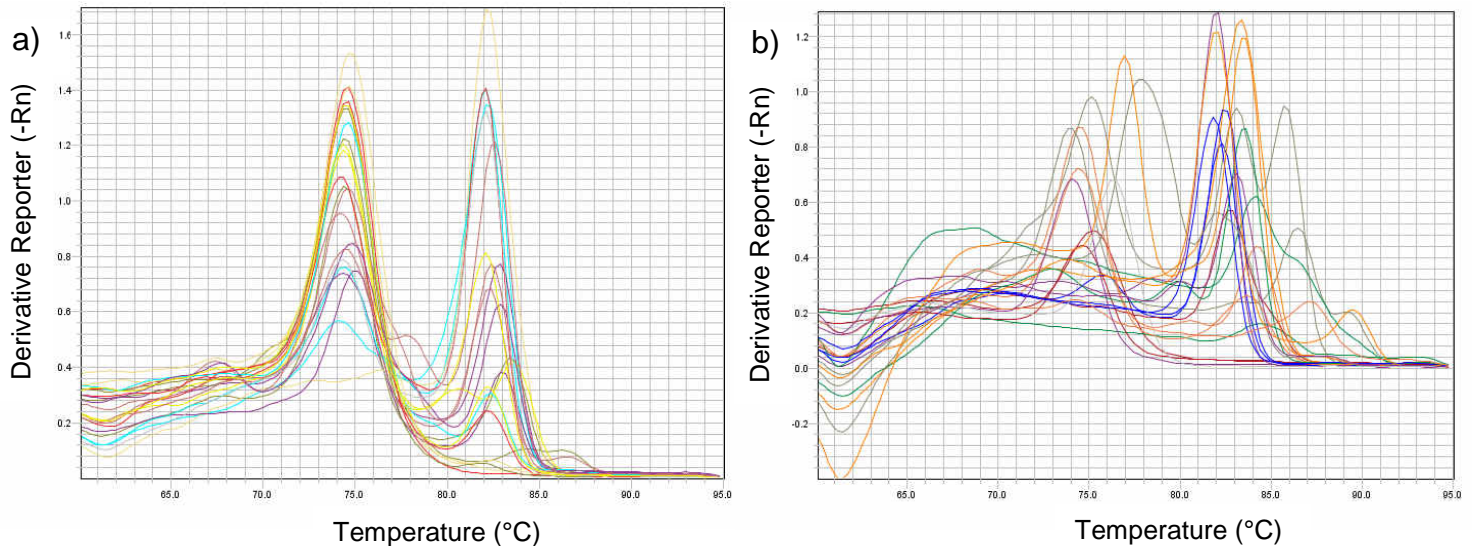
Figure A3.4. Melt curve plots, taken from QuantStudio™ 12K Flex Software, showing the multiple Tm peaks (two - three) of GPx for a) female and b) pup samples. Melt curves for all samples are presented at once on each plot.

Discussion: unexplained distinct third band during integrity analysis of RNA

There were a number of surprising technical issues associated with this experiment, including the presence of an unexplained third band during integrity

analyses of total RNA (for all samples) and poor performance of multiple primer pairs (leading to their exclusion from further analyses).

We were unable to explain the distinct third band, located below the 28S rRNA band, despite attempts to confirm it's identify via sample analysis using the Bioanalyzer Instrument (Agilent Technologies, California, U.S.A), during which RNA samples were heated to denature and remove secondary structures, and cloning, which failed. The only certainty is that this band is not a result of gDNA contamination, as it was still present after treatment to remove gDNA from the RNA samples.

It is likely that the third band may still be the result of an RNA secondary structure. Most RNA forms extensive secondary structures (via intramolecular base pairing) that are separated on native gels resulting in multiple bands, also preventing RNA from migrating precisely according to size (Spohr *et al.*, 1976). Secondary structures can be denatured and eliminated chemically; chemical denaturation also prevents secondary structure re-formation more effectively than heat treatment alone. In this case, the heat conditions may not have been sufficient to denature the secondary structure (i.e. the temperature of the heat denaturation step employed during the Bioanalyzer protocol (70-72°C) may not be above the melting temperature of the secondary structure, especially if it is highly structured). Running grey seal leukocyte RNA on a formaldehyde denaturing gel may remove the third band and also improve gel image quality (denaturing gels are more likely to produce crisp, single bands compared to native agarose gels).

There are other potential explanations of the third band, including bacterial contamination of the grey seal leukocyte RNA sample or the presence of a parasite.

Total RNA from a mixture of mammalian and bacterial cells run on an agarose gel will display three-four distinct high molecular weight bands: 28S, 23S, 18S, 16S rRNA. This follows the atypical banding structure seen here, with the exception of a visible 16S fourth band. However, it is unclear what the source of the bacterial contamination would be; it would have to be a massive bacterial load in every animal to show up on an agarose gel as a separate band in the first place, and it is present in all samples, including those of very young pups (from five days old). Since all our animals were in healthy condition, a high bacterial load in the plasma and a latent infection in all animals seems unlikely.

Various studies have found evidence of parasites in RNA samples from agarose gels. These exhibit third and fourth bands alongside the typical mammalian 28S and 18S rRNA bands. Daily *et al.* (2004) found evidence of the malarial parasite *Plasmodium falciparum* in blood of infected humans when total RNA was electrophoresed on a formaldehyde agarose gel; this appeared as four bands, with evidence of the parasitic RNA below the 28S and 18S rRNA bands. Malla *et al.* (2011) saw evidence of trichomonad parasite *(Trichomonas vaginalis)* and associated viral double stranded (ds) RNA (that together induce trichomoniasis) on RNA agarose gels of swab cultured vaginal human cells. This again appeared as three distinct bands. However, both these studies were conducted using subjects that were known to be infected with the specified parasite and, again, the extra band is present in all grey seal leukocyte samples. It seems very unlikely that such young pups would have had the opportunity to amass such a large parasite load, which would almost certainly lead to their death (which was not the case here). We may also expect evidence of parasite load when examining whole-blood smears. For example, larval stages of the heart worm *Acanthocheilonema spirocauda* have been

observed in whole-blood smears of harbour seals (*Phoca vitulina*; Leidenberger *et al.*, 2007).

Unexplained distinct third band during integrity analysis of RNA: cloning failure

There are a number of reasons why the cloning protocol may have failed. It may have been prudent to repeat the procedure in an effort to ascertain whether the failure was a result of technical skill. There are various changes to the protocol that may increase the likelihood of success, including: using another *E. coli* strain or other forms of transformation e.g. getting electro rather than chemically competent cells. The blunting reaction that was performed prior to ligation, as suggested by the manufacturer of the cloning kit used here, could have been the problem, since the T overhang can be useful and is, in some cases, TA cloning is reported to be a simpler and more efficient cloning strategy (Hadjeb and Berkowitz, 1996; Yao *et al.*, 2016).

Primer failures

Sequencing of amplicons to identify primer identity revealed alignments with isoforms is not abundant in leukocytes. GPx-3 is expressed in immune cells at very low levels which accounts for the low efficiency. As this isoform is expressed at low levels (if at all in grey seal leukocytes as the C_T values were 30+, mostly over 32) it is likely that the primers formed dimers and reacted to bind with themselves, which led to non-specific amplification and accounts for the multiple peaks seen.

For all other primers the isoforms are expressed in leukocytes, therefore I am uncertain why they would function so poorly. However, the unexplained third band may be driving poor primer function. Though there are multiple cells types, various other studies consider leukocytes as a whole and adipose tissue has the potential to

be made up of multiple cell types including macrophages and the primers worked optimally during qPCR experiments with blubber samples. The cell-type number here (indicated by differential WBC counts) was also consistent.

As a result of poor primer function in these samples, the suite of markers for which there is reliable data is reduced. A larger suite of markers or different isoforms of the current genes may improve primer function. For example, GPx-3 (the isoform used here and identified as such by Sanger sequencing) expression in leukocytes can be very low and in selenium deficient mice GPx-3 was not detectable in leukocytes (Kipp *et al.*, 2012). However, isoform GPx-1 is generally expressed abundantly in all cells so may be more suitable for use here. Therefore, if primers were redesigned to account for these changes we may have more successful primer function. This may also allow a more meaningful interpretation of the current data presented here. It may also be informative to investigate the relative gene expression of immune function markers (in blubber tissue also), for example the pro-inflammatory cytokines interleukin 10 (IL-10) and tumor necrosis factor alpha (TNF-α). Though primers were designed for these genes and appeared to amplify one specific amplicon, efficiencies were again poor (measured using pooled breeding female and pup RNA) most likely due to very low expression (very high C_T values of 30+ in both blubber and leukocytes; data not presented). Designing primers for non-model organisms without fully sequenced genomes is a challenge as gene sequences available on the NCBI database may be predicted or partial. Sequences from related species may be all that is available and though alignment softwares allow detection of conserved regions of genes, the likelihood that the primers may not function adequately is increased. Multiple design attempts may be required before specific, efficient primer pairs are achieved.

Table A4.1. Milk composition (% ± SD), milk calorific content, lactation duration and breeding strategy of grey seals compared to a range of other representative mammalian species. [1]Milk composites taken from Mellish *et al.* (1999) and represent mature milk (~day 10 of lactation); [2]Values, taken from Baker *et al.* (1990) reported without SD; [3]Mature milk composition (mid-late lactation) taken from Eisert *et al.* (2013); [4]taken from Jenness and Sloan (1970) but stage of lactation and SD not specified; * unconfirmed reports suggest some breeding females may forage during lactation; [5]Riet-Sapriza *et al.* (2012); [6]Dosako *et al.* (1983); [Δ]Costa and Gentry (1986); [7]Baker *et al.* (1963 a; b; 1967) and Cook *et al.* (1970); [ΔΔ]Hedberg *et al.* (2011); [8]Composition details of mature human milk (not colostrum) taken from Jenness (1979) [Handbook of Milk Composition], SD not reported; [9]Ben Shaul (1962); [ΔΔΔ] Król and Speakman (2003); [**]König and Markl (1987).

	Milk composition								
Species	Fat (%)	Protein (%)	Lactose (%)	Ash (%) (mineral constitue-nts)	Water (%)	Total solids (dry matter; %)	Calorific content	Lactation duration	Capital or income breeder? (C=capital; I=income)
Grey seal (*Halichoerus grypus*)[1]	53.7 ± 1.22	9.1 ± 0.17	0.7 ± - [2]	0.8 ± - [2]	30.7 ± 0.61	69.3 ± 0.61	23.3 ± 0.48 MJ kg^{-1}	18-21 days	C

Appendix 4: accompanying Chapter 1, General Introduction
Milk composition table

Species									
Weddell seal (*Leptonychotes weddellii*)[3]	54.0 ± 0.80	10.1 ± 0.16	1.0[4]	0.75 ± 0.02	33.8 ± 0.82	-	23.3 ± 0.25 MJ kg^{-1}	40-50 days	C*
New Zealand sea lion (*Phocarctos hookeri*)[5]	21.3 ± 8.1	9.4 ± 2.4	Absent	0.48 ± 0.06	67.9 ± 8.8	32.1 ± 8.8	10.3 ± 3.2 MJ kg^{-1}	~10 months	I
Northern fur seal (*Callorhinus ursinus*)[6]	46.6	12.4	Absent	0.6	59.6	61.0	19.9 MJ kg^{-1}Δ	~4 months	I
Polar bear (*Ursus maritimus*)[7]	33.1	10.9	0.3	1.4	52.4	-	15.0 MJ kg^{-1}ΔΔ	~2 years	I
Human (*Homo sapiens*)[8]	3.8	1.0	7.0	0.2	87	-	2.51 – 3.14 MJ kg^{-1}	6 months (exclusive	I

								) – 2 years (mixed)	
Mouse (*Mus musculus*)[9]	13.1	9.0	3.0	1.3	70.7	-	10.5 ± 1.0 MJ kg$^{-1\Delta\Delta\Delta}$	~ 23 days[**]	I

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