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Causes of individual variation in fasting
fuel allocation and consequences for
diving behaviour in grey seal pups
(*Halichoerus grypus*)

Thesis submitted in partial fulfilment of the degree of
Doctor of Philosophy

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I, Kimberley Ann Bennett, hereby certify that this thesis, which is approximately 75,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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Signature of candidate .

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b. Between *timepoint* (df = 37). Bold font indicates a significant difference between *timepoint* within each sex within each group. * denotes a significant difference between males and females within group. Red font indicates a significant difference in the change in [cortisol] between *timepoint* between the FED group and the group highlighted. ~ denotes a significant interaction between sex, *timepoint* and the FED group with the group indicated.

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Appendix 3

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*, # and ~ denote a significant difference in the change in [TT4] between CONTROL and SALINE, CONTROL and DEX and SALINE and DEX, respectively ($p < 0.05$). AIC = 1629.828; n (individuals) = 29; n (observations) = 170.

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List of abbreviations

α -MSH	α -Melanocyte stimulating hormone: hypothalamic appetite suppressing neuropeptide
<i>a</i>	Refers to analysis in which all pups with SRDLs were included
Ab	Antibody
ACTH	Adrenocorticotrophic hormone: pituitary hormone that acts on adrenal glands to elevate corticosteroid production and secretion
ADX	Adrenalectomised
Ag	Antigen
AgRp	Agouti-related peptide: hypothalamic orexigen
AIC	Aikaike's information criterion
ANOVA	Analysis of variance
ATII	Alveolar type II cell: produces PTHrP in response to stretch to stimulate leptin secretion by LFs
ATP	Adenine triphosphate
β -HBA	β -hydroxybutyrate: ketone body; product of fat metabolism
%(B/B ₀)	Amount of radiotracer bound to Ab as a percentage of amount present in zero standard
BC pups	Pups for which body composition information was known
BUN	Blood urea nitrogen: urea product of protein catabolism present in blood
CART	Cocaine-and-amphetamine regulated transcript: appetite suppressing neuropeptide
CBG	Cortisol binding globulin
cDNA	Complementary DNA
<i>CHT</i>	Cumulative handling time
CNS	Central nervous system
COM	Combined group containing those animals from CONTROL and SALINE groups for which body composition information was known at departure
<i>condition</i>	Mass/ length ratio used as a condition index when body composition information was not available
CONTROL	Animals that received no additional treatment in 2002 study
cpm	Counts per minute
CRH	Corticotropin releasing hormone: Hypothalamic neuropeptide that induces ACTH release from pituitary and acts as an anorexigen
%CV	Co-efficient of variation
<i>d</i>	Refers to analyses that included only those pups with SRDLs for which body composition information at departure was available
<i>(d)</i>	At departure
<i>d95</i>	95 th percentile of dive duration within four day time bins
<i>day</i>	Time bins to categorise days postweaning
<i>day1</i>	Day after departure that dives of that depth or duration were performed
<i>day10</i>	Day after departure that <i>SI5</i> was within 10% of <i>SI5</i> _{min}
<i>day90</i>	Day after departure that <i>d95</i> or <i>%dive</i> was within 90% of <i>max</i> values
DEE	Daily energy expenditure
%DEE _{fat}	Percentage contribution of fat to DEE

DEX	Animals that received 50 μ g kg ⁻¹ IM dose of dexamethasone at ten days after weaning in 2002 study
DFL	Daily rate of fat loss
%dive	95 th percentile of proportion of time spent in “dive” within four day time bins
DM	Departure mass
DMG	Daily mass gain during suckling
DML	Daily mass loss
DML _(f)	Daily mass loss between first and last capture after weaning
DML _(fish)	Daily mass loss between first and last capture after weaning in the FED group including the mass of the fish
DMSL	Mass specific mass loss
DPL	Daily protein loss
duration	One minute duration bin for dives
D ₂ O	Deuterium oxide
Δ [cortisol]	Absolute change in cortisol concentration between <i>S1</i> and <i>S2</i>
Δ fat %	Daily change in percentage fat content
early	First <i>timepoint</i> – first capture postweaning
EIA	Enzyme immunoassay
EDTA	Ethylenediaminetetraacetic acid
FED	Animals that were fed herring for first five days after weaning in 2001 study
FFA	Free fatty acid
_{first}	Subscript refers to first dives performed of that depth/ duration
GC	Glucocorticoid
GR	GC receptor
group	Treatment group
HE	Human equivalent
HIF	Heat increment of feeding (also referred to as SDA); increase in metabolic rate as a result of feeding
HIGH	Animals sampled at three day intervals throughout the postweaning fast
hour	Time of day that blood sample was taken
HPA	Hypothalamo-pituitary-adrenal: axis responsible for GC secretion
HRPO	Horseradish peroxidase
IEMA	Immunoenzymetric assay
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IRMA	Immunoradiometric assay
late	Final <i>timepoint</i> – day of release from the pen
LF	Lipofibroblast: lung cell that secretes leptin
LM	Linear model
LME	Linear mixed effect model
LOW	Animals sampled three times throughout the postweaning fast
LO/UN	Combined group containing those animals from LOW and UNKNOWN groups for which body composition information was known at departure
LQ	Location quality
MANOVA	Multivariate analysis of variance
max	Subscript refers to maximum 95 th percentile of dive duration or

	percentage time in dive achieved within three months after departure from the colony
<i>max depth</i>	25m depth bin
mid	Second <i>timepoint</i> - ~ 13 days postweaning
min	Subscript refers to minimum 5 th percentile of postdive surface interval achieved within three months after departure from the colony
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
MW	Molecular weight
NOAA	National Oceanic and Atmospheric Administration
NPY	Neuropeptide Y: powerful appetite-stimulating hypothalamic neuropeptide
<i>ob</i>	Gene that encodes leptin
ObRb	Cell surface leptin receptor
PCB	Polychlorinated biphenyls
PMP	Phenolphthalein monophosphate
POMC	Pro-opiomelanocortin: precursor to α -MSH
ppm	Parts per million
PRL	Prolactin
<i>prop</i>	Time category representing one tenth of total fast duration
PTHrP	Parathyroid hormone related protein stimulates leptin secretion in lung
QC	Quality control
%R	Percentage recovery of known amount of unlabelled hormone in spiked sample
rate of Δ [cortisol]	Rate of change in cortisol concentration between <i>S1</i> and <i>S2</i>
RIA	Radioimmunoassay
<i>S1</i>	First blood sample taken at each <i>timepoint</i>
<i>S2</i>	Second blood sample taken ~ 5 minutes after <i>S1</i> at each <i>timepoint</i>
SALINE	Animals that received IM dose of saline solution at ten days after weaning in 2002 study
<i>sample time</i>	Time taken to obtain blood sample from first physical contact with the animal
s.d.	Standard deviation
SDA	Specific dynamic action (also referred to as HIF); increase in metabolic rate as a result of feeding
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
s.e.	Standard error of the mean
<i>SI5</i>	5 th percentile of post-dive surface interval within four day time bins
SMRU	Sea Mammal Research Unit
SRDL	Satellite Relayed Data Logger
T3	Tri-iodothyronine: considered more active of two major TH
T4	Thyroxine: considered less active form of TH
tADL	Theoretical aerobic dive limit: dive duration beyond which net lactate accumulation is expected to occur, based on calculated body oxygen stores and metabolic rate
TBA	Total body ash
TBF	Total body fat
%TBF	Body fat as a percentage of body mass
TBGE	Total body gross energy

TBP	Total body protein
%TBP _U	Percentage of initial body protein stores utilised by departure
TBW	Total body water
TE	Total energy available to FED animals from the food they received
TEE	Total energy expended by departure
TF	Total fat available to FED animals from the food they received
TFU	Total fat (kg) utilised by departure
TG	Triglycerides
TH	Thyroid hormones
<i>timepoint</i>	Time category: early, middle or late fast
TMB	3,3',5,5'-tetramethylbenzidine
TP	Total protein (kg) available to FED animals from the food they received
TPU	Total protein utilised by departure
TRH	Thyrotrophic releasing hormone: hypothalamic hormone that stimulates TSH secretion
Tris	Tris(hydroxymethyl)aminomethane
TSH	Thyroid stimulating hormone: pituitary hormone that stimulates TH production by thyroid gland
TT3	Total T3: both protein bound and free T3
TT4	Total T4: both protein bound and free T4
TW	Total water available to FED animals from the food they received
UCP	Uncoupling protein: mitochondrial membrane protein responsible for uncoupling proton conductance from ATP production
UHF	Ultra High Frequency
UNFED	Pooled group including HIGH, LOW and UNKNOWN groups
UNKNOWN	Pups in 2001 study that were not sampled during suckling from females that had not been handled
<i>w</i>	Refers to analyses that included only those pups with SRDLs for which body composition information at weaning was available
^(w)	At weaning
WBC	White blood cell
WM	Weaning mass
[<i>x</i>]	Concentration of <i>x</i>

Abstract

Grey seal pups are weaned abruptly after a short, intensive nursing period, during which they lay down fat and protein. Fat stores are crucial for insulation when they first go to sea and protein is essential for muscle and oxygen store development. Both components also provide them with energy to sustain them during an extended postweaning fast and their first days and weeks at sea until they can forage effectively. There is thus a trade off between the requirements for fat and protein in metabolism and for other functions. It is crucial for the survival of grey seal pups that they begin to feed before these reserves become critically depleted. The ability to do so depends on the size of their fat and protein stores at weaning, energy partitioning during the postweaning fast and the development of adequate diving and foraging capabilities. The management of fat and protein stores through energy partitioning and appropriate timing of departure are key to survival but the mechanism by which this management is achieved is unknown.

To investigate the mechanism that controls energy partitioning and timing of departure from the colony, changes in body mass and composition and levels of hormones that are involved in energy balance in other mammals were measured during the postweaning fast of wild grey seal pups. The impact of initial energy reserves and hormone levels on energy use and departure was investigated directly, by manipulation of energy reserves and hormone levels, using supplementary feeding and hormone treatment. This is the first time such intervention studies have been performed in wild fasting pups. The impact of the stress of repeated handling on hormone levels and energy utilisation was also examined. The movement patterns and development of maximum diving capabilities of pups after departure were investigated using satellite telemetry.

Leptin was present in grey seal serum but could not be measured accurately using two currently available immunoassays. Prolactin was not detected in postweaned pup serum and was eliminated as a potential candidate for control of fuel use and timing of departure from the colony. Cortisol and thyroid hormones (TH) were measured in serum but cortisol could not be measured in grey seal saliva.

Cortisol and TH are potentially involved in energy expenditure and fuel partitioning during fasting. Cortisol and total thyroxine levels decreased from weaning to midway

through the fast. Both hormones increased in response to both natural and artificially induced changes in fuel availability. Dexamethasone, a potent cortisol analogue, caused a short-term increase in mass loss, and induced reversible and short-lived changes in cortisol levels and immune function, but its impact on energy partitioning was unclear. The effects of the drug were not mediated through TH. There were no changes in TH or cortisol consistently associated with the timing of departure and dexamethasone did not induce departure. Neither hormone therefore seemed likely to be involved directly in the cue to leave the breeding colony. Handling regime did not impact significantly on cortisol secretion, TH levels or long-term fuel use in grey seal pups.

Fatter animals, or those provided with additional food, relied more heavily on fat to meet energetic requirements than leaner pups. There was no difference in energy partitioning between the sexes. Grey seal pups had a greater tolerance to protein depletion than terrestrial mammals but may have reached critical protein levels soon after departure from the colony. They were therefore under considerable pressure to begin to feed very soon after they have gone to sea, despite their extensive fat reserves.

Pups showed marked inter-individual differences in movement patterns, which were very different from those of adults. Initially they undertook coastal movement, but showed wide dispersal from the colony within their first few months at sea. They began to exhibit adult-like repeated trips from known haul-outs to discrete offshore areas 4-5 months after departure. They were able to reach almost all areas and depths available in the North Sea, but were constrained in their ability to remain submerged, in terms of dive duration, post-dive surface interval and percentage of time spent diving over extended periods, compared with adults. Their maximum ability to remain submerged when they first went to sea was related to the duration of the postweaning fast and thus the degree of development on land. In contrast, maximum diving capabilities achieved during the first three months at sea did not vary substantially between animals and increased with time since departure, and were thus likely to be a product of diving-induced development of oxygen stores and cardiovascular control. Overall, larger body size and longer fast duration conferred increased diving capabilities, which may present one mechanism for increased survivorship in bigger animals.

Chapter 1

General Introduction

1.1. Ecological context

1.1.1. Population trends and first-year survival

The grey seal (*Halichoerus grypus* Fabricius) is one of only two seal (Order: Pinnipedia; Family: Phocidae) species to breed around the British Isles and is the largest of the ten extant species of the subfamily Phocinae (northern phocids). There are three distinct populations; in the northwest Atlantic in Canada, in the northeast Atlantic, largely in Scotland, and in the Baltic Sea (Bonner and Thompson, 1981). The UK stock is currently estimated at between 97,900 and 123,000 individuals, which represents roughly 40% of the world population (Special Committee on Seals (SCOS), 2003).

The UK grey seal population has been increasing annually at a rate of 5-6% for at least the last four decades, although the rate of increase has slowed in recent years from 5.2% between 1992 and 1996 to 2.8% between 1997 and 2001 (SCOS, 2002). The population is predicted to continue to increase at approximately 1% per annum for the next ten years (SCOS, 2003). This increase has largely been attributed to protective legislation and a reduction in levels of human occupation on remote islands around Scotland (Harwood and Prime, 1978; Summers, 1978; SCOS 2003).

First-year survivorship has a major impact on the population dynamics of marine mammals in general (Sinclair, 1996). Juvenile survivorship has been identified as one of the major factors that determines the rate of increase in the UK grey seal population (Harwood and Prime, 1978). Survival of seals during their first year of life is low and extremely variable. For instance, survival rate of Weddell seal

(*Leptonychotes weddelli*) pups into their second year of life is half that of adults over the course of a year (Burns, 1999). Mean percentage survival to age one in northern elephant seal pups (*Mirounga angustirostris*) is only 37% and most first-year mortality occurs during the first trip to sea (Le Boeuf et al., 1994). First year survivorship probability in grey seal pups is 62% for female pups but only 19% for males (Hall et al., 2001). To understand the mechanisms that may be responsible for the increase in grey seal numbers around the UK requires an investigation of factors that contribute to first-year survival.

The first year survivorship estimates for grey seals are based on data from a small sample of animals in one year within a limited area (Hall et al, 2001; Hall et al, 2002) and may only reflect the conditions that were prevalent in that year or in that locality. There is evidence for substantial inter-annual variability in first-year survivorship in northern elephant seals, which is related to climatic and oceanographic changes (Le Boeuf et al, 1994). There is likely to be similar variation in first-year survival in grey seals between areas and from year to year. The factors internal to grey seals that can influence pup survival have been shaped by evolution over a much longer time-scale and may be unable to accommodate relatively unpredictable, short-term or local variation in conditions.

1.1.2. First-year survival: maternal investment and metabolic fuel utilisation

Despite potential problems with the reliability of generalising from estimates of survival based on a single year, the pattern that seems to emerge is an increase in the probability of survival with body mass and condition. For instance, first year

survivorship increases with body mass and size of energy reserves at weaning in grey seal (Hall et al, 2001; Hall et al, 2002), southern elephant seal (*M. leonina*; McMahon et al, 2000; Biuw, 2003) and northern fur seal (*Callorhinus ursinus*; Baker and Fowler, 1992) pups. Similarly, the probability of survival into the second year of life declines markedly below a critical weaning weight in Weddell seal pups (Burns, 1999). Mass of grey seal pups at weaning can vary by a factor of two, and a similar magnitude of size difference in weaned southern elephant seal pups leads to an increase in probability of survival from 54% in the lightest animals to 72% in the heaviest (McMahon et al, 2000).

The availability of energy reserves at weaning in phocids is constrained by the net transfer of energy from mother to pup during suckling (Arnbom et al, 1997; Mellish et al, 1999a). Like many other phocids, female grey seals usually produce a single pup each year, which they feed on high-fat (>50%) milk during a brief, intensive nursing period (Mellish et al, 1999a). The pups suckle for roughly 18 days and gain mass at a rate of up to 2.5 kilograms per day (Fedak and Anderson, 1982; Anderson and Fedak, 1987; Mellish et al, 1999b), mainly as subcutaneous blubber, but also as lean body mass. This high investment is energetically costly for the females, which show an average daily mass loss of 3.8kg per day and lose 40% of their post-partum mass by the time they leave the colony (Fedak and Anderson, 1982; Anderson and Fedak, 1987; Pomeroy et al, 1999). Higher investment in offspring in any given year has fitness consequences for grey seal females since it results in reduced pupping success in the following year (Pomeroy et al, 1999).

Although the costs to the mother (Fedak and Anderson, 1982; Anderson and Fedak, 1987; Arnbom et al, 1997; Mellish et al, 1999b; Pomeroy et al., 1999) and the survival consequences to the offspring (McMahon et al, 2000; Hall et al, 2001; Hall et

al, 2002) of maternal investment have been investigated in phocid seals, the mechanisms underlying enhanced survival probability in larger pups are unknown.

Grey seal pups are weaned abruptly and then undergo a protracted fast, which lasts from ten days to more than four weeks before these naïve animals go to sea to learn to forage without parental guidance (Reilly, 1991). Pups are completely reliant on the energy reserves laid down whilst suckling to sustain them during both the postweaning fast and the initial phase at sea before they begin to feed. The physiological and behavioural transition from fasting to foraging is a crucial developmental period and is therefore likely to have a large impact on first-year survival. Energy availability, fuel use and the development of diving and feeding capabilities will largely determine how pups cope with this transition. The relationships between body mass and condition, fasting energy expenditure and partitioning and the development of diving behaviour in grey seal pups will be examined in this thesis.

1.2. Energy availability and utilisation

If pups are to maximise their chance of survival, they must carefully regulate the rate at which their finite fat and protein depots are utilised and must leave the colony at an appropriate time to balance developmental requirements with current and future metabolic needs. This requires co-ordination of physiological and behavioural responses to information from fuel reserves and a mechanism that anticipates future metabolic demands. To gain a better understanding of the impact of body condition on first-year survival in grey seal pups, more information is needed about the regulatory

mechanisms and information flow underlying decisions about energy partitioning, timing of departure from the colony and behavioural decisions of pups once they go to sea.

1.2.1. Fasting fuel use

1.2.1.1. Physiological constraints and trade offs

A large fraction of the finite fat and protein stores available to fasting animals can be mobilised to meet energetic requirements without compromising tissue integrity and function (Dulloo and Jacquet, 1999). The remainder is comprised of components that are essential for life, including structural and functional proteins and lipids. Fast duration is ultimately constrained by the size of dispensable fat and protein reserves and the rates at which they are utilised. Stored fat is important for grey seal pups as an energy reserve, both while fasting on land and at sea as they learn to forage, and is vital for insulation against cold water temperatures. Protein plays a smaller but significant role as a metabolic fuel, and is a major component of body tissues. Fasting animals thus face a trade off between the use of fat and protein as metabolic fuel. The different premiums placed on these tissue types are likely to depend on the individual needs and body size and composition of the animal.

1.2.1.2. The general fasting model

In the general model of fasting in mammals and birds three distinct phases are typically recognised. They are based on characteristic transitions in the primary metabolic fuel source, alterations in metabolic rate and associated changes in the rate of mass loss (Le Maho et al, 1981; Cherel et al, 1988c; Cherel et al, 1988a; Cherel et al, 1988b; Castellini and Rea, 1992; Cherel et al, 1992).

Phase I, the postabsorptive state, begins three to four hours after the digestion of a meal. It is a brief period of metabolic adjustment to the fasted state and is characterised by a fall in metabolic rate and rapid loss of body mass, largely caused by emptying of the gut (Brady et al, 1977; Le Maho et al, 1981; Cherel et al, 1988c; Cherel et al, 1988a; Cherel et al, 1988b; Nordoy et al, 1990; Castellini and Rea, 1992; Cherel et al, 1992; Lydersen et al, 1997). Glycogen reserves in liver and muscle are utilised while fat deposits are mobilised in preparation for increased reliance on lipid metabolism in phase II, which begins when glycogen reserves are exhausted.

Energetic requirements during phase II are largely met by lipid catabolism and protein oxidation is minimised (Brady et al, 1977; Le Maho et al, 1981; Cherel et al, 1988b and c; Cherel et al, 1992). For animals that normally rely on carbohydrate as the major metabolic fuel, this represents a substantial metabolic shift. Extensive protein catabolism can eventually cause severe impairment of organ function, including metabolic acidosis, renal failure and cardiac arrest (Castellini and Rea, 1992). Protein reserves are smaller and less energy-dense than fat stores, thus the relative contribution of these two energy sources to metabolism is adjusted to avoid the consequences of exhaustion of protein reserves, which would otherwise be

encountered well before substantial depletion of fat reserves. Animals remain in phase II for as long as possible since reliance on fat breakdown substantially slows protein depletion. The contribution of fat to energy expenditure is positively correlated with initial adiposity (Cherel et al, 1992; Atkinson et al, 1996; Dulloo and Jacquet, 1999). Fatter rats can spare protein more effectively and can remain in phase II up to five times longer than lean conspecifics of the same lean mass (Cherel et al, 1992).

Phase II is characterised by a low rate of change in body mass (Le Maho et al, 1981; Cherel et al, 1988b and c; Nordoy et al, 1990; Cherel et al, 1992). This is caused both by a lower metabolic rate and the increased reliance on fat, which contains less intracellular water and is more energy dense than an equivalent volume of protein. Metabolic rate is a function of lean body mass (Kleiber, 1975) and thus a reduction in metabolic rate is expected as a result of lean tissue loss. However, the fast-related fall in metabolic rate is greater than can be accounted for by mass loss alone and is achieved through minimising activity, and possibly a depression of cellular metabolism (Reilly, 1991). A reduction in metabolic rate causes an overall reduction in the rate of tissue utilisation and therefore extends the possible duration of the fast.

Some protein use during phase II is still necessary for efficient fat metabolism (Adams et al, 1992) and provision of glucose to obligate glucose consuming organs through hepatic gluconeogenesis. During phase II fasting or semi-starvation 8-23%, 10-20% and 4-7% of energy requirements in rodents (Cherel et al, 1992), humans (Dulloo and Jacquet, 1999), and penguins (*Aptenodytes sp.*; Groscolas and Robin, 2001), respectively, are met through protein oxidation.

The onset of phase III occurs before complete exhaustion of fat reserves and the threshold adiposity at which it begins varies between species (Robin et al, 1988;

Belkhou et al, 1991; Cherel et al, 1992; Friedl et al, 1994; Robin et al, 1998; Groscolas and Robin, 2001). The depletion of fat reserves to a low critical threshold necessitates a further metabolic shift towards greater reliance on protein catabolism, which drives an increase in the rate of change in body mass (Le Maho et al, 1981; Cherel et al, 1988b and c; Cherel et al, 1992; Groscolas and Robin, 2001). The contribution of protein to energetic needs may reach 55% during phase III in fasting penguins (Groscolas and Robin, 2001) and may rapidly reduce protein reserves to critical levels.

The transition from phase II to phase III is thought to be an adaptive mechanism that provides a “refeeding signal” to avoid critical depletion of fat (Cherel et al, 1992; Groscolas and Robin, 2001). Rats and penguins show a marked increase in locomotor activity, indicative of increased motivation to seek food, coincident with reduced fat utilisation and elevated protein catabolism (Cherel et al, 1988a; Koubi et al, 1991; Cherel et al, 1992; Robin et al, 1998; Groscolas and Robin, 2001).

Animals can suffer a fatal loss of protein while they still possess extensive fat reserves (Cherel et al, 1992) but can tolerate a substantial loss of body protein before tissue wastage becomes irreversible. For example, penguins can be successfully re-fed after three weeks in phase III of fasting (Cherel et al, 1988a). Terminal starvation in humans begins when 30-50% of the body protein has been utilised (Garrow et al, 1965). Similarly, dogs starve to death when 66% of their initial protein reserves have been utilised (Garrow, 1959). The onset of terminal starvation occurs, irrespective of any remaining fat deposits, when the labile protein reserve is exhausted and catabolism of proteins integral to tissue structure and function begins.

1.2.1.3. *Fasting in seals*

The pattern of fuel utilisation in fasting seals largely conforms to the general fasting model. However, pinnipeds primarily utilise fat for metabolism, even during periods of active feeding (Kirby and Ortiz, 1994), such that the onset of fasting does not require a major metabolic shift in these animals (Castellini et al, 1987). The combination of negligible carbohydrate intake, reliance on fat metabolism and conservation of protein throughout the short suckling period are thought to pre-adapt phocid seal pups to prolonged fasting (Houser and Costa, 2001).

Grey seal pups undergo a 45% reduction in basal metabolic rate within ten days after weaning which is accompanied by a 50% fall in mass specific mass loss (Nordøy et al, 1990). A similar pattern occurs in fasting northern elephant seal pups in which re-feeding is accompanied by an increase in metabolic rate, indicating that the reduction in metabolic rate during fasting is directly related to the fast rather than to developmental changes (Rea and Costa, 1992).

Sparing of protein is especially important during the postweaning fast since it diverts amino acids away from oxidation and into lean tissue development and reorganisation. Protein sparing is so effective in northern elephant seal pups that they lose a similar amount of lean mass during a six week fast to the amount they gain during a single day of suckling (Houser and Costa, 2001). In captive grey seal pups, the amount of lean body tissue utilised per day decreases by 66% over 50 days of fasting (Nordøy et al, 1990). Captive grey seal pups fasting for 31 days show a 20% reduction in cross sectional area of skeletal muscle (Nordoy and Blix, 1985). Based on this rate of muscle protein utilisation, protein mass would not be depleted by a critical amount (30-50%; Garrow, 1959; Garrow et al, 1965) until approximately 60

days of fasting in these animals (Nordoy and Blix, 1985). However, accurate estimates of body composition changes during fasting in wild seals are needed to verify this.

Energetic needs during fasting are met mainly by free fatty acids (FFA), glycerol and ketone bodies (Castellini et al, 1987; Nordoy and Blix, 1991), and to a lesser extent by amino acids derived from protein catabolism. Most glucose is recycled (Davis, 1983) and directly contributes less than 1% to the metabolic rate in northern elephant seal pups (Keith and Ortiz, 1989). Despite heavy reliance on fat during the postweaning fast captive grey seal pups do not suffer increased thermal stress as a result of a reduction in blubber thickness, even after 31 days of fasting (Nordoy and Blix, 1985).

Previous work has shown that the contribution of fat to energy expenditure is extremely high in fasting seal pups. Grey seal pups meet ~94% of their energy needs during the postweaning fast from the utilisation of subcutaneous fat and only 6% from protein stores during phase II of fasting (Nordøy and Blix, 1985; Worthy and Lavigne, 1987; Nordoy et al, 1990; Reilly, 1991). Similarly, proteins contribute up to 4% to total energy supply during fasting in northern elephant seal (Kirby and Ortiz, 1994; Houser and Costa, 2001) and harp seal (*Phoca groenlandica*) pups (Nordoy et al, 1993). Although the contribution of protein to energetic needs is small in proportional terms, it may represent a significant daily loss of absolute lean tissue mass due to the low energy density of protein compared with fat.

The relatively consistent values of fat contribution to energy expenditure reported in grey seals seem to contrast with the flexibility in the contribution of fat to energy expenditure exhibited by rats (Cherel et al, 1992), polar bears (*Ursus maritimus*; Atkinson et al, 1996) and human subjects (Dulloo and Jacquet, 1999). The

earlier studies on fasting seals did not focus on the variability in fuel allocation between individuals. Where variation in energy partitioning has been investigated in other pinnipeds, including elephant seals (Carlini et al, 2001; Biuw, 2003; Noren et al, 2003a) and Subantarctic fur seals (*Arctocephalus tropicalis*; Beauplet et al, 2003), there is a positive relationship between initial adiposity and the contribution of lipid to overall energy use. As in humans (Dulloo and Jacquet, 1999), the variability of this contribution is greater between lean individuals than between fat subjects (Biuw, 2003). Since pups must adjust their fuel allocation strategy to meet current and future metabolic and developmental requirements, it is this individual variability that is likely to impact on the probability of future survival.

A further source of inter-individual variability in fuel use pattern may arise as a consequence of sex differences in growth patterns and physiological requirements related to the extreme sexual dimorphism generally seen in pinnipeds. Sex differences in fuel allocation strategy occur in otariid pups, which, in contrast to phocids, undergo a protracted preweaning period that consists of intensive suckling bouts of several days punctuated by fasting periods when their mothers return to sea to forage. Males divert a greater proportion of energy gained during suckling into somatic growth compared to females, which prioritise fat storage (Arnould et al, 1996; Arnould et al, 2001; Donohue et al, 2002; Beauplet et al, 2003). Such sex differences in fuel allocation are not apparent during suckling in elephant seal pups and are not generally seen during fasting (Carlini et al, 2001; Noren et al, 2003a), unless very lean animals are included in the sample (Biuw, 2003). In this case males, again, seem to utilise more fat and spare protein, whereas females retain fat at the expense of their protein reserves (Biuw, 2003). Male grey seal pups have a substantially lower probability of first-year survival than females, despite being heavier and in better condition at

weaning, and the effect of an increase in condition on survivorship is greater for males than for females (Hall et al, 2001; Hall et al, 2002). Differences in fuel allocation between males and females may contribute to this disparity in survivorship. The impact of weaning body composition and sex on fuel allocation during fasting has not been explored in grey seals but requires examination to understand how the interplay between sex and body condition influences first-year survival in these animals.

1.2.2. Departure from the colony

1.2.2.1. Trade off between developmental and metabolic requirements

Timing of departure from the colony is crucial for the survival of grey seal pups. The land-based postweaning fast is likely to be important for remodelling the tissues laid down rapidly during the intensive nursing phase. Growth and development can only occur by restructuring existing tissue, since the pups have no external sources of nutrition whilst fasting. Developmental needs therefore rely on the same tissues as those that provide the metabolic fuel required to sustain the fast. There is likely to be a trade off between these conflicting demands. The signal that prompts departure from the colony in seal pups must incorporate information from fuel reserves and aspects of development to ensure that departure occurs at an appropriate time.

Seal pups undergo growth and development of the skeleton, nervous system and musculature whilst fasting (eg. Pattersonbuckendahl et al, 1994). The fast is important for development of motor skills and co-ordination, and the physiological

capabilities necessary for diving, including blood and muscle oxygen stores, the ability to tolerate prolonged periods of apnoea and the capacity to selectively direct blood flow to maximise oxygen delivery to vital organs, whilst minimising oxygen use by non-essential tissues (Arnbom et al., 1993; Thorson and Le Boeuf, 1994; Falabella et al, 1999; Noren et al, 2003b). The fast may also be necessary for establishing social interactions (Modig et al, 1997). If pups depart from the colony before they have attained some minimum degree of development they may compromise their survival because they are ill-equipped to dive and forage.

The decision to leave the colony must balance developmental considerations with future fuel requirements (Arnbom et al, 1993). After departure from the colony, pups remain reliant on their endogenous fuel reserves until they can meet their energetic requirements through food intake. Finding food for the first time in a relatively unpredictable environment is a major challenge and will depend critically on diving and learning capabilities as well as prey distribution. Pups should therefore terminate the fast well in advance of the exhaustion of fuel depots. Animals that are too lean at departure may be unable to find food before they deplete their blubber layer to a point at which its insulative capacity is also compromised. In addition to the metabolic demands of foraging, these animals must oxidise fat to meet thermoregulatory requirements. This results in a positive feedback process that hastens the onset of terminal starvation and is facilitated by the cold water temperatures experienced by British grey seals.

There is thus a conflict between the need to undergo development and the need to begin foraging before energy stores are depleted. The consequences of this trade-off are greater for leaner pups, which may have to compromise development in order to try to feed before energy reserves are exhausted and blubber insulation is

reduced. Post-moult starveling elephant seal pups depart from the colony, presumably in response to low fuel availability, before they have had an opportunity to practice diving behaviour close to the rookery (Houser and Costa, 2003). As a result they may be poorly equipped to survive at sea and many of them subsequently strand themselves (Houser and Costa, 2003).

As discussed below, oxygen storage capacity has a large impact on diving capability and develops throughout the postweaning fast (Thorson and Le Boeuf, 1994; Noren et al, 2000; Noren et al, 2003b). Although blood oxygen stores in grey seal pups are comparable with those in yearlings by 24 days postweaning, the land-based fast can be as short as nine days and its duration is positively correlated with percentage fat at weaning (Noren et al, 2003b). Fatter pups can thus afford to develop blood oxygen stores whilst fasting on land, whereas leaner animals depart from the colony when their oxygen stores are less developed and this may compromise their ability to dive and to forage (Noren et al, 2003b).

1.2.2.2. Phase II/phase III transition

Departure from the colony in post-moult starveling northern elephant seal pups seems to be related to entry into phase III of fasting (Houser and Costa, 2003). However, it is unclear whether healthy phocid seal pups naturally reach phase III whilst fasting on land. In contrast to fasting penguins, healthy seal pups have substantial fat reserves even at the end of the postweaning fast, presumably because blubber reserves are the main form of insulation as well as fuel in these animals. Free ranging elephant seal pups are roughly 45% fat at departure from the colony (Carlini et al, 2001; Noren et al, 2003a; Biuw, 2003) compared to the 5-20% adiposity seen at

departure in penguins (Robin et al, 1988; Cherel et al, 1992; Robin et al, 1998; Groscolas and Robin, 2001). However, despite possessing sizeable fat reserves, some southern elephant pups may approach critical levels of protein depletion whilst fasting on the colony, without displaying classical signs of entry into phase III (Biuw, 2003). An increase in blood urea nitrogen (BUN) levels, an indirect measure of the rate of protein oxidation and an indication of the onset of phase III, begins at around 38 days of fasting in captive grey seal pups, at a time when they still have 53% of their initial fat stores and significant protein reserves at their disposal (Nordoy et al, 1992). If departure from the colony in fasting seal pups is prompted by a signal that occurs at the onset of phase III, this phase must begin at a much higher relative fat content than it does in other mammals.

Many of the typical changes in biochemical parameters and weight loss that occur in other species at the transition into phase III do not occur simultaneously in fasting seal pups. Few studies have demonstrated an increase in protein utilisation in the latter stages of fasting in healthy phocid pups (Nordøy et al, 1992). The increase in protein utilisation after 38 days of fasting in captive grey seal pups (Nordoy et al, 1992) is not accompanied by other signs of entry into phase III, including a fall in FFA levels (Nordoy and Blix, 1991), or a rapid loss of body mass (Nordoy et al., 1990). Wild grey seal pups do not generally fast for as long as 38 days (Reilly, 1991). It is possible that they are exposed to more extreme environmental conditions and therefore incur higher energetic costs, which may lead to entry into phase III sooner than captive seals (Nordoy and Blix, 1985). Grey seal pups may leave at or before the onset of phase III. The signal that prompts them to leave the colony is unknown.

1.2.3. Information from fuel reserves

Irrespective of whether cues that prompt fasting seal pups to leave the colony occur at the transition into phase III, it is clear that the signal to depart must incorporate information from fuel reserves. This information must be anticipatory since seal pups cannot afford to wait for a signal that indicates an absolute depletion of fuel before they go to sea. Fuel allocation must also be based, at least in part, on information derived from energy availability. Endogenous signals that link energy stores with fuel use and feeding behaviour are well documented in many terrestrial mammals and birds. They incorporate a variety of inputs, including circulating levels of metabolites and/or hormones involved in energy balance. Despite substantial work in this area, the role of such signals in seals is poorly understood and requires further investigation.

1.2.3.1. Involvement of metabolites in fuel use and foraging

Changes in metabolite levels associated with major shifts in fuel use observed during the course of fasting could provide important information about the status of individual fuel reserves.

1.2.3.1.1. Glucose

The onset of feeding behaviour in animals that have a carbohydrate-based diet is partially controlled by circulating glucose levels (Louis-Sylvestre and Le Magnen, 1980; Campfield et al, 1996; Bray, 2000; Campfield and Smith, 2003). A transient

drop in blood glucose precedes hunger sensations and is associated with meal initiation. Circulating glucose levels are higher in phocids than in terrestrial mammals (Schweigert, 1993), especially during fasting (Nordøy and Blix, 1991) and it has been suggested that hyperglycaemia initiates and maintains postweaning hypophagia (Keith and Ortiz, 1989). Glucose levels show a slow decline throughout fasting in grey and northern elephant seal pups (Nordøy and Blix, 1991; Costa and Ortiz, 1982). The implication is that the inhibition on feeding behaviour is reduced as levels fall. However, high glucose levels before weaning do not prevent suckling. The high circulating concentrations of glucose in fasting seal pups are not defended when challenged by insulin injection, and persistently (>1 hour) low glucose levels do not elicit a behavioural response (Kirby and Ortiz, 1994). The lack of a physiological or behavioural response to artificially altered circulating glucose levels and the chronically high glucose levels and low levels of glucose utilisation during fasting strongly suggest that changes in glucose levels do not provide a hunger signal that triggers food seeking behaviour in seal pups. The high glucose levels in fasting pups may, instead, provide the carbon backbone for amino acid synthesis for lean tissue building (Keith and Ortiz 1989).

1.2.3.1.2. Products of fat and protein utilisation

Alterations in fat metabolism are associated with changes in circulating concentrations of FFA and the ketone bodies (breakdown products of partial fat oxidation produced as an alternative fuel for tissues that normally use glucose), D- β -hydroxybutyrate (β -HBA) and acetoacetate. Levels increase during phase II and decline, often abruptly, in phase III, whilst reciprocal changes occur in levels of BUN,

creatinine and alanine, which are indices of muscle tissue breakdown (Le Maho et al, 1981; Costa and Ortiz, 1982; Cherel et al, 1988a, b and c; Castellini and Rea, 1992; Cherel et al, 1992; Nordoy et al, 1992; Kirby and Ortiz, 1994; Houser and Costa, 2001).

Lipolytic flux and FFA levels in blood do not reflect the size of adipose reserves (Bernard et al, 2002a) and do not change significantly between phase II and phase III. They are therefore unlikely to control fuel use or promote foraging (Bernard et al, 2002a). Instead, entry into phase III is initiated by a reduction in the rate of FFA oxidation (Bernard et al, 2002b). This causes a fall in circulating ketone bodies, which are thought to play a role in nutrient sensing and regulation of metabolism during fasting (Robinson and Williamson, 1980). However, the fall in β -HBA occurs as a result of reduced FFA oxidation, and is therefore downstream of the mechanism that controls fuel allocation during fasting.

β -HBA reduces food intake and has been proposed to act as a satiety signal (Bray, 2000). Although there is a strong correlation between the termination of fasting and a rapid decline in β -HBA levels in fasting penguins (Cherel et al, 1988a, b and c; Robin et al, 1998), changes in ketone body production do not prompt departure from the colony in seal pups. β -HBA levels show a sharp downturn after eight weeks of fasting in northern elephant seal pups (Kirby and Ortiz, 1994) but the change in concentration is relatively small (Kirby and Ortiz, 1994; Castellini and Costa, 1990; Nordoy and Blix, 1991) compared with the 80% reduction seen in fasting penguins at the onset of phase III (Cherel et al, 1988 b and c). Pups depart from the rookery around the same time as the fall in β -HBA, but may leave before, during or after the decrease (Castellini and Costa, 1990). Captive grey seal pups show a slight reduction in β -HBA concomitant with the increase in protein-based metabolism that occurs after

38 days of fasting (Nordøy and Blix, 1991). Since wild grey seal pups do not generally fast for such extended periods, departure must occur before this signal occurs. The inconsistency in the occurrence of this signal across all animals and the lack of immediacy in the response to it suggest that this biochemical shift in itself is insufficient to cause the pups to go out to sea.

1.2.3.2. Integrated fuel utilisation and nutrient sensing

Changes in individual circulating metabolite levels reflect the availability of only one fuel store and, furthermore, cannot provide anticipatory information regarding the status of that reserve. Individual metabolite concentrations alone therefore seem unable to provide satisfactorily integrated information about total body fuel stores on which to base decisions about energy partitioning and departure from the colony. The co-ordination of fuel utilisation during fasting and the appropriate timing of departure seems to require a system in which information from different energy reserves is fed into a central integrative mechanism that controls both fuel utilisation and behaviour.

Cross-talk between different fuel sources allows co-ordination of the rate and pattern of their oxidation. There is a reciprocal relationship between the availability of fat and glucose and the rate of utilisation of these two fuel types. Elevation of glucose levels suppresses the rate of FFA oxidation and high FFA levels can reduce glycolysis (Watford, 2000; Bernard et al., 2003a). Futile glucose cycles are often control points for such changes in fuel allocation (Keith and Ortiz, 1989; Rossetti, 2000). Penguins, like seals, primarily utilise fat rather than carbohydrates to meet their energy needs, but glucose still plays an important role in the control of the rate of FFA oxidation

(Bernard et al, 2003a) and this may also be the case in seals. High glucose levels in fasting pups combined with the low rate of glucose oxidation may indicate that most glucose is involved in futile cycling and could be crucial in fuel partitioning. It is interesting to note that glucose cycling decreases as the postweaning fast progresses in northern elephant seals (Champagne et al., 2003).

The existence of a mechanism that integrates information from the rate of glucose and fat utilisation and controls feeding behaviour is suggested by the synergistic increase in food intake in rats in response to inhibition of glucose and fat metabolism (Friedman and Tordoff, 1986). One possible mechanism that acts as a fuel sensor and integrates information from both fat and glucose availability is the hexosamine pathway, a minor pathway of glucose utilisation within the cell (Wang et al, 1998; Rossetti, 2000). An intracellular increase in fructose-6-phosphate that occurs as a result of either hyperglycaemia or an inhibition of glycolysis by high FFA availability, causes increased flux through the hexosamine pathway. The end products of this pathway, UDP-N-acetyl- hexosamines, reduce glucose transport into the cell, and thereby act as a signal of cellular satiety (Rossetti, 2000). UDP-N-acetyl- hexosamines are used to glycosylate transcription factors for target genes that are responsive to nutrient availability. Some of these genes encode hormones and thus increased flux through the hexosamine pathway can potentially relay information about the energy status of the cell to the rest of the body (Rossetti, 2000). It is likely that seals possess a nutrient sensing mechanism that integrates information from different fuel sources and is coupled to systems that regulate fuel use and stimulate foraging behaviour.

1.2.3.3. Hormonal signals in fuel use and foraging

The information derived from nutrient sensors must be relayed to the rest of the body if the animal is to respond appropriately to its changing energy status. A critical component of this integrative mechanism is likely to be the endocrine system. A hormonal messenger secreted in response to an intracellular nutrient-sensing pathway can communicate information about the long-term availability of fuel stores to the rest of the body. Peripheral and central receptors can then effect an appropriate response by modifying both fuel utilisation and behaviour patterns. The management of changes in fuel utilisation and developmental processes throughout the postweaning fast and the timing of departure from the colony are likely to be orchestrated by hormonal signals.

A wide range of hormones play a major role in long-term regulation of energy balance in other mammals, and are therefore likely to be involved in fuel allocation and the transition from fasting to feeding in grey seal pups. However, because seals undergo large cyclical changes in body fat content throughout their lives, the control of energy balance may be radically different from many mammals that do not routinely undertake intensive foraging bouts and extended periods of fasting. Leptin, glucocorticoids (GCs), thyroid hormones (TH) and prolactin (PRL) were chosen as the focus for this study.

1.2.3.3.1. Leptin

In rats, humans and many other terrestrial mammals, leptin acts as a sensor of adiposity and provides a link between peripheral energy stores, energy expenditure

and higher centres in the brain that control food intake (Campfield et al, 1995; Halaas et al, 1995; Pelleymounter et al, 1995; Collins et al, 1996; Ahima and Flier, 2000; Reidy and Weber, 2000). It is secreted primarily by adipocytes in proportion to their triglyceride content, such that plasma concentrations correlate with available fat stores (Ahima and Flier, 2000; Spiegelman and Flier, 1996). The mechanism by which the fat cells detect their nutritional status and use it to control leptin secretion is not fully understood, but seems to involve flux through the hexosamine pathway (Wang et al, 1998; Considine et al, 2000; McClain et al, 2000; Rosetti, 2000).

Leptin promotes lipid utilisation (Halaas et al, 1995; Shimabukuro et al, 1997; Barzilai et al, 1997; Van Dijk et al, 1999; Reidy and Weber, 2000) by upregulation of key lipolytic enzymes (Reidy and Weber, 2000) and by activating expression and action of mitochondrial uncoupling proteins (UCPs) (Scarpace et al, 1997; Arvaniti et al, 1998b; Legradi et al, 1997). UCP 1 transports protons across the mitochondrial membrane, thereby avoiding ATP synthesis and dissipating energy as heat (Brand et al, 1999; Matthias et al, 1999; Stuart et al, 1999). UCP 2 and 3 are thought to be fatty acid transporters involved in the control of lipid metabolism (Brand et al, 1999; Cadenas et al, 1999). In addition, leptin may reduce fat mass by inducing apoptosis of fat cells (Reidy and Weber, 2000) and decreasing the preference for dietary fat (Arvaniti et al, 1998b).

Leptin has a pivotal role in the signalling of energy status to hypothalamic nuclei that mediate food intake (Campfield et al, 1995; Halaas et al, 1995; Pelleymounter et al, 1995; Stephens et al, 1995). It inhibits the secretion of neuropeptide Y (NPY), and agouti-related peptide (AgRP), which are powerful appetite stimulants (Mizuno and Mobbs, 1999), and promotes the secretion of anorexigenic neuropeptides, including pro-opiomelanocortin (POMC), α -melanocyte

stimulating hormone (α -MSH), cocaine-and-amphetamine regulated transcript (CART) and corticotropin-releasing hormone (CRH) (Van Dijk et al, 1999; Ahima and Flier, 2000; Vergoni and Bertolini, 2000).

Low leptin levels elicit appropriate modifications to behaviour and metabolism in response to reduced food availability (Saladin et al, 1995; Spiegelman and Flier, 1996; Friedman and Halaas, 1998; Ahima and Flier, 2000). They cause suppression of the gonadal and thyroid (Ahima and Flier, 2000; Legradi et al, 1997) axes to minimise energy expenditure, and activation of the hypothalamo-pituitary-adrenal (HPA) axis, which induces food-seeking behaviour (Spiegelman and Flier, 1996).

The role of leptin in pinnipeds remains unclear. In the few studies that have measured circulating leptin in pinnipeds, reported levels are low and unrelated to adiposity (Gurun et al, 2001; Ortiz et al, 2001b; Ortiz et al, 2001a; Arnould et al, 2002; Ortiz et al, 2003a). It has therefore been suggested that leptin is not involved in fuel regulation in these animals. However, both grey and harbour (*Phoca vitulina*) seals express leptin in blubber (Hammond et al, in press), which suggests that it performs a similar function in fat regulation in seals as it does in other mammals. This study examines the methods that have been used to measure leptin in seals in an attempt to investigate the role of this hormone in energy resource management in grey seal pups.

1.2.3.3.2. Glucocorticoids

GCs control energy intake, deposition and utilisation. They are instrumental in maintaining an adequate substrate supply in the face of constant changes in energy demand and are a major component of the mechanism that allows an animal to respond to and cope with stress (Sapolsky et al, 2000). “Stress” is the umbrella term applied to an almost infinite array of psychological, physical or physiological challenges, and can range from immediate and potentially damaging insults, such as sudden attack and injury by a predator, to persistent adverse conditions, such as extended periods of food deprivation. Circulating GC concentrations are elevated rapidly and dramatically within minutes of exposure to an acute stressor (Sapolsky et al, 2000). Basal levels increase less rapidly in response to chronic stress, such as fasting (Bergendahl et al, 1996; Friedl et al, 2000).

Both basal and stress-induced GC levels have a major impact on fuel use and feeding behaviour. Changes in GC levels alter the allocation of different fuel types to energy expenditure. GCs enhance the gluconeogenic capacity of the liver and increase the provision of substrates by facilitating the mobilisation of fat (Divertie et al, 1991; Samra et al, 1998; Djurhuus et al, 2002; Djurhuus et al, 2004) and protein reserves (Simmons et al, 1984; Legaspi et al, 1985; Tatarani et al, 1996; Weiler et al, 1997; Mantha and Deshaies, 2000). The specific effects of GCs on fuel utilisation are dependent on the levels of other hormones, including insulin, which often opposes the effects of GCs (Divertie et al, 1991; Strack et al, 1995; Samra et al, 1998; Mantha and Deshaies, 2000; Djurhuus et al, 2002; Djurhuus et al, 2004).

Basal levels of GCs are crucial in appetite regulation (Green et al, 1992) through their stimulatory action on NPY-producing neurones (Debons et al, 1986; Chen and Romsos, 1996; Rohner-Jeanrenaud, 1999) and suppression of CRH secretion (Santana et al, 1995; Rohner-Jeanrenaud, 1999). The large increase in GCs in fasting rats (Cherel et al, 1992), penguins (Cherel et al, 1988a and b; Robin et al, 1998) and humans (Friedl et al, 2000) that coincides with entry into phase III is thought to contribute to the refeeding signal (Challet et al, 1995; Robin et al, 1998).

In most mammals, including pinnipeds, the major GC secreted by the adrenal cortex is cortisol (St Aubin and Dierauf, 2001) in response to the pituitary hormone, adrenocorticotrophic hormone (ACTH). Secretion of ACTH in turn is activated by release of CRH from the hypothalamus. Stimulation of the zona fasciculata/reticularis cells by ACTH causes storage vacuoles containing cholesterol esters to move alongside the mitochondria and increases the activity of both cholesterol esterase and steroidogenic acute regulatory protein (Martin, 1976). This initiates *de novo* synthesis of cortisol by facilitating uptake of cholesterol by the mitochondria. ACTH also promotes the activity of the steroidogenic enzymes bound to the inner mitochondrial membrane that hydroxylate cholesterol to give the GC precursor, pregnenolone. This is modified in the smooth endoplasmic reticulum to 11-deoxycortisol before it is shuttled back to the mitochondria for final conversion to cortisol. GCs bind to receptors in CRH and ACTH-producing neurones to inhibit secretion of the two hormones until GC concentrations fall back down to levels where the receptors are no longer activated.

The actions of GCs on target tissues are mediated by two distinct receptor populations that allow basal and stress-induced GC levels to produce different, and sometimes opposite, effects on metabolism and food intake (Tempel and Leibowitz,

1994; Saplosky et al, 2000). Mineralocorticoid receptors (MR) have a tenfold higher affinity for GCs than GC receptors (GR). GR are activated at the higher GC concentrations usually only experienced during acute stress, when MR are fully saturated. In general, the actions of basal GC levels are mediated via MR, whereas GR control the response to elevated GC levels (Saplosky et al, 2000).

Seasonal and diel variation in cortisol levels have been investigated in harbour seals (Gardiner and Hall, 1997). Cortisol concentrations have also been examined in other phocids in relation to contaminant burdens, immune function and handling stress (eg. Gulland et al, 1999; Engelhard et al, 2002). However the role of GCs in fasting pinnipeds is not well defined. An increase in cortisol is seen in northern elephant seal pups (Ortiz et al, 2001a and b; Ortiz et al, 2003a; Ortiz et al., 2003c) and adult female Subantarctic fur seals (Guinet et al, 2004) with time spent fasting. This suggests that, as in other mammals, GCs may play an important role in fuel use and initiation of foraging in fasting seals. However, harp and grey seal pups fasting in captivity do not show a change in cortisol levels (Nordoy et al, 1990; Nordoy et al, 1993). The disparity between species may reflect differences in metabolic requirements or methodological approach during sampling, since handling and restraint are likely to be stressful.

1.2.3.3.3. Thyroid hormones

TH (thyroxine (T4) and triiodothyronine (T3)) are typically central to the control of energy expenditure and development (Oppenheimer, 1979; Zhang and Lazar, 2000). They are secreted in response to stimulation by the pituitary hormone

thyroid-stimulating hormone (TSH) (Hadley, 1992), which is in turn activated by the hypothalamic neurohormone thyrotrophin-releasing hormone (TRH). TH downregulate TRH and TSH secretion, thereby regulating their own release from the thyroid in a classic negative feedback loop.

T₄, which is generally considered the less biologically active of the two compounds (Oppenheimer, 1979), comprises the majority of TH secreted from the thyroid. T₃ in the circulation is therefore derived mainly from extrathyroidal conversion from T₄ by monodeiodinase enzymes (Hadley, 1992).

TH bind to several different cellular receptor types, which have a substantially greater affinity for T₃ than T₄ (Hadley, 1992; Zhang and Lazar, 2000). Nuclear receptors, consisting of TH-responsive promoter sequences on target genes, are found in all cell types and allow TH to influence long term changes in transcription and translation. A cytosolic receptor exists that is thought to concentrate the hormone and TH receptors located on the inner mitochondrial membrane are responsible for TH effects on oxygen consumption. These extranuclear receptors mediate the more immediate effects of TH. The distribution of these receptors reflects the physiological responsiveness of the tissue type to TH (Hadley, 1992).

TH regulate growth and differentiation (Hadley, 1992; Nilsson et al, 1994; Bernal and Nunez, 1995; Dainiak et al, 1978), elevate resting metabolic rate (Guerra et al, 1996; Greco et al, 1998; Branco et al, 1999; Jekabsons et al, 1999) and enhance the rate of fat breakdown (Cheikh et al, 1994).

TH influence feeding behaviour in reindeer (*Rangifer tarandus tarandus*), rats and woodchucks *Marmota monax*, both directly, and indirectly through their impact on metabolic rate (Ryg and Jacobsen, 1982; Oppenheimer et al, 1991; Concannon et

al, 1999), and may also stimulate foraging in harbour seals (Renouf and Noseworthy, 1991; Boness et al, 1994; Haulena et al, 1998).

The thyroid axis is responsive to nutritional status (Flier et al, 2000; Harris et al, 2001). In terrestrial mammals and birds, food restriction causes a decline in TH levels (Moshang Jnr. et al, 1975; Crosson and Ibbertson, 1977; May, 1978; Spencer et al, 1983; ChereI et al, 1988b and c; Yen et al, 1994; Byerley and Heber, 1996; Fuglei, et al, 2000), and a fall in receptor density (Schussler and Orlando, 1978), which facilitates energy conservation by reducing activity levels and metabolic rate. However, fast-adapted marine mammals do not show a reduction in TH during imposed food restriction (Ortiz et al, 2000) or the normal course of fasting (Ortiz et al, 2001a).

Given the importance of TH in metabolism and development in other mammals and their responsiveness to nutritional status, it is possible that they play a role in the control of fuel allocation and the timing of departure from the colony in fasting seal pups.

1.2.3.3.4. Prolactin

Prolactin (PRL) is a peptide hormone that regulates energy balance in a number of mammalian species through its impact on lipolysis and food intake. It is secreted from lactotrophs in the anterior pituitary in response to a wide variety of stimuli including TRH, leptin, oxytocin, light and specific sounds and smells (Freeman et al, 2000). The effects of PRL are mediated by cell surface protein receptors that are present in wide variety of tissues and have a sex specific

distribution. The number of lactotrophs and responsiveness to PRL increase with age (Becuvillalobos et al, 1992).

PRL is best known for the stimulation of milk production and increase in appetite in response to suckling (Freeman et al, 2000). It also maintains pregnancy, promotes sexual behaviour and stimulates parental care of offspring. However, its role is not limited to reproductive physiology and behaviour. It plays a major part in the regulation of fat deposition and mobilisation (Kopelman, 2000) as well as the regulation of water and solute homeostasis and immune function (Freeman et al, 2000).

Chronic PRL treatment induces a reduction in adipose tissue mass in non-lactating rabbits (Fortun et al, 1994). PRL injection rapidly increases lipolysis in rabbits, (FortunLamothe et al, 1996), dogs (Winkler et al, 1971), humans (Berle et al, 1974) and lambs (Pearce et al, 2003). An increase in lipolysis is not seen in isolated rabbit adipocytes, suggesting that other signals are required in addition to PRL to induce lipolysis (FortunLamothe et al, 1996).

PRL increases appetite either by a direct impact on NPY (Strader and Buntin, 2001; Garcia et al, 2003) or AgRP (Strader and Buntin, 2003) secretion in the hypothalamus, or indirectly by stimulation of GC release (Bray, 1985; Freedman et al, 1985). An increase in PRL is associated with the hyperphagia exhibited by many mammals during lactation (eg. Garcia et al, 2003) and by breeding birds (eg. Strader and Buntin, 2003). Virgin or gonadectomised female rats and young male reindeer also show an increase in food intake and weight gain in response to PRL injection (Ryg and Jacobsen, 1982; Gerardo-Gettens et al, 1989; Byatt et al, 1993; Suave and Woodside, 1996; Heil, 1999). Elevated PRL levels are associated with a large increase in food intake, reduced body weight, negative energy balance, and, by

inference, an increase in basal metabolic rate in male wood chucks (Concannon et al, 1999).

PRL levels and sensitivity to the hormone can be responsive to changes in nutritional status. In humans, PRL concentrations increase with body mass in both children and adults (Kopelman, 2000). PRL levels are decreased by food restriction in Soay sheep (Rhind et al, 2000) but not in red deer (*Cervus elephus*; Rhind et al., 1998). Food restricted rats show a reduction in latency to increased food intake, suggesting that starvation increases sensitivity to PRL (Sauve and Woodside, 1996).

The role of PRL in the reproductive cycle and during lactation of pinnipeds has been investigated (Boyd, 1991; Mellish et al, 1999). It plays a major role in the mobilisation of fat reserves for milk production in these animals. It is possible that it is also involved in fuel partitioning in fasting pups and could play a role in the onset of foraging if it increases appetite in these animals.

1.3. Development of diving and foraging capabilities

It is crucial for the survival of grey seal pups that they develop adequate diving and foraging skills. The impact of body size and condition on diving and swimming capabilities is one possible mechanism through which variation in maternal investment may influence the survival probability of grey seal pups. Although there is substantial information about the diving and foraging behaviour of adult grey seals in the North Sea (Thompson et al, 1991; McConnell et al, 1992; Hammond et al, 1993; McConnell et al, 1999), and elsewhere (Sjoberg et al, 1995), little is known about the

development of physiological diving capabilities and behaviour of these animals at sea in their first year of life.

1.3.1. At-sea behaviour of grey seals

The behaviour of grey seals in the UK has been investigated at sea using Time-Depth Recorders (TDRs) and Satellite Relayed Data Loggers (SRDLs) deployed on individual animals (McConnell, 1986; Thompson et al, 1991; Hammond et al, 1992; McConnell et al, 1992; Fedak and Thompson, 1993a and b; Hammond et al, 1993; McConnell et al, 1999). These devices record information such as swim speed, depth and location, to provide a detailed picture of diving and foraging behaviour in time and space.

Adult grey seals show substantial interindividual variation in their behaviour, but there are some broad generalisations that can be made about their movement patterns and diving characteristics. They undertake both long distance trips (>100km) between known haul out sites, and repeated, short duration trips (less than three days) from the same haul out site to discrete foraging grounds within 40km offshore (Thompson et al, 1991; McConnell, et al, 1992; Hammond et al, 1993; McConnell et al, 1999). They spend up to 50% of the time at or close to haul out sites, roughly 30% travelling between haul-outs and only 12-14% on the short foraging trips (Thompson et al, 1991; Hammond et al, 1992; McConnell et al, 1992; McConnell et al, 1999). Feeding may not only occur during the intensive periods in foraging grounds, but also close to haul out and on long distance trips.

Long distance travel generally consists of directed, rapid movement with a high frequency of v-shaped dives, that involve active swimming (Thompson et al, 1991; McConnell et al, 1999). Foraging behaviour tends to be characterised by slow, less directed movement and square-bottom dives, usually to the sea-bed, with little swimming activity at the bottom of the dive (Thompson et al, 1991). Foraging occurs in areas with gravel/sand sediment, the preferred habitat of sandeels (*Ammodytes marinus*) (McConnell et al, 1999), which make up a large proportion of the grey seal diet (Hammond and Prime, 1990; Hammond et al, 1994a and b).

1.3.2. Physiological constraints on diving ability: size and age

Diving animals face many constraints that may limit their diving capability. Since seals are reliant on their tissue oxygen stores whilst submerged, their ability to remain at depth crucially depends on the size and rate of utilisation of those stores.

1.3.2.1. Development of oxygen storage capacity

In diving animals, oxygen is stored bound to haemoglobin in the blood and myoglobin in muscle tissue (Kooyman, 1989). Oxygen storage capacity scales with body mass and thus increases as pups grow as a result of blood volume expansion and development of muscle mass (Thorson and Le Boeuf, 1994). In addition, increases in haematocrit, haemoglobin, mass-specific blood volume and myoglobin concentration result in the expansion of mass-specific oxygen storage capacity as seal pups develop either during suckling or during the land-based postweaning fast (Bryden and Lim,

1972; Kodama et al, 1977; Thorson and Le Boeuf, 1994; Noren et al, 2000; Noren et al, 2003b). By departure from the colony, phocid pups possess roughly 75% of the mass-specific oxygen storage capacity of adult females (Thorson and Le Boeuf, 1994).

1.3.2.2. Oxygen utilisation

The maximum dive duration beyond which net lactate accumulation is expected to occur as a result of a switch from aerobic to anaerobic metabolism is termed the theoretical aerobic dive limit (ADL) (Kooyman et al., 1980). Since anaerobic metabolism yields only one eighteenth of the ATP produced by aerobic pathways and leads to tissue lactate accumulation, which has to be cleared subsequently by aerobic metabolism, it is preferable to dive aerobically (Kooyman et al, 1983; Fedak and Thompson 1993a; Boyd, 1997). Oxygen consumption must therefore be regulated carefully during diving to maximise time submerged. Minimising activity whilst submerged can conserve oxygen (Fedak and Thompson 1993a and b). The metabolic rate of captive seals during active voluntary diving is lower than when resting at the surface (Sparling and Fedak, 2004).

Management of oxygen reserves whilst submerged involves selective vasoconstriction (Davis et al, 1983; Ostholm and Elsner, 1999) which redistributes the blood supply such that heart and brain are perfused. The blood supply to other tissues is sporadic to allow them to reduce oxygen utilisation and make use of myoglobin stores, which would otherwise act as an oxygen sink by sequestering haemoglobin-bound oxygen and reducing its availability for metabolism. The degree of perfusion in

different tissue types depends on the length and circumstances of the dive (Davis et al, 1983; Kooyman, 1989; Butler and Jones, 1997; Ostholm and Elsner, 1999). Heart rate is modulated to maintain blood pressure. In adult grey seals freely diving in the wild (Fedak and Thompson, 1993) or in the laboratory (Reed et al, 1994) heart rate shows a bimodal pattern with tachycardia at the surface and often extreme bradycardia as low as four beats per minute during dives. Anticipatory changes in heart rate occur prior to descent and ascent and an increase in physical activity whilst submerged is not necessarily associated with alterations in heart rate (Butler and Jones, 1997). These responses to diving clearly require a considerable degree of cardiovascular control.

The ability to reduce metabolic rate develops during the postweaning fast in northern elephant seal pups (Thorson and Le Boeuf, 1994). The duration of sleep apnoea and the ability to regulate cardiorespiratory and vascular responses to breath-holding also increase during the postweaning fast in elephant seals (Blackwell and Le Boeuf, 1993; Castellini, 1994; Falabella, 1999). A similar increase in cardiovascular control occurs with age in Weddell seal pups (Burns, 1999) and is likely to occur in grey seal pups.

Metabolic rate during diving may be influenced by drag and buoyancy, which are related to body size and composition and impact on diving behaviour in juvenile northern elephant seals and adult grey seals (Webb et al, 1998; Beck et al, 2000; Biuw et al, 2003). Larger grey seals experience greater drag due to larger surface area (Beck et al, 2000). Buoyancy is negatively related to mass and positively related to fat content (Beck et al, 2000). Descent rate is significantly faster in less buoyant (e.g. leaner and larger) grey and northern elephant seals, such that fatter animals of any given mass must work harder to attain depth (Webb et al, 1998; Beck et al, 2000).

However, extra costs of positive buoyancy on the descent phase may be compensated, at least in part, on the return to the surface. Since grey seals tend to be benthic feeders, the cost of higher buoyancy may be apparent if animals must work harder to maintain depth as a result and therefore utilise more oxygen, than leaner, less buoyant conspecifics.

Juvenile animals, including grey seal pups, have elevated mass-specific metabolic rates compared to adults (Boily and Lavigne, 1997) and metabolic rate decreases with age in Weddell seals (Ponganis et al, 1993), harp, grey and northern elephant seal pups (Worthy and Lavigne, 1987; Thorson and Le Boeuf, 1994).

1.3.3. Behavioural and survival consequences of constraints on diving ability

Juvenile seals are often unable to remain submerged for as long as adult seals due to lower oxygen stores, as a result of their smaller body size and lower mass specific oxygen storage capacity, combined with a higher mass-specific metabolic rate (Burns and Castellini, 1996; Burns, 1999; Thorson and Le Boeuf, 1994). It has been suggested that juveniles should compensate for their limited diving capabilities either by rapidly acquiring the physiological characteristics needed to forage effectively, utilising prey resources that are easier to exploit e.g. at shallower depths or easier to catch and handle, diving more frequently or spending more time in the water (Burns, 1999).

Adult seals usually dive well within their theoretical ADL, which is estimated from assumptions about the size of body oxygen reserves and metabolic rate (Kooyman et al, 1980; Kooyman et al, 1983; Boyd, 1997). However, juveniles often

have a much lower theoretical ADL than adults and often need to reach or exceed the ADL to reach the prey depth or remain there for long enough to feed (Thorson and Le Boeuf, 1994; Burns, 1999). This may contribute to low juvenile survival (Burns, 1999).

Young Weddell, and elephant seals also show differences in diving and foraging strategies associated with body size (Burns et al, 1997; Hindell et al, 1999; Irvine et al, 2000). Smaller animals perform shorter dives. Smaller Weddell seals perform deeper dives than larger conspecifics, whereas the opposite is true in elephant seals (Hindell et al, 1999; Irvine et al, 2000).

Since increased time spent fasting results in greater oxygen storage capacity and cardiovascular control, but increased body size is associated with greater diving capabilities, there may be a trade off between spending time on land and going to sea at a higher body mass. Smaller pups are more likely to experience the costs of this trade off. Body size, composition and development during fasting may therefore influence diving capabilities of grey seal pups and provide a mechanism by which maternal investment influences survival.

1.4 Overview of thesis structure

This thesis is intended to provide an insight into some of the possible mechanisms that may underpin the relationship between body size and condition and first-year survival in grey seal pups. The way in which grey seal pups dispose of their endogenous fuel reserves involves a complex interplay between current and future demands both whilst fasting and at sea. Fasting fuel allocation and its regulation are

likely linked to the size and availability of energy reserves. Together these factors may shape the development of foraging behaviour and ultimately limit survival as a result of the limitations they place on fuel reserves and the physiological and physical constraints on diving capabilities.

A description of the fieldwork and statistical methods used in several of the chapters of the thesis is given in chapter 2. The validation studies of the hormone assays used later in the thesis are detailed and their utility in fasting seal pups are discussed in chapter 3.

The approach taken in this thesis was to manipulate directly both energy availability (using supplementary food) and GC levels (using a synthetic cortisol analogue) and determine the impact of these treatments on both the hormones that may be involved in fuel allocation and fuel allocation itself. In addition, the effects of handling frequency on fuel use and hormone levels were investigated to assess the impact of different handling regimes.

Chapter 4 describes changes in GC and TH levels during fasting and in response to acute and chronic stress and supplementary feeding. These changes are discussed in terms of their implications for the role of these hormones in fasting fuel use and for the planning of future sampling regimes.

Chapter 5 describes the testing of the synthetic GC, dexamethasone, in a captive setting to determine its efficacy and duration of action, followed by its impact on hormone levels and white blood cell number in wild fasting pups.

The effect of handling frequency, supplementary feeding and GC manipulation on fasting fuel use is investigated in chapter 6, in addition to natural variability in fuel allocation strategy in relation to individual covariates, such as sex

and energy reserves. The impact of dexamethasone on fast duration is also examined to establish whether high cortisol levels can prompt departure from the colony. The number of days available at sea before starvation occurs is calculated to determine the impact of fuel use strategy on the probability of survival post departure for individual animals. This provides an estimate of the margin of time that seal pups of different sizes and fasting strategies have available to them once they have left the colony.

The impact of initial body reserves, fasting fuel use strategy and sex on the development of foraging and diving skills is investigated in chapter 7. Movement patterns of individual grey seal pups are also described to provide information about the geographical areas used and behaviours exhibited by naïve animals in their first months at sea. Differences in the development of maximum diving capabilities over time are related to sex and size and body composition at weaning and departure.

The final chapter provides an integrated discussion of the key findings of the thesis and places them in a wider context, offering suggestions for future research.

Chapter 2

General materials and methods

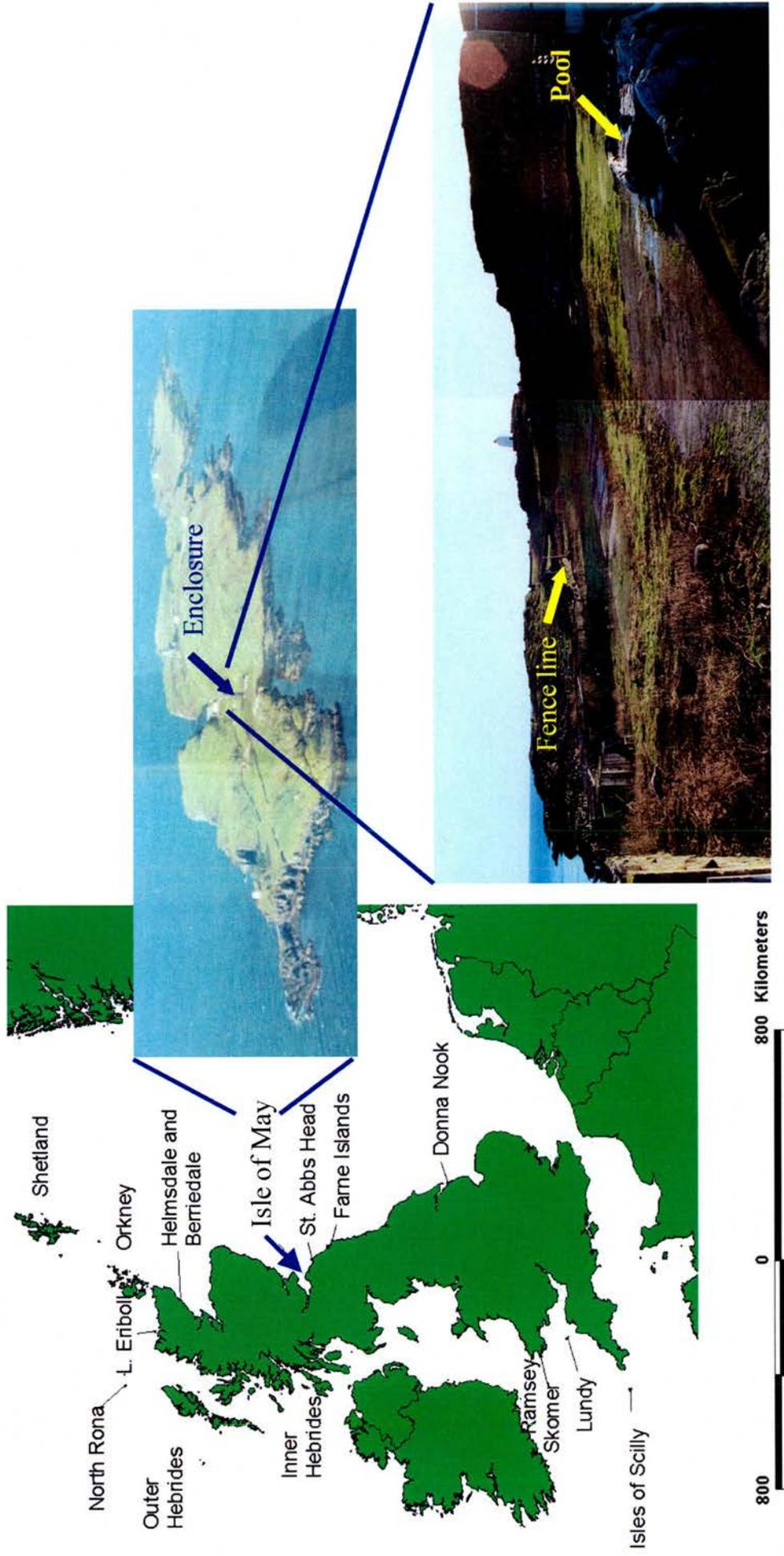
2.1. General restraint, handling and sampling

Field-based studies were performed on the Isle of May (Figure 2.1), in the mouth of the Firth of Forth, Scotland (56° 11'N, 2° 33'W) during October-December 2001 and 2002. The captive study described in chapter 5 was performed at the Sea Mammal Research Unit (SMRU) at the Gatty Marine Laboratories, University of St Andrews. All capture and handling procedures were performed under Home Office project licence #60/2589 and conformed to the Animals (Scientific Procedures) Act 1986.

All seals were caught on land. Pups and juveniles were physically restrained in a heavy-duty plastic “pup bag”. Body mass was measured using a 50kg (accuracy \pm 0.2kg) or, when necessary, a 100kg (accuracy \pm 0.5kg) Salter spring balance. Body length (to the nearest cm) was measured from the tip of the nose to the end of the tail when the animal was straight and flat on the ground. Length was used to calculate condition index (mass/ length; Hall et al, 2001; Hall et al, 2002).

Adults were anaesthetised with an intramuscular injection of 1mg kg⁻¹ zoletil¹⁰⁰ (Virbac, France). At each capture a blood sample was taken and animals were weighed. Blood samples were obtained as quickly as possible (usually within two minutes) after first contact with the animal to try to minimise the effect of stress on hormone levels. In the field-based experiments, blood samples were taken in the morning, where possible between 09:00 and 12:00, to minimise the effects of circadian rhythms on hormone measurements. Blood samples were taken from the extradural vein into sterile 10ml plain (serum) or heparin treated (plasma) vacutainers (Becton Dickinson), using either 19 gauge, 2 inch or spinal needles, whichever was most appropriate for the size of the animal.

Figure 2.1. Photograph and location of Isle of May on map of major grey seal breeding sites in the UK. The position of the temporary outdoor enclosure is indicated. View of enclosure to approximately south-east towards fence line that marks the extent of the pen. The freshwater pool is shown.



Animals were given an intramuscular injection of 1ml 10kg⁻¹ slow-release oxytetracycline (terramycin: Pfizer Ltd., Sandwich, Kent, UK) in the dorsal pelvic region when the blood sampling site appeared hot or raised, or when there were other visible signs of infection. The antibiotic injections were not administered more frequently than once every three days. All puncture sites were disinfected with Savlon before sampling or injection, and sprayed with topical terramycin™ (oxytetracycline: Pfizer Ltd) immediately afterwards to reduce infection risk.

2.2. Field based studies

2.2.1. Study animals

A total of 58 wild grey seal pups were included in the experiments described in this thesis. Table 2.1. provides summary information for these animals. Daily observations of mother-pup pairs allowed weaning date to be determined. Pups were assumed to be weaned when the female was not observed in attendance for one day.

2.2.2. Temporary captivity

Pups were captured as soon after weaning as possible and penned in a large outdoor enclosure, roughly 115 x 80m, shown in Figure 2.1. Study pups were penned to allow them to be located rapidly and to avoid daily disturbance of large areas of the colony. The large enclosure was thought to minimise the animals' awareness of being confined to avoid stress associated with captivity

Table 2.1: Summary information for field-study animals. * denotes those animals that were fitted with an SRDL prior to release. ? indicates where information is unknown.

Year	Group	Sex	Flipper tag	Animal name	Female brand/tag	Birth date	Wean date	Wean mass (kg)	Departure date	Fast duration (days)	
2001	FED	M	*50895	Leonardo	2B	28/10	19/11	52.1	15/12	26	
			51145	Newton	6U	30/10	19/11	47.2	15/12	26	
			51146	Roger	7J	31/10	20/11	42.1	?	?	
			*50900	Mawson	DO	31/10	17/11	40.7	12/12	25	
		F	*50899	Yeti	C9	30/10	23/11	66.1	01/01	39	
			51148	Bernie	4B	01/11	23/11	39.8	09/12	17	
	HIGH	M	*51142	Hubble	H7	29/10	15/11	50.5	06/12	21	
			51141	Jenner	F2	30/10	17/11	45.2	17/12	30	
			*50891	Alfie	5U	23/10	09/11	44.2	04/12	25	
			50893	Felix	2U	28/10	15/11	43.3	07/12	22	
		F	*50894	Queenie	50457	31/10	20/11	51.4	18/12	28	
			50892	Charlie	V-tail	27/10	12/11	40.8	07/12	25	
	LOW	M	*50896	Ikea	1B1	29/10	15/11	39.6	17/12	32	
			50887	Bertie	3B	22/10	11/11	49.3	03/12	22	
			*51144	Eric	OH	29/10	15/11	48.1	09/12	24	
			52021	Kelvin	3H	31/10	15/11	46.5	02/12	19	
		F	50888	Galileo	4U	25/10	16/11	46.3	07/12	21	
			51147	Oppenheimer	OJ	29/10	18/11	51.6	14/12	26	
	UNKNOWN	M	50889	Daisy	D1	24/10	12/11	41.4	09/12	27	
			52411	Shackleton	?	29/10	20/11	39.6	11/12	22	
			52104	Telford	-	?	20/11	53.7	?	?	
		F	*52039	Volt	-	?	21/11	38.2	11/12	20	
*52103			Zap	-	?	26/11	49.7	?	?		
52045			Watson	-	?	22/11	46.6	17/12	25		
2002	CONTROL	M	52102	Xena	-	?	22/11	46.1	?	?	
			52101	Uranus	-	?	20/11	45.0	15/12	25	
			52050	Ailsa	-	?	26/11	28.6	07/12	11	
		SALINE	M	*54010	Captian Caveman	H3	07/11	28/11	57.9	17/12	19
				52782	Foghorn Leghorn	52015	23/10	09/11	49.8	27/11	18
				52794	Homer	6J	28/10	13/11	42.5	17/12	34
	52785			Custard	C2	21/10	08/11	38.9	30/11	22	
	F		*54009	Dumbo	40638	10/11	28/11	38.3	10/12	12	
			*52262	Ulysses	OH	01/11	22/11	38.0	06/12	14	
	DEX	M	52799	Muttley	9H	28/10	16/11	46.7	01/12	15	
			52485	Quincy	6U	04/11	18/11	39.8	04/12	16	
			*54016	Zebedee	DO	05/11	23/11	38.8	09/12	16	
			52777	Dastardly	7B	23/10	10/11	52.3	29/11	19	
		F	52776	Asterix	1H	16/10	04/11	51.6	27/11	23	
			52784	Elmer Fudd	3B	23/10	09/11	45.7	24/11	15	
	CONTROL	M	*52800	Woody	5B	31/10	23/11	45.3	05/12	12	
			54017	Big Ears	9B	10/11	26/11	40.3	12/12	16	
			*52258	Scooby	2B	31/10	20/11	51.4	03/12	13	
		F	52260	Taz	2U	01/11	20/11	43.9	13/12	23	
			*52261	Olive Oil	7J	31/10	18/11	41.8	13/12	25	
			52788	Ivorina	3W	28/10	15/11	40.2	09/12	24	
	DEX	M	54014	Noddy	54015	?	17/11	35.4	09/12	22	
*52257			Kermit	4U	28/10	16/11	56.0	29/11	13		
52787			Mr. Benn	OB	20/10	08/11	48.4	01/12	23		
F		52259	Paddington	49450	31/10	19/11	43.7	04/12	15		
		52484	Alistair	3H	03/11	22/11	40.3	07/12	15		
		54005	Victor	44004	05/11	23/11	36.5	15/12	22		
CONTROL	M	*52480	Yogi	C9	01/11	23/11	61.0	23/12	30		
		*52482	Lassie	H7	30/10	16/11	41.2	06/12	20		
	F	54004	Roobarb	40799	06/11	20/11	40.6	07/12	17		
		52793	Grotbags	OJ	28/10	13/11	38.4	27/11	14		
52481	Xanadu	4B	03/11	20/11	36.4	14/12	24				

Weaned pups frequently utilise natural fresh and seawater pools whilst fasting, possibly for thermoregulation, fluid balance or the development of breath-hold capabilities underwater. Animals were therefore provided with an artificial freshwater pool, 6 x 4 x 0-0.5m, in the north-west corner of the pen.

2.2.3. Individual identification

The marking systems used to identify study pups are shown in Figure 2.2. At first capture, the sex and stage of each pup was recorded and the animal was fitted with a unique, five-digit cattle ear tag in the interdigital webbing of one rear flipper

Figure 2.2: Grey seal pup with flipper tag (A), highly visible paint identification letter (B) and SRDL (C).



Pups from branded or tagged females were first caught during suckling, whereas pups from unknown females were first captured after weaning.

Each pup was given a unique letter mark on its back in a highly visible yellow outdoor emulsion paint to enable rapid individual identification at relatively long distances. They were assigned letters in alphabetical order corresponding to the order in which they entered the pen. This method of marking reduced search time and eliminated the need to approach and handle animals unnecessarily to check identity. On the day of release from the pen, a subset of animals was also fitted with satellite relayed data loggers (SRDLs) described in chapter 7.

2.2.4. Release and departure

Animals were released between Kirkhaven and the Loan, near the top of Logan's Road as indicated on Figure 2.3. After the release of the first animal, the south end of the island was searched daily between Kettle Ness and the South Ness. The presence/absence of all study animals and their position on the colony was noted each day after release. Released pups still present on the colony were re-sampled every three days until the end of the study period (18/12/2001 or 16/12/2002) when most animals had departed from the colony. Departure was assumed to occur the day after the last sighting of the animal. This was checked independently, using data from those animals fitted with SRDLs (chapter 7).

2.2.5. Feeding and saliva collection

For both feeding (2001: chapter 4) and saliva collection (2002: chapter 3) pups were restrained manually and the mouth held open using a leather gauntlet and a chain-mail glove.

For the feeding experiment, frozen herring (Lunar Freezing, Peterhead, Scotland) were allowed to defrost in fresh water overnight then divided into portions of whole, intact fish and weighed to the nearest 0.2kg in individual plastic bags. Each pup received its entire meal during one handling episode. Pups were fed each fish head first and allowed to relax and breathe between each fish. The whole procedure took approximately five minutes. Fish that were rejected and damaged by the animals were reweighed and subtracted from the initial mass of the portion to allow final meal weight to be determined.

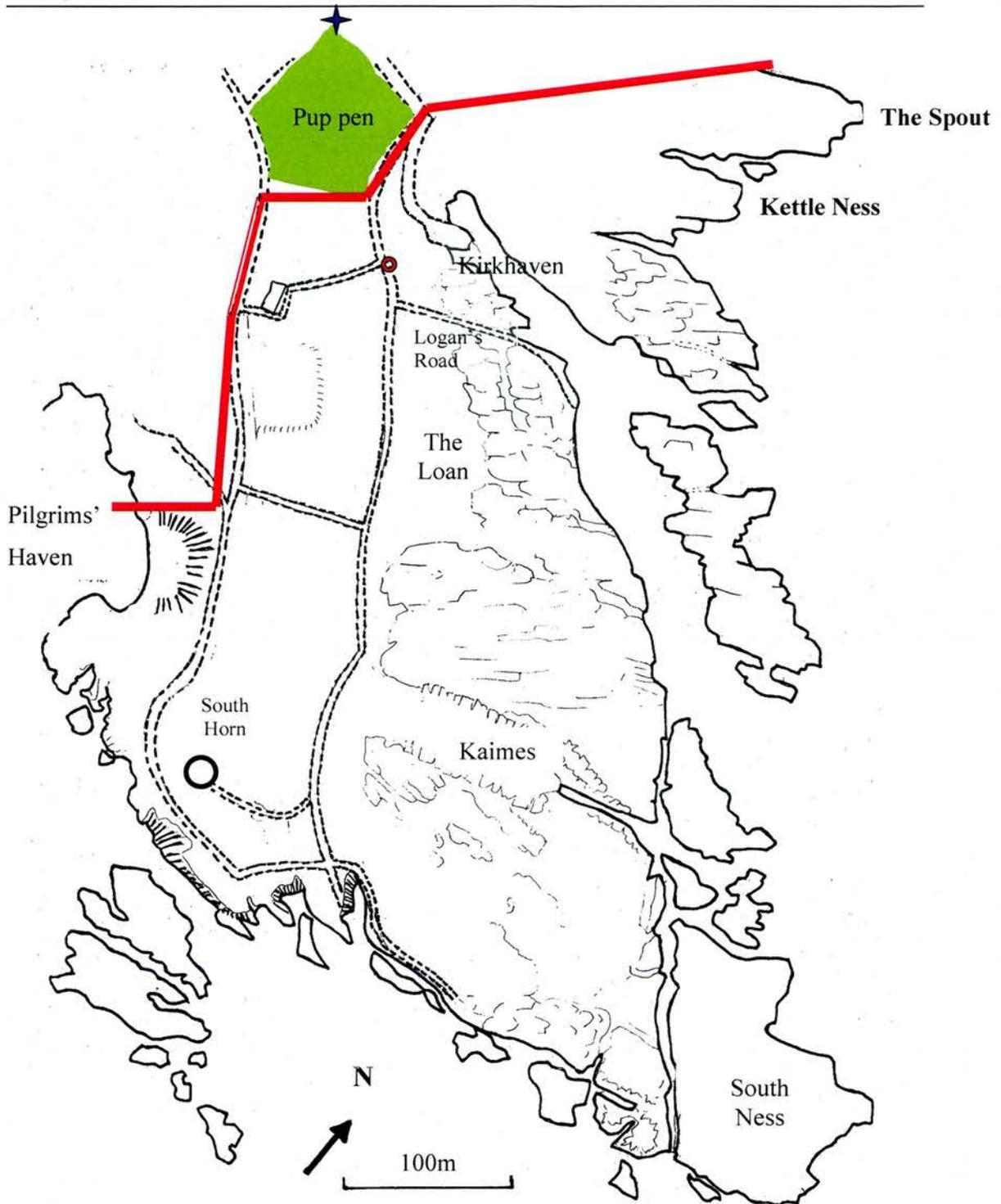


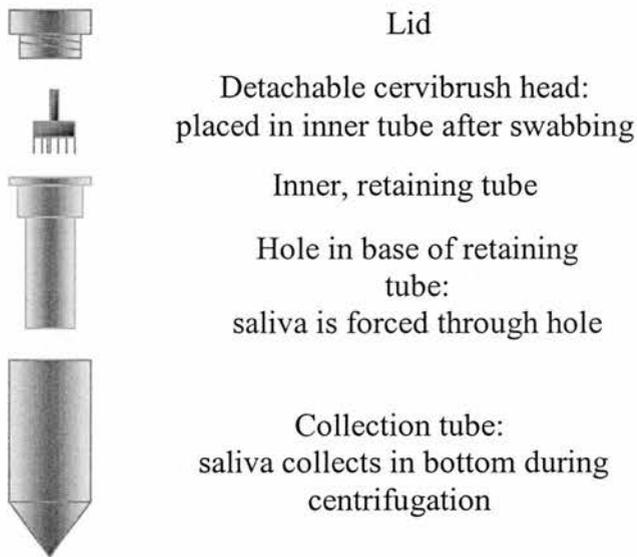
Figure 2.3: Sketch map (based on map produced by Scottish Natural Heritage) of southern portion of Isle of May, indicating location of pup enclosure (green area) and release site (red circle). The red line marks the northern extent of the area searched daily for the presence of released pups. The blue cross indicates the viewpoint for the photograph in Figure 2.1.

The Salivette® system (Sarstedt, Numbrecht, Germany) commonly used to collect saliva from humans (Kirschbaum and Hellhammer, 1994) was modified for use in seals as shown in Figure 2.4. Instead of using a chewable cotton roll, the inside of the mouth was swabbed several times with a cervibrush and the head of the cervibrush placed inside the inner, retaining tube. A total of 11 saliva samples were collected from six pups; one on three occasions, three on two occasions and two on one occasion.

2.3. Blood and saliva sample processing and storage

Blood samples were centrifuged in a swing-out bench top centrifuge at 2000g for 15 minutes, as soon as possible, and within ten hours, after sample collection. The retaining tube of the salivette® was placed inside the collection tube and spun in a microcentrifuge for 15 minutes at $\sim 2000 \times g$, which forced the saliva from the cervibrush through the small hole in the base of the retaining tube and into the bottom of the centrifuge tube. Aliquots of the serum, plasma (approximately 250 μ l) or saliva (variable amounts) were transferred to 500 μ l microtubes using disposable glass Pasteur pipettes, and stored at -20°C until analysis.

Figure 2.4: Modified salivette® system to collect saliva from grey seal pups



2.4. Statistical analysis

Statistical analyses were performed using MINITAB (Minitab 13.32, Minitab Inc, 2000) or R (R 1.9.1, R Development Core Team, 2003; Ihaka and Gentleman, 1996). Anderson-Darling tests were used to check that continuous data were normally distributed, and values were log or arcsine transformed where appropriate.

In most instances in this thesis, the data consisted of repeated measures, either from individual animals or blood samples, and were therefore not independent. In addition, missing data were common in all experiments because the number of samples obtained from an individual was dependent on the duration of its postweaning fast and thus not all animals were represented at each sampling point. Indeed, missing data were integral to the design of the experiment in 2001 described in chapters 4 and 6.

Linear mixed effects models (LMEs), which include both fixed and random effects terms (Chatfield, 1989; Crawley, 2002) were therefore used in many of the analyses. LMEs incorporate the covariance between successive data points in the model structure, thus accounting for individual differences in the response without over-parameterising the model (Chatfield, 1989; Crawley, 2002).

Fixed effects are expected to cause a consistent response across all individuals from a population. Random effects are not reproducible between replicate studies because they depend on, and may be biased by, the response of the particular individuals chosen for the experiment. The size of the effect of individual on the response variable is unimportant in the context of the analysis. A random term was included in each model to allow the intercept to vary for each individual animal or sample. Analysis of variance (ANOVA) was conducted to test whether the inclusion of this random intercept term significantly improved upon the simpler linear model containing only the fixed effects terms.

Forward and backward stepwise selection was used to establish which fixed effects should be retained in the models. Models were fitted using maximum likelihood estimates to allow the use of ANOVA to compare Akaike's An Information Criterion (AIC) of models containing different fixed effects. In instances when there appeared to be a difference in variance between factors, e.g. treatment group, sex or time variable, ANOVA was used to assess whether weighting each factor by variance significantly improved the model.

Overall model fit was assessed both by AIC and visually using plots of residuals against fitted values, residuals against the fixed effects included in the model, and fitted versus observed values. In instances where forward and backward selection gave different results, the best model was decided upon using these criteria.

Contrasts in R were set to the default “treatment” contrasts such that each fixed effect factor was compared to the level of that factor included in the intercept term. The factor level included in the intercept was changed such that each factor level was compared to every other factor level in each model.

Chapter 3

Immunoassay validation and storage stability of hormones

3.1. Introduction

3.1.1. Summary

Hormones known to regulate long-term energy balance in other species, including leptin, cortisol, thyroid hormones (TH) and prolactin (PRL), are likely to perform a similar role in seals. Accurate measurement of serum concentrations of these hormones is vital to understanding their role in the regulation of fasting fuel utilisation and timing of departure from the colony in grey seal pups.

This study confirms the presence of a protein in grey seal serum that has the same molecular weight as other mammalian leptins, and similar immunoreactive properties to leptin of a terrestrial carnivore. This chapter then describes the performance of the immunoassays used to measure hormone levels in serum and saliva in subsequent chapters. The impact of a delay between obtaining and freezing blood samples on cortisol measurements is also investigated to ensure that the way that samples are processed under field conditions provides reliable results.

3.1.2. Immunoassays

Immunoassays are widely used to measure hormone concentrations in a variety of body fluids (Gosling, 2000). These procedures utilise the specific binding properties between an antigen (Ag) and one or more antibodies (Ab) raised against Ag. Many immunoassays are reagent-limited techniques (enzymeimmunoassays (EIA) and radioimmunoassays (RIA)), in which Ag competes with a fixed

concentration of tracer-labelled Ag for access to limited binding sites on the Ab.

Alternatively, in immunometric assays (immunoenzymetric (IEMA) and immunoradiometric assays (IRMA)), Ab is present in excess and there is no competition for binding sites, to allow all the Ag present to bind to the Ab.

The Ab-Ag complex is detected by the coupling of either Ab or Ag to a tracer. The tracer in EIAs and IEMAs is an enzyme that produces a colour change reaction when exposed to its substrate, whereas the tracer in RIAs and IRMAs is a radioisotope. The amount of radioactivity or degree of colour change in the reaction vessel at the end of the assay is proportional to the amount of Ab-Ag complex formed. The response of the assay to known Ag concentrations is used to construct a standard curve, from which the concentration of Ag in unknown samples is calculated (Gosling, 2000).

3.1.3. Use of immunoassays in non-target species

Commercially available immunoassay kits intended for diagnostic or research purposes in humans or domesticated animals are often used to measure hormone levels in other species (St Aubin, 2001). However, if the detection Ab has been raised against Ag from another species it is possible that the reaction between Ab and study species Ag will be sub-optimal. This may arise from species differences either in the structure of the binding site, or epitope, on Ag, or in the carrier proteins and other macromolecules in the serum, which can interfere with the formation of the Ag-Ab complex (Ezan and Grassi, 2000; O' Fegan, 2000).

Many steroid, thyroid and small polypeptide hormones have a relatively simple chemical structure. Antibodies raised against the human versions of these

hormones often readily cross-react with the equivalent hormones from other animals (St Aubin, 2001). Larger peptide hormones differ widely in the degree of similarity between species. Small differences in protein sequence can have a large effect on the 3-dimensional structure of a protein and may alter the shape of binding sites on the molecule. Large proteins contain both conserved and variable regions. The degree to which an Ab will cross react with Ag from different species depends on the similarity between species of the epitope against which the antibody has been raised.

Despite similarities in the structure of hormones from phylogenetically distinct vertebrate groups, there are numerous differences between species in other serum constituents, including carrier proteins, enzymes and triglycerides (Ezan and Grassi, 2000). These large molecules can interfere with the binding of Ab to Ag, producing so-called matrix effects. Matrix effects can severely impair immunoassay function by preventing detection of the hormone of interest or increasing measurement variability to unacceptable levels (O' Fegan, 2000).

A difference in the response of the assay to Ag from the target and study species precludes the use of the target species standard curve to quantify Ag in the study species. For measurements to be meaningful it is therefore important to verify that the assay responds to Ag from the species of interest in the same way as it does to the Ag against which the detection Ab has been raised. The immunoassays used here are intended and optimised for use in human or canine samples and their performance characteristics are well defined for their target species (BioChem ImmunoSystems a, b and c; Linco Research Inc., 2000; Orion-Diagnostica, 2001). It is necessary to ensure that they can detect and measure accurately hormone concentrations in grey seal serum.

3.1.4. Hormone measurements in seals

Of the hormones investigated in this study, cortisol and TH are structurally identical between species. They have been measured reliably in seal serum and plasma using commercially available immunoassay kits intended for diagnostic or research purposes in humans (St. Aubin, 2001).

PRL and leptin are peptide hormones that show a high degree of protein sequence similarity between the terrestrial species for which they have been sequenced (eg. Hope et al, 2000; Iwase et al., 2000a; Sasaki et al, 2001; Zhao et al, 2003; Hammond et al, in press). Seal PRL has been measured reliably in assays in which the detection Ab has been raised against human PRL (Boyd, 1991; Gardiner, 1994)

The monoclonal detection Ab in one commercially available multi-species leptin RIA (Linco Research Inc, St Charles, Missouri), cross-reacts with leptin in several laboratory, companion and domesticated mammals, including rat, mouse, cow, horse, cat, pig and sheep (Linco Research Inc., 2000), and in little brown bat (*Myotis lucifugis*; Widmaier et al, 1997). This RIA has also been used in species for which leptin has not been characterised, including brown bear (*Ursus arctos arctos*; Hissa et al, 1998), mink (*Mustela vison*; Nieminen et al, 2000) and raccoon dog (*Nyctereutes procyonoides*; Nieminen et al, 2002). The assay has also been used to quantify leptin in several pinniped species for which leptin has not been positively identified. These include northern elephant seals (Ortiz et al, 2001a and b; Ortiz et al, 2003a) and Antarctic fur seals (*Arctocephalus gazella*: Arnould et al, 2002). The RIA seems to perform in a similar way in response to sequential dilutions of northern elephant seal serum as it does to human leptin standards (Ortiz et al, 2001a and b), which indicates

the presence of a leptin-like protein in the blood of these animals. However, the undiluted serum samples used in the validation appeared to contain much higher leptin concentrations, based on comparison of binding values with the standard curve, than any of the leptin measurements reported in these animals. Furthermore, the complementary DNA (cDNA) and protein of phocine leptin has recently been identified in grey and harbour seals, and shows some sequence differences in regions that are normally conserved in terrestrial mammals, which may alter the shape of binding sites on the protein (Hammond et al, in press). It is therefore necessary to demonstrate that the multi-species RIA can be used to quantify leptin reliably in grey seal serum.

An IEMA, developed to measure canine leptin using a species-specific Ab (Iwase et al, 2000b), may be better able to detect leptin in seal serum than the multi-species RIA. Canine leptin has 76 -88% amino acid sequence identity with leptin from other mammals (Iwase et al, 2000a). However, anti-mouse and anti-human-leptin Abs do not cross-react strongly with canine leptin (Iwase et al, 2000b). This is probably due to differences in the amino acid sequence that cause alterations in protein structure. Canine leptin has eight amino acid substitutions at positions that are normally highly conserved (Iwase et al, 2000a). It is likely that the greater differences between the sequence of phocid leptin and that of terrestrial mammals may impair or prevent binding between phocid leptin and currently available Abs raised against leptin from other species. The leptin sequence from grey and harbour seals is more similar to canine leptin than it is to leptin of other mammal groups, for which the protein has been sequenced (Hammond et al, in press).

The anti-canine leptin IEMA uses a polyclonal detection Ab, which binds to a variety of epitopes on the leptin molecule. This may circumvent difficulties caused by

species differences in leptin sequence and increase the probability of the formation of the Ab-Ag complex.

Leptin levels in carnivores (Iwase et al, 2000b), and pinnipeds in particular (Gurun et al, 2001; Ortiz et al, 2001a and b; Arnould et al, 2002; Ortiz et al, 2003a), seem particularly low when compared with terrestrial non-carnivores. These low levels may be a genuine reflection of actual leptin concentrations, rather than a product of poor Ag detection by the kit Ab. This means, however, that measurements of phocine leptin from the multi-species RIA lie close to the detection limit of the assay (1ng ml^{-1}), where small changes in concentration do not produce a large response. This lack of sensitivity in the range where leptin concentrations seem to lie makes it less likely that small, but biologically significant, changes in leptin concentration will be detected. If possible, an assay should be used in which measurements lie on the linear section of the standard curve, where small changes in Ag concentration produce a substantial difference in the assay response and therefore provide more precise results. The canine leptin IEMA is designed to measure leptin at much lower concentrations ($0.2\text{-}12.8\text{ng ml}^{-1}$) than the multi-species RIA ($1\text{-}50\text{ng ml}^{-1}$). The ability of the anti-canine leptin IEMA to detect and measure phocine leptin was therefore investigated as a potential alternative to the multi-species kit.

3.1.5. Salivary cortisol

Circulating cortisol levels increase dramatically, in seals (Gardiner and Hall, 1997; Engelhard et al, 2002) as in other mammals (Dohler et al, 1977; Hadley, 1992; Saplosky et al, 2000), in response to acute stress, which includes both 'natural' stress

and that caused by human activities, such as capture and handling. Most blood samples can be obtained rapidly after initial contact with animals that are physically restrained. However, time taken to obtain the blood sample (Dohler et al, 1977; Gardiner and Hall, 1997; Engelhard et al, 2002), the requirement for prior chemical immobilisation (Engelhard et al, 2002) and the amount of movement (Kanaley and Hartman, 2002) and/or anxiety (Alpers et al, 2003) prior to sampling can all introduce a degree of variability in the cortisol measurements obtained from blood samples. A non-invasive and reliable method for the measurement of baseline cortisol levels in samples collected under field conditions from wild seals is clearly desirable.

Cortisol metabolites can be measured in faeces and urine (e.g. Miller et al, 1991; Palme et al, 1996). Faecal cortisol levels have been measured in captive harbour seals (Gulland et al., 1999) and Steller sea lions (*Eumatopias jubatus*; Hunt et al, 2004; Mashburn and Atkinson, 2004) However, these techniques have limited application in wild, fasting seals because these animals do not defecate or produce significant quantities of urine after the first few days of fasting. It is also difficult to assign particular scat samples to specific individuals when the animals are free-ranging.

Salivary cortisol measurements are routinely taken from human patients and volunteers (Kirshbaum and Hellhammer, 1994) and are also used in captive mammals (Bradshaw et al, 1998; Pell and McGreevy, 1999; Millspaugh et al, 2002) as a non-invasive and reliable alternative to blood sampling. Since the acinar cells that line salivary glands prevent entry of proteins into the saliva, peptide and protein-bound hormones cannot be measured using this technique (Kirschbaum and Hellhammer, 1994).

There are a number of potential advantages to the measurement of cortisol in seal saliva rather than in blood. Cortisol takes much longer to diffuse passively into saliva than it does to enter the blood stream by active secretion from the adrenals. Cortisol levels in saliva samples are therefore less prone to transient variability caused by acute stress, and are a better reflection of true baseline levels of the hormone than measurements from serum or plasma samples (Kirschbaum and Hellhammer, 1994). A further advantage to the use of saliva, especially in field-based studies, is the ability to store samples at ambient temperature for up to four weeks without significant sample degradation (Kirschbaum and Hellhammer, 1994).

This study explored the feasibility of obtaining saliva samples from fasting grey seal pups, and tested an immunoassay that can be used to measure cortisol in human saliva for its ability to detect and quantify cortisol in grey seal saliva.

3.1.6. Sample degradation

Hormones in blood samples taken under field conditions may degrade before the samples are stored. Blood taken in the morning is often not processed until early evening, up to 12 hours after sampling. Although the unprocessed samples usually remain below 10°C, there is the possibility that enzyme action, temperature and light could degrade hormones present in the blood. As a result, measured hormone levels may not be an accurate reflection of the hormone levels present in the blood at the time of sampling. It is necessary, therefore, to establish whether hormone levels change as a function of time taken to process the sample before storage.

3.1.7. Experimental aims

The aims of this chapter are to establish whether the immunoassays chosen for this work can reliably measure the hormones of interest in grey seal serum and saliva. The existence of phocine leptin in grey seal serum is demonstrated and the ability of two leptin immunoassays to quantify this protein is compared. The effects of timing of sample processing on serum cortisol measurements are also described.

3.2. Materials and methods

All serum and saliva samples for use in assay validation were obtained from grey seal pups and adult females as described in chapter 2.

3.2.1. Identification of leptin in grey seal serum

3.2.1.1. *Partial purification and concentration of phocine leptin*

Adult female grey seal serum was fractionated by gel filtration (Wallace, 2000) on a Sephadex G100 (Sigma chemicals, UK) column (16 x 300mm) equilibrated and eluted with 50mM Tris(hydroxymethyl)aminomethane (Tris), 1mM ethylenediaminetetraacetic acid (EDTA), 0.05% Triton X-100, pH7.5 at 20°C.

To determine where a known leptin protein would elute from the column, 1ml pooled adult female grey seal serum was spiked with 200µl (~1kBq) ¹²⁵I-human leptin (multi-species RIA, Linco Research Inc., St Charles, Missouri), mixed and incubated for two days at 4°C to allow equilibration with carrier proteins. This was applied to the column and filtered at a speed of 5ml hr⁻¹ and 1.5ml fractions were collected. Each fraction was counted for one minute in a gamma counter (Minaxi Auto-Gamma 5000 series, Packard). All fractions containing ¹²⁵I-human leptin were indicated by the presence of counts above background.

Total protein was measured in 25µl from each fraction using the Lowry method (Lowry et al., 1951). Those fractions with counts above background and high total protein values were assumed to reflect the bound portion of the hormone,

whereas fractions with counts above background and low protein values were assumed to contain free leptin.

Six ml of pooled unspiked adult female grey seal serum was concentrated to 1ml in a vacuum desiccator over anhydrous silica gel for several hours at room temperature and fractionated as described above. Fractions 29-31, 32-34 and 35-37 were pooled to give three pooled fractions (pooled fractions I-III). Pooled fractions I and II were equivalent to those that had contained free ^{125}I -human leptin in the spiked serum (likely positive for phocid leptin). Pooled fraction III was equivalent to those in which counts from the spiked serum had not been above background (likely negative for phocid leptin). Pooled fractions I-III were dried in a vacuum desiccator and reconstituted in 0.5ml elution buffer.

3.2.1.2. Protein separation and detection

Pooled fractions I and III, canine leptin and broad spectrum molecular weight markers (6.5-200kDa: BioRad) were separated by SDS-PAGE using the method devised by Schagger and von Jagow (1987) to resolve proteins of 1-100 kDa.

Protein bands were detected by silver staining as follows: gels were agitated in fixative 1 (40% methanol, 10% acetic acid) for twenty minutes at room temperature and in fixative 2 (40% ethanol) for ten minutes. The gels were washed twice for five minutes in deionised water. Oxidiser (0.02% $\text{Na}_2\text{S}_2\text{O}_4$) was added for one minute, followed by a further two 20-second washes in deionised water. The gels were then agitated in chilled 0.1% AgNO_3 for twenty minutes and washed again in deionised water before the addition of developer (0.05% CH_2O ; 3% Na_2CO_3) for thirty seconds. The gels were then covered in developer and agitated until the protein bands had

developed sufficiently. Developer was discarded and stop solution (5% acetic acid) added for five minutes.

3.2.2. Immunoassay procedures

Both the commercially available multi-species RIA (Linco Research, Inc, XL-85K) and the ultra-sensitive canine leptin IEMA, developed by Morinaga Institute of Biological Science (MIBS), Yokohama, Japan, were evaluated for their ability to detect leptin in grey seal serum. Cortisol concentrations ([cortisol]) in both serum and saliva were measured using Spectria ^{125}I -cortisol radioimmunoassay (Orion Diagnostica, Espoo, Finland). Serum TH and PRL concentrations ([TH] and [PRL]) were determined using Serozyme magnetic solid phase EIAs and IEMAs, respectively (BioChem ImmunoSystems (UK) Ltd., Woking, Surrey).

The protocols are described in the manufacturers' instructions (BioChem ImmunoSystems, a, b and c; Linco Research Inc., 2000; Orion-Diagnostica, 2001) and the design of each assay is outlined in appendix 1. The protocol of the canine leptin IEMA was modified by the manufacturers from a previously published assay (Iwase et al, 2000b). All samples, standards and quality controls (QC) were assayed in duplicate following the assay protocols. Changes to the original protocols are described below.

3.2.2.1. *Leptin RIA*

Modifications to the manufacturer's protocol were introduced for the leptin RIA to allow use with available equipment and to permit measurements of

concentrations less than the lowest standard concentration supplied. Extra standards at the lower end of the range (0.1 and 0.5ng ml⁻¹) were made up immediately prior to use, by dilution of the kit standards. Initially, 1.5ml microcentrifuge tubes and polystyrene LP4 tubes were used in the assay to test pellet stability in different tubes. Pellet formation was found to be sufficiently stable in the microcentrifuge tubes and these were used in all subsequent assays. Centrifuge steps were performed in unrefrigerated bench-top fixed-angle microcentrifuges. At the end of the assay, each microcentrifuge tubes was placed inside a PONY vial and counted for one minute in a gamma counter (Minaxi Auto-Gamma 5000 series, Packard).

3.2.2.2. TH and PRL

Assay procedures for TH and PRL measurement were adapted from the kit protocols for use in 96 well flat-bottom microtitre plates (Dynex technologies). Quarter volumes of all reagents were used and incubation times extended accordingly, (Gardiner, 1994) (Table 3.1).

Table 3.1. Summary of modified TH and PRL assay volumes and incubation times. A - G are reagents (A: sample, standard or QC; B: conjugate; C: anti-Ag antibody; D: separation reagent; E: wash buffer; F: PMP; G: stop solution).

Assay	Step 1: Ab- Ag complex formation ⇒				Step 2: Separation ⇒			Step 3: Colour development		
	µl			Incubation time (mins)	µl D	Incubation time (mins)	µl E	µl F	Incubation time (mins)	µl G
	A	B	C							
TT4	12.5	50	50	45	50	15	125	75	30	250
TT3	25	25	25	45	50	20	125	75	30	250
PRL	25	50	-	45	50	15	125	75	20	250

The plate was covered and shaken for two minutes on a plate shaker before each incubation step and after the addition of wash buffer. After the addition of stop solution, the plate was placed on the magnetic separator and the magnetic particles allowed to sediment for five minutes. 150µl from each well was transferred into the equivalent well on a clean microplate and the absorbance in each well was measured at 570nm, with 490nm used as reference, in a spectrophotometer (Dynex technologies). For any given sample, the serum required for both TH assays was drawn from the same aliquot of serum and the assays were performed within 24 hours of defrosting the aliquot to minimise any effects of sample degradation. Serum was stored at 4-8°C between assays.

3.2.3. Calculation of results

Mean absorbance for duplicate wells was calculated in the EIAs and IEMAs. For both RIAs, mean counts per minute (cpm) were calculated for duplicate tubes. In the leptin RIA, the mean cpm for non-specific binding tubes was subtracted from the mean cpm from all other tubes. For both RIAs the final mean cpm for each tube was expressed as a percentage of radiolabel bound to the antibody compared to the zero standard (%(B/B₀)) as follows:

$$\% (B/B_0) = \frac{(\text{standard or sample count})}{0\text{-standard count}} \times 100$$

A standard curve was constructed for each assay run from the standard absorbance or $\%(B/B_0)$ values using Curve Expert 1.34. Sample [Ag] was calculated from the equation of the line.

3.2.4. Serum stripping

Grey seal serum was stripped to use as assay diluent for the cortisol RIA. Twenty-five mg ml⁻¹ activated charcoal (Fisons, 4040/60) was added to pooled female grey seal serum in plastic 7ml centrifuge tubes. The tubes were gently shaken continuously overnight at 4°C and then centrifuged for 15 minutes at 4°C and 4000rpm in a swing out bench top centrifuge. The supernatant was decanted and the pellet discarded. The centrifuge and decanting steps were repeated four times. After the final centrifuge, the serum was passed through a 1µm pore polydisc SPF disposable filter (Whatman 6724-5000) to remove all remaining charcoal particles from suspension. Stripped serum was stored frozen in 1ml aliquots at -20°C until use.

3.2.5. Assay performance

Accuracy was assessed both by testing for linearity of assay measurements at a variety of sample dilutions and by the amount of analyte recovered from samples spiked with known analyte concentrations (O' Fegan, 2000). Precision was assessed by determining inter and intra assay coefficients of variation (%CV) of sample measurements.

3.2.5.1. Linearity

Dilutions of selected serum samples, pooled serum or pooled saliva were assayed in duplicate to ensure that the measurements obtained were independent of the effective sample volume used. Samples were chosen to represent a range of initial

analyte concentrations. Pooled fractions I-III were also sequentially diluted and assayed on one occasion in the leptin IEMA. Dilutions were prepared in U-bottom microtitre plates. The number of dilutions performed was dependent on reagent availability (Table 3.2). Each dilution was assayed in duplicate. For each sample the $\%(B/B_0)$ values (leptin RIA and cortisol) or absorbance values (canine leptin IEMA, TH and PRL) obtained were plotted alongside those for the standard curve, and the slopes of the two lines were compared visually to assess parallelism to the standard curve.

Table 3.2: Details of dilution factors, number of occasions on which dilutions were assayed, total number of samples and assay diluent used (provided in the kits except for stripped seal serum). Pooled fractions I-III were also diluted in the same way in the leptin IEMA. * indicates that sample pools were used

Assay	Dilution factors	Occasions	Total samples used	Assay diluent
Leptin RIA	1, 1.33, 2, 4	2	6	Assay buffer
Leptin IEMA	1, 2, 4, 8, 16	3	3 *	Stripped canine serum
Serum cortisol	1, 2, 4, 8, 16	5	7	Stripped grey seal serum
Salivary cortisol	1, 1.33, 2, 4, 8	1	1 *	Assay buffer
TT4	1, 2, 4, 8	2	7	T4-free human serum
TT3	1, 2, 4, 8	2	7	T3-free human serum
PRL	1, 1.33, 2, 4, 8	2	6	PRL-free human serum

A more rigorous test of linearity established whether the measured [Ag] at each dilution was independent of the volume of sample used (O’Fegan, 2000). Effective sample volume was calculated for each dilution (effective volume = sample volume x dilution factor) and measured [Ag] at each dilution was corrected for effective sample volume. Corrected [Ag] was plotted against effective volume. This

was performed for all assays, except salivary cortisol and pooled fractions I-III in the leptin IEMA, due to insufficient sample and reagent, respectively.

3.2.5.2. Spike recovery

The recovery of known quantities of standard from spiked serum samples (%R) was used to determine assay accuracy, except for the canine IEMA in which there was insufficient reagent available. Mixtures of 1:1 sample to standard were prepared in U-bottom microtitre plates. Samples with high and low measured [Ag], and the zero standard or stripped serum were spiked with high (leptin = 50ng ml⁻¹; cortisol = 500nmol l⁻¹; TT4 150ng ml⁻¹; TT3= 5ng ml⁻¹; PRL =22.71ng ml⁻¹;) and low (leptin = 20ng ml⁻¹; cortisol = 50nmol l⁻¹; TT4= 40ng ml⁻¹; TT3 = 0.5ng ml⁻¹; PRL = 9.11ng ml⁻¹) standards. The appropriate volume of the mixture was assayed in duplicate. Expected values (0.5 x unspiked sample measurement + 0.5 x [standard]) were calculated and %R was determined as follows:

$$\%R = \frac{\text{observed value} \times 100}{\text{expected value}}$$

3.2.5.3. Precision

Intra-and inter-assay %CV were calculated for a subset of grey seal serum samples. The number of samples selected and the number of repeats performed was limited by the amount of reagents available for each assay and are given in Table 3.3. There was insufficient sample to perform this test for salivary cortisol measurements. To determine the variation due to kit modifications intra and inter %CV for quality

controls were calculated where available (leptin RIA, TH and PRL assays). %CV was calculated as follows:

$$\%CV = \frac{\text{standard deviation of sample measurements}}{\text{mean of sample measurements}} \times 100$$

Table 3.3: Number of samples, duplicates of each sample and separate occasions used to calculate inter and intra %CV for each assay. * denotes assays where %CV was also calculated using the kit QC to compare with grey seal serum values.

Assay	Intra %CV			Inter %CV	
	samples	duplicates	occasions	samples	occasions
Leptin RIA *	1	10	1	10	3
Leptin IEMA	1	3	1	-	-
Cortisol	5	3	4	5	4
TT4 *	3	3	3	3	3
TT3 *	3	3	3	3	3
PRL *	3	3	1	3	4

3.2.6. Hormone measurements

[Ag] and TT3:TT4 ratio (another measure of TH dynamics) in the serum samples used for the validation studies are reported for those assays deemed appropriate for use in grey seal serum, to compare with values reported in the literature.

3.2.7. Storage stability

Five vacutainers of blood were taken from each of three female grey seals (one juvenile and two adults) on a single occasion in the captive facility at SMRU. The samples were kept in laboratory light and temperature conditions until they were centrifuged, aliquotted and stored. One tube from each animal was processed at 0, 2, 4, 8 and 12 hours after obtaining the sample, to reflect the maximum possible range of time taken to store samples under field conditions. [Cortisol] was measured using the RIA described above.

3.2.8. Statistical analysis

Results from sequential dilutions of the same sample, and samples from the same individual, are non-independent. Linear mixed effects models (LMEs) that included sample as a random effect, were thus used to determine whether measurements were independent of volume in each assay (Crawley, 2002). For each assay, the effect of effective sample volume on corrected [Ag] was assessed. One effective sample volume was removed, starting with the smallest, and the regression repeated until the slope of the line was no longer significantly different from zero. The smallest effective volume at which the slope of the line was no longer significantly different from zero was assumed to be the limit of linearity for the assay.

An LME that included individual as a random effect was used to determine whether cortisol measurements were influenced by time taken to process the blood sample.

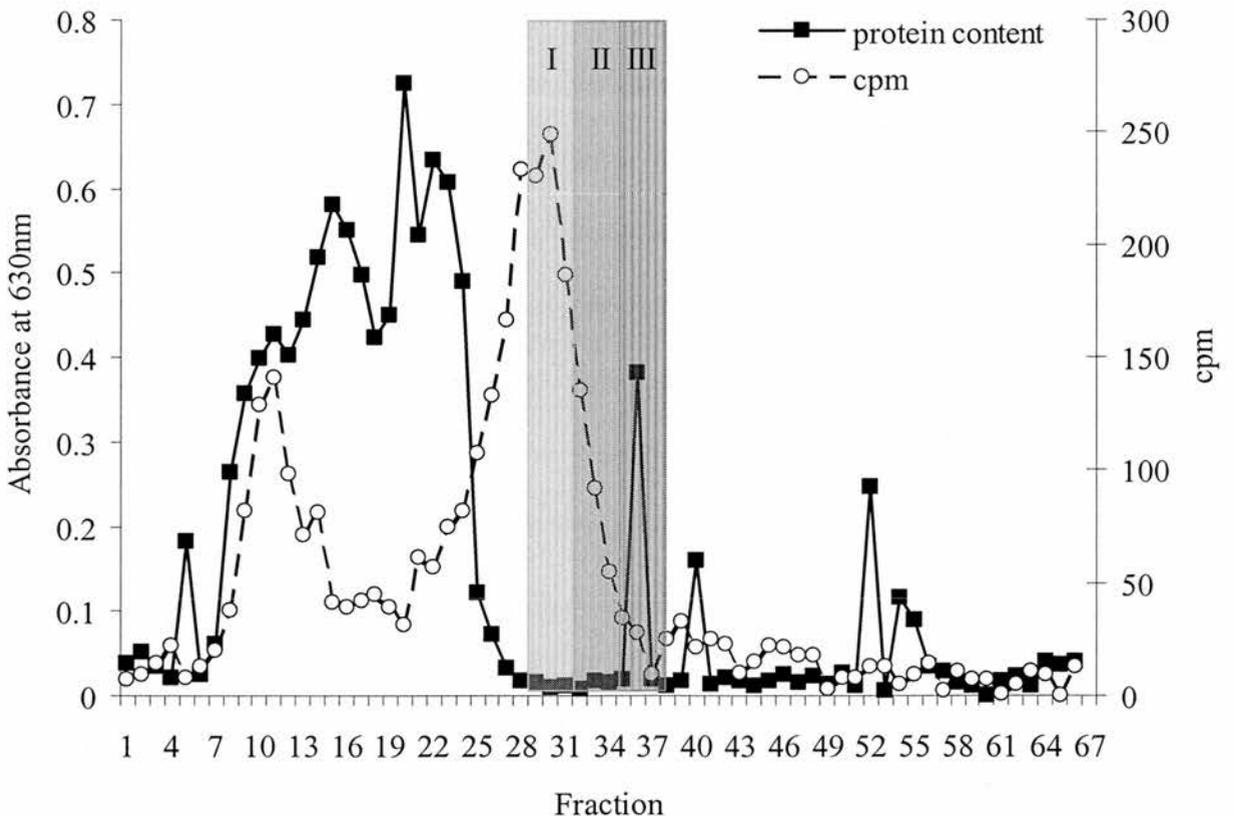
3.3. Results

3.3.1. Identification of leptin in grey seal serum

3.3.1.1. Partial purification of phocine leptin

Total protein content and cpm of the fractionated spiked grey seal serum are shown in Figure 3.1. ^{125}I -human leptin in seal serum eluted two peaks: the first in the same fractions (9-14) as the major protein component of the plasma, and the second in fractions 21-34, which contained very little protein.

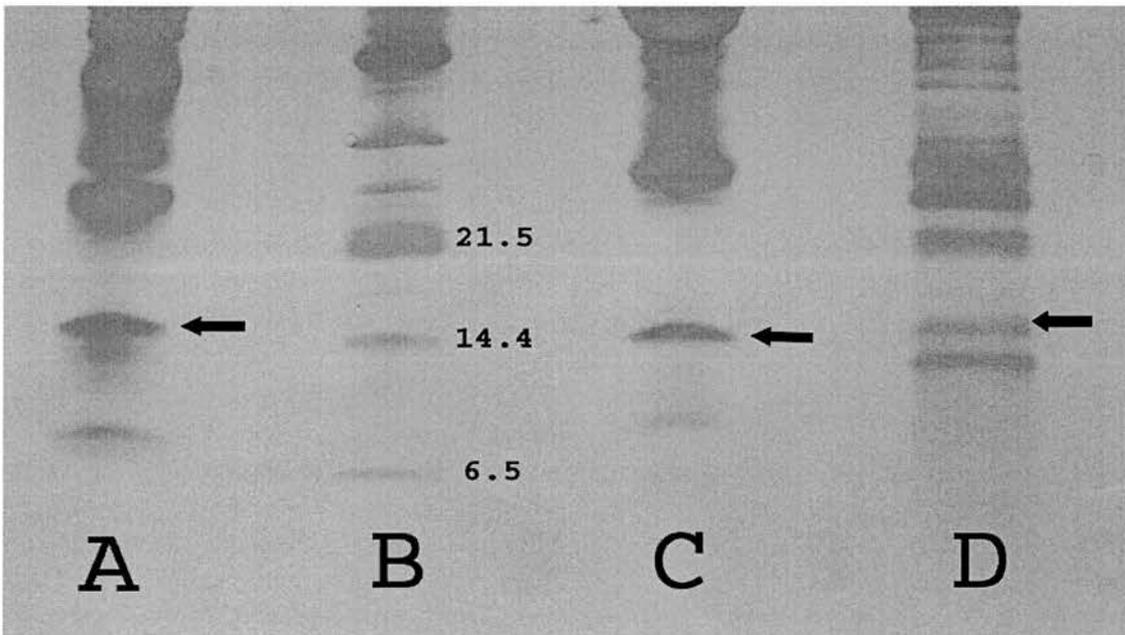
Figure 3.1: Absorbance values (proportional to protein content) and cpm (proportional to ^{125}I -human leptin concentration) for fractionated ^{125}I -human leptin-spiked grey seal serum. Pooled fractions I-III are indicated by shading.



3.3.1.2. Protein separation and detection

Figure 3.2 shows the protein bands separated by SDS-PAGE from canine leptin, broad-band molecular weight markers, and pooled fractions I and III. A protein band of ~ 16kDa is clearly visible in lanes A (canine leptin standard) and C (pooled fraction I). Lane D (pooled fraction III) also contained a band of a similar size but, as discussed below, this fraction did not react with an anti-canine leptin antibody and was thus unlikely to be leptin.

Figure 3.2: SDS PAGE gel showing protein bands from canine leptin standard (A), broad range molecular weight markers (molecular weight of smaller markers shown) (B), pooled fraction I (C), and pooled fraction III from grey seal serum (D). Arrows indicate protein bands of the predicted molecular weight for leptin in canine standard and grey seal fractions.



3.3.2. Assay performance

3.3.2.1. Linearity

Linearity was assessed visually using Figures 3.3-3.9a., and measured [Ag] for each effective sample volume are shown in Figures 3.3-3.9.b. The dilution/ effective sample volume at which the slope of the linear regression was significantly different ($p < 0.05$) from zero (the limit to linearity) is indicated for each assay.

It was difficult to assess visually whether $\%(B/B_0)$ values for sample dilutions were parallel to the standard curve in the leptin RIA because the values were close to the lower working limits of the standard curve (Figure 3.3.a.). Leptin measurements were linear up to a dilution factor of 1.33.

Absorbance values for dilutions of pooled fractions I and II were parallel to the standard curve in the leptin IEMA. For dilutions of raw samples, absorbance values were not parallel to the standard curve, and leptin values were not independent of volume, (Figure 3.4.a. and b) and the assay showed no response to pooled fraction III.

Figure 3.3. a. Parallelism of $\%(B/B_0)$ values of grey seal serum samples with the leptin RIA standard curve (mean \pm standard deviation) b. Leptin measurements (human equivalent) from sequential dilutions of grey seal serum as a function of effective sample volume. Stars indicate those dilutions/ effective sample volumes for which the response of the assay to seal serum was not linear.

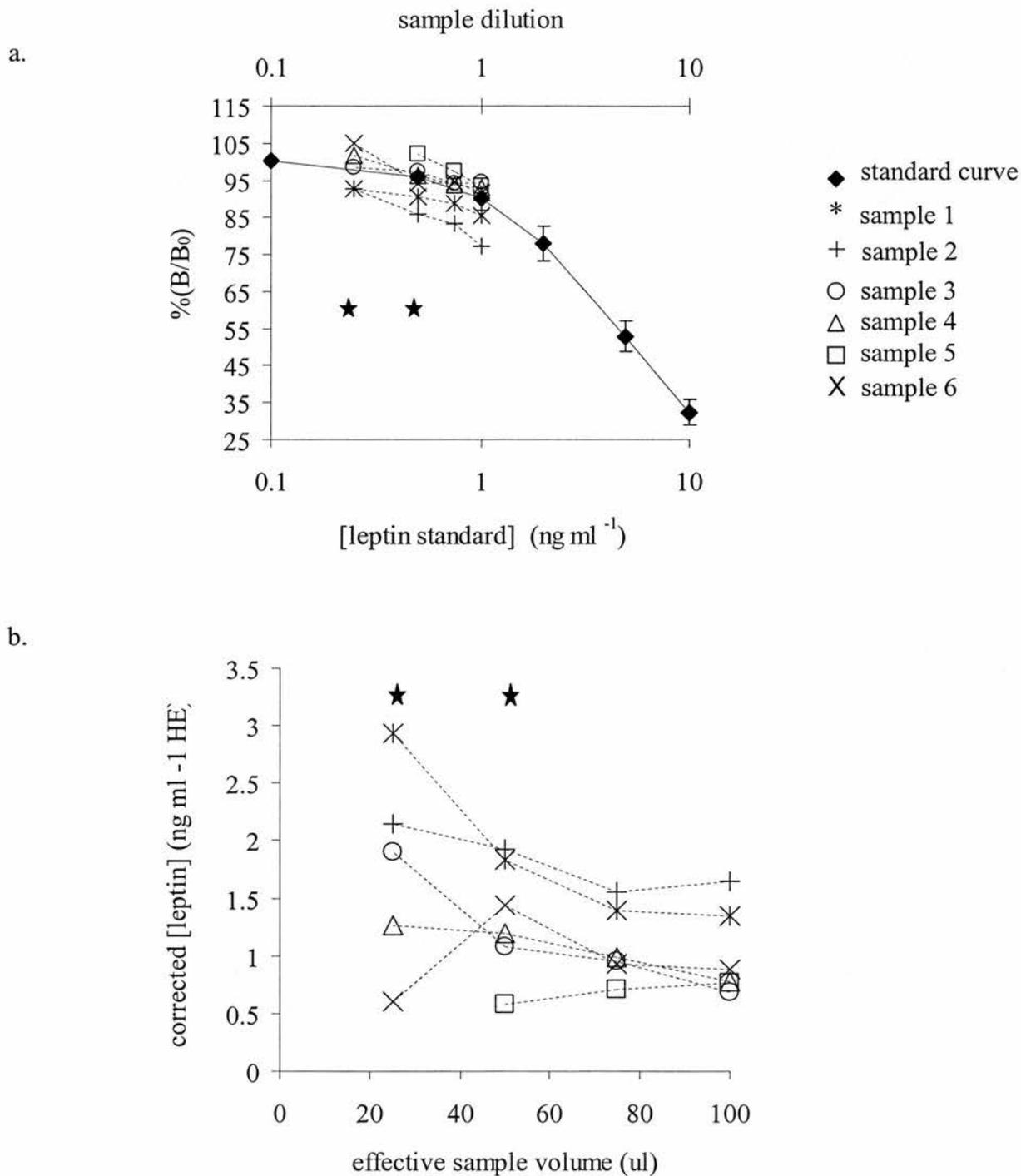
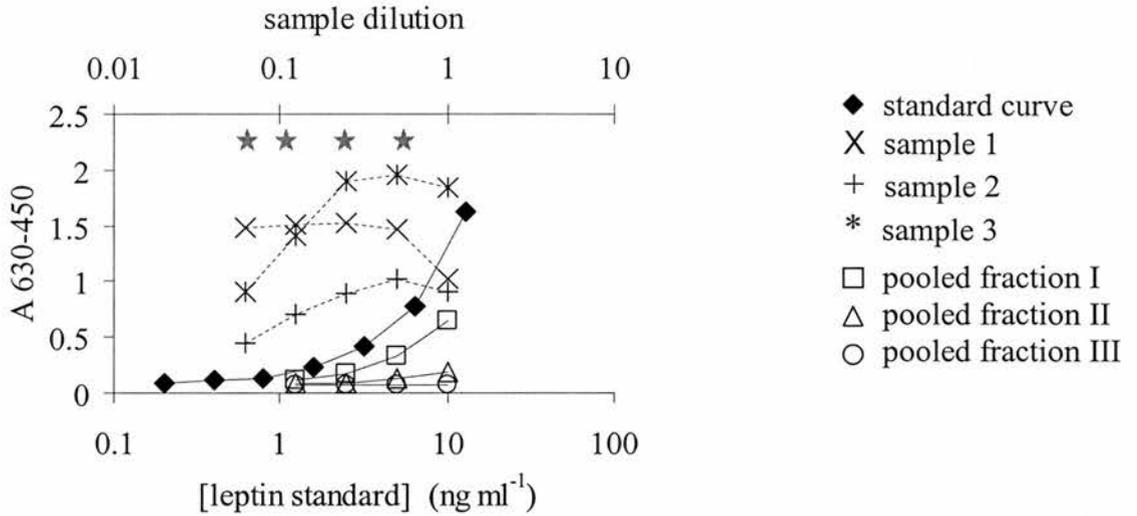
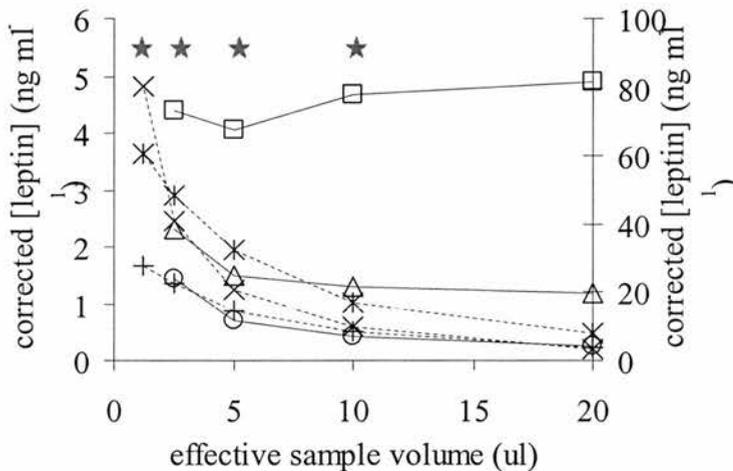


Figure 3.4: a. Parallelism of absorbance values of samples and pooled fractions I-III with the leptin IEMA standard curve b. Leptin measurements (canine equivalent) from sequential dilutions of grey seal serum (primary y axis) and pooled fractions I-III (secondary y axis) as a function of effective sample volume. Stars indicate those dilutions/ effective sample volumes for which the response of the assay to raw seal serum was not linear

a.



b.



%(B/B₀) values for serum sample dilutions were parallel to the standard curve in the cortisol RIA (Figure 3.5.a.) and cortisol values were independent of volume to a dilution factor of eight (Figure 3.5.b). However, %(B/B₀) values for dilutions of saliva samples were not parallel to the standard curve or independent of volume in the cortisol RIA (Figure 3.6).

Figure 3.5: a. Parallelism of $\%(B/B_0)$ values of serum samples with the cortisol RIA standard curve b. Cortisol measurements from sequential dilutions of grey seal serum in stripped serum as a function of effective sample volume. Stars indicate those dilutions/ effective sample volumes for which the response of the assay to seal serum was not linear.

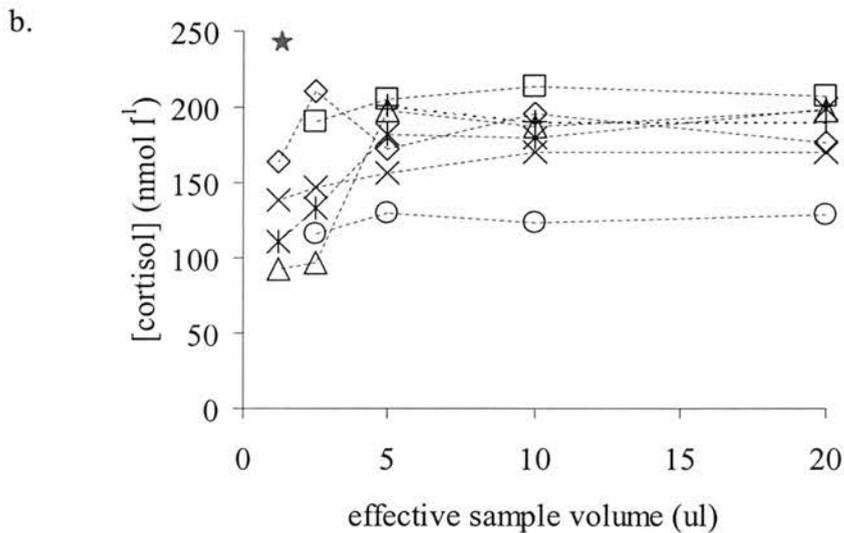
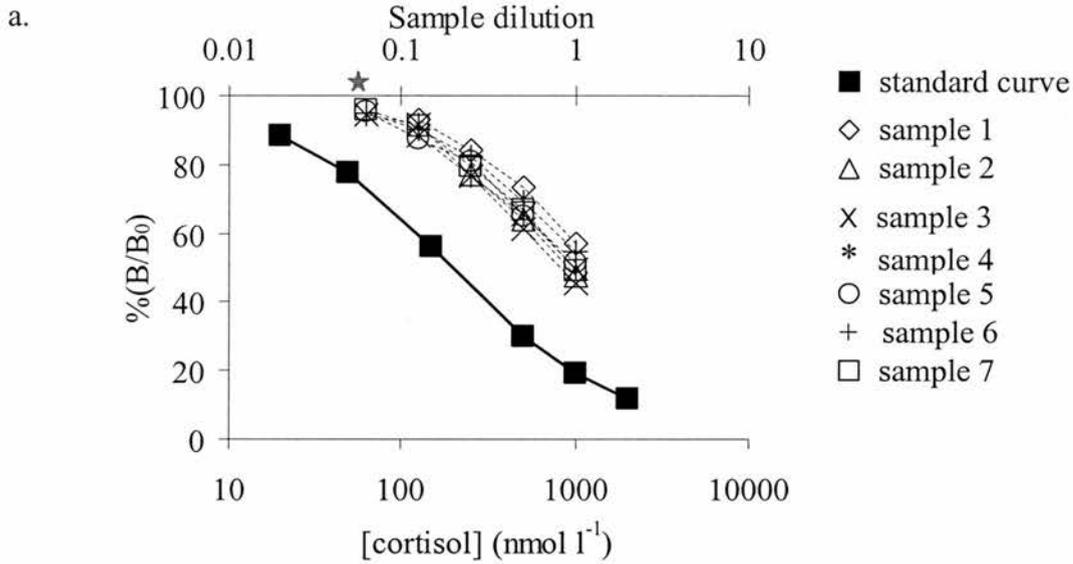
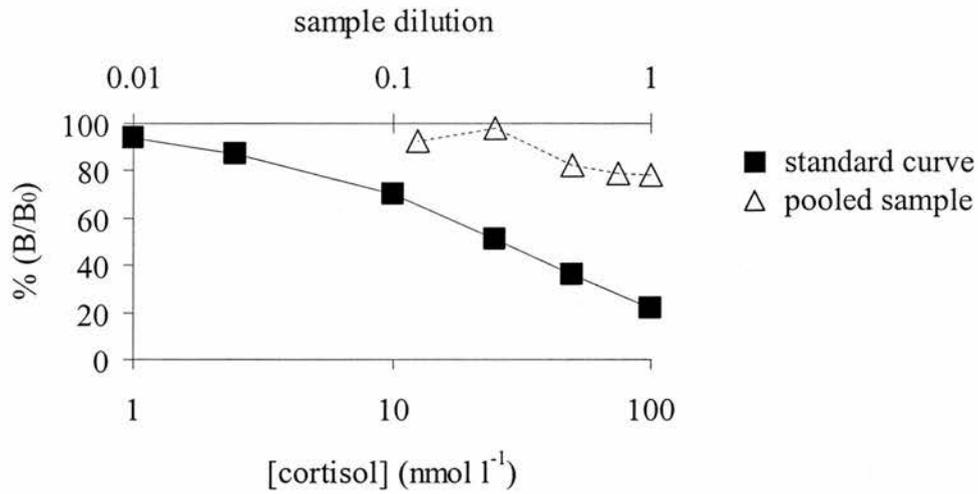
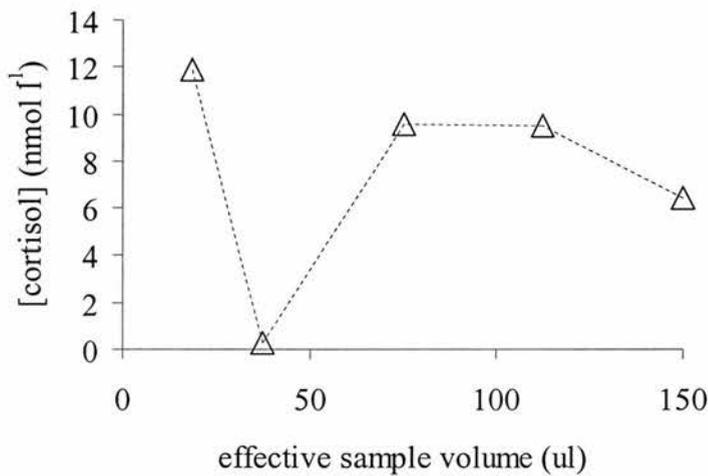


Figure 3.6: a. Parallelism of $\%(B/B_0)$ of saliva samples with the cortisol RIA standard curve b. Cortisol measurements from sequential dilutions of grey seal saliva in assay buffer as a function of effective sample volume.

a.



b.



Absorbance values for dilutions of samples seemed parallel to the standard curve in the TH EIAs and PRL IEMA (Figures 3.7-3.9a). TT4 values obtained were independent of volume to a dilution factor of four (Figure 3.7b). TT3 and PRL values were independent of volume to a dilution factor of 1.33 (Figure 3.8-9b).

Figure 3.7: a. Parallelism of absorbance values of serum samples with the TT4 standard curve b. TT4 measurements from sequential dilutions of grey seal serum as a function of effective sample volume. The response of the assay to seal serum was linear for all dilutions/ effective sample volumes.

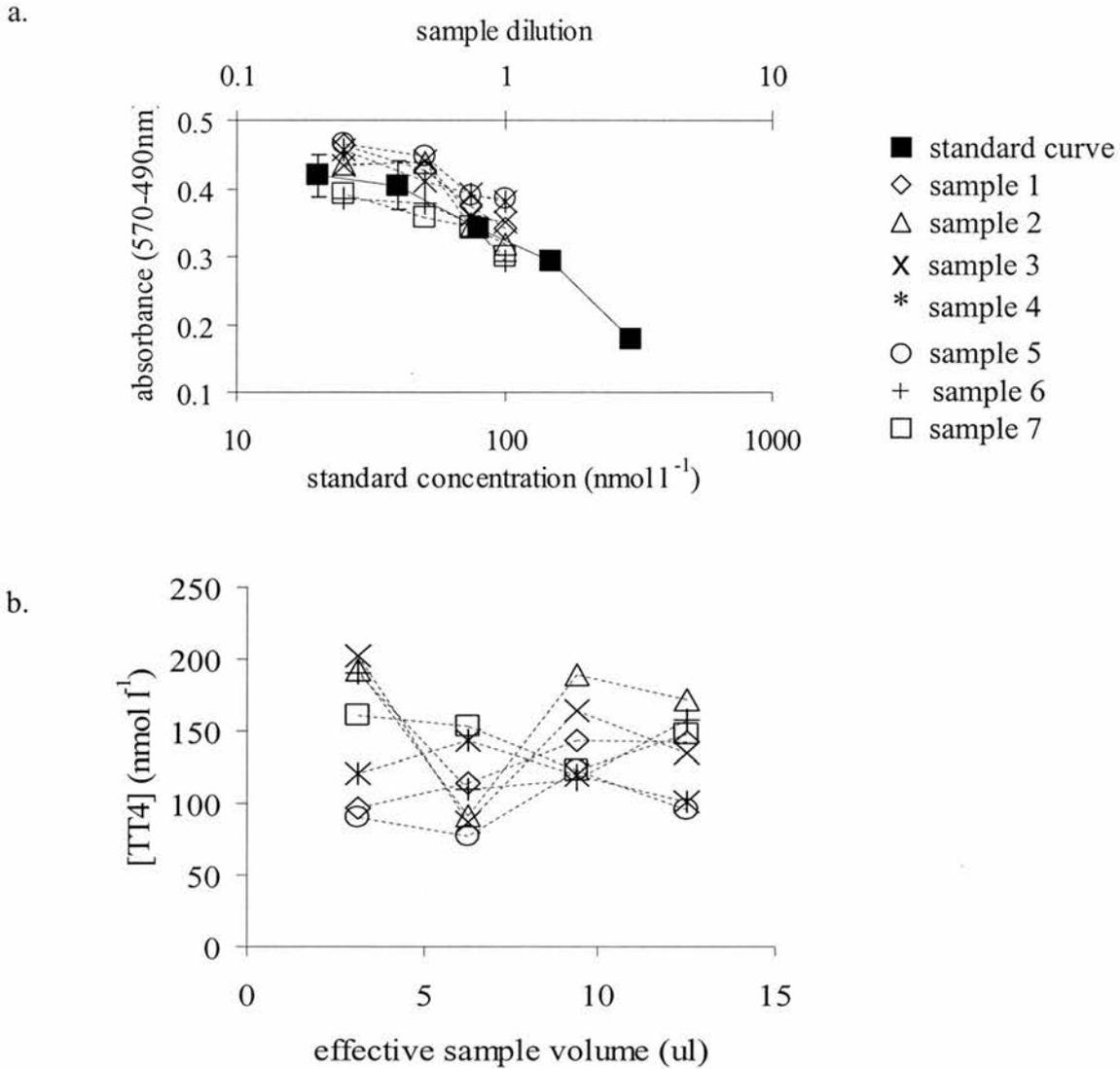


Figure 3.8: a. Parallelism of absorbance values of serum samples with the TT3 standard curve b. TT3 measurements from sequential dilutions of grey seal serum as a function of effective sample volume. Stars indicate those dilutions/ effective sample volumes for which the response of the assay to seal serum was not linear.

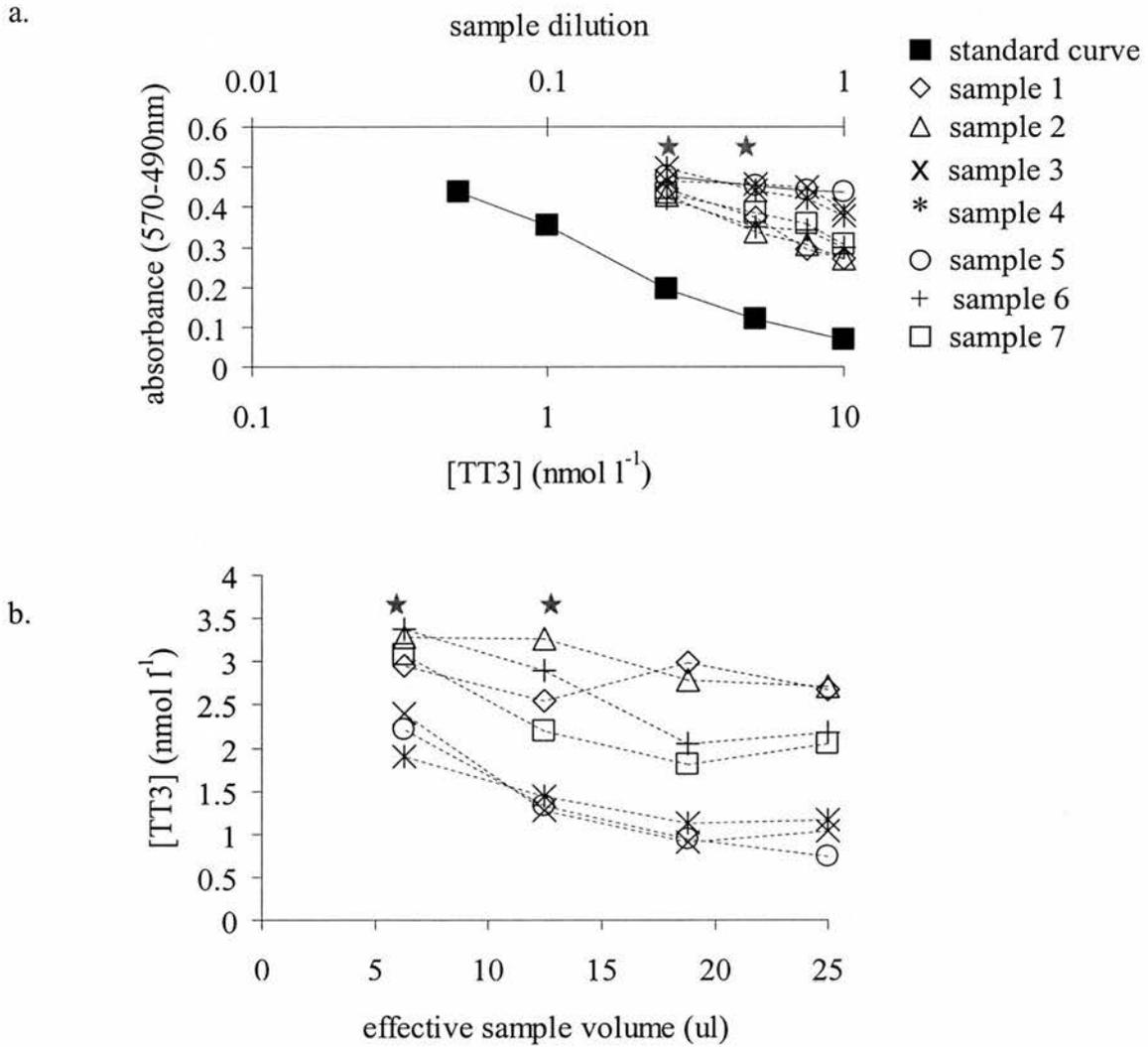
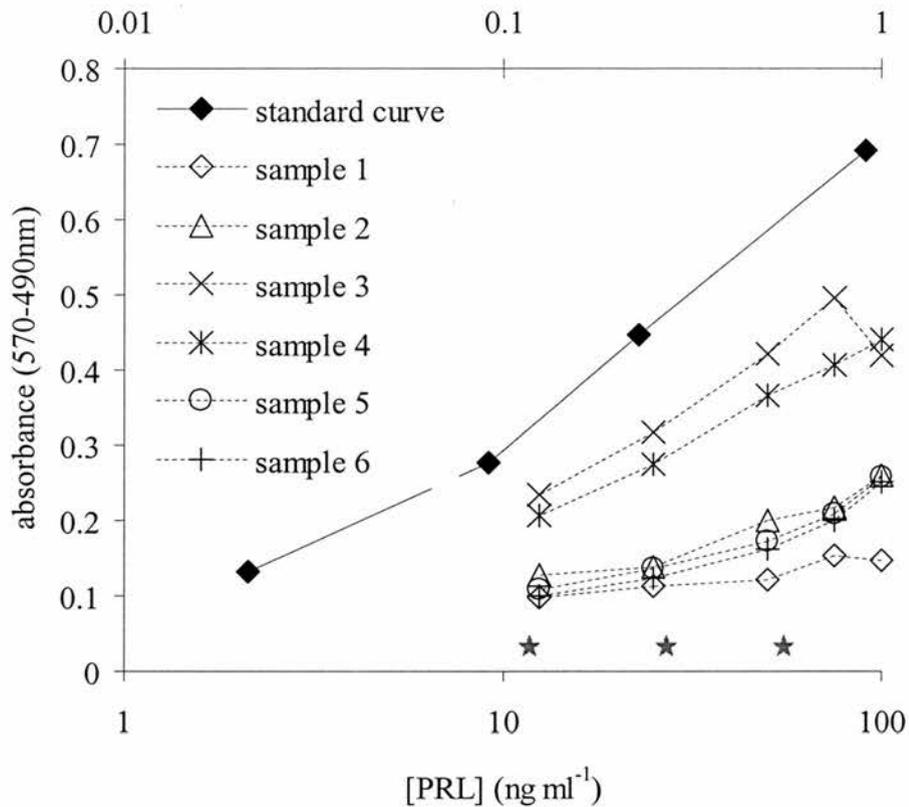
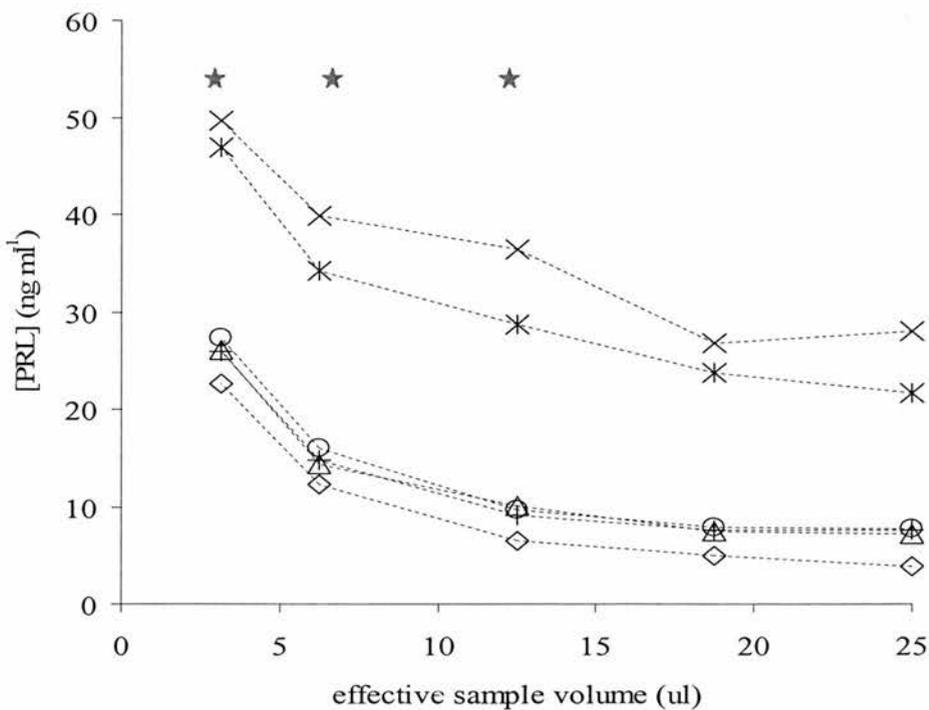


Figure 3.9: a. Parallelism of absorbance values of serum samples with the PRL standard curve b. PRL measurements from sequential dilutions of grey seal serum as a function of effective sample volume. Stars indicate those dilutions/ effective sample volumes for which the response of the assay to seal serum was not linear.

a.



b.



3.3.2.2. Spike recovery and precision

R% values for spiked grey seal serum are given in Table 3.4 and assay precision for sample and QC [Ag] measurements are shown in Tables 3.5-3.7.

Table 3.4: %R for each assay (except canine IEMA and salivary cortisol). TT3 n = 4, TT4 n = 4, PRL n = 3. Mean measured values \pm standard error (s.e.) are given in ng ml⁻¹ human equivalent (HE) for [leptin], nmol l⁻¹ for [cortisol] and [TH], and ng ml⁻¹ for [PRL].

Assay	Sample	Standard	Measured value \pm s.e .	n	Range %R	Mean %R
Leptin RIA	Low	High	30.61 \pm 1.64	8	89.92 - 143.89	115.07
	Low	Low	10.67 \pm 1.00	12	14.40 - 128.34	96.10
Cortisol	High	High	317.64 \pm 0.81	3	91.29 - 92.03	91.64
	High	Low	109.33 \pm 1.14		87.74 - 91.73	89.91
	Low	High	226.20 \pm 6.89		82.19 - 90.62	85.77
	Low	Low	35.46 \pm 0.26		90.80 - 93.19	91.63
	Stripped serum	High	217.31 \pm 4.70		84.06 - 90.91	87.39
	Stripped serum	Low	19.57 \pm 0.51		77.64 - 88.32	82.75
TT4	High	High	184.97 \pm 2.95	2	87.87 - 109.44	98.65
	High	Low	94.27 \pm 15.67		80.62 - 89.27	84.94
	Low	High	153.70 \pm 7.97		126.12 - 144.62	135.37
	Low	Low	56.01 \pm 12.96		133.51 - 143.54	138.52
	Zero standard	High	119.38 \pm 8.90		114.45 - 132.90	123.67
	Zero standard	Low	29.49 \pm 6.56		89.07 - 140.05	114.56
TT3	High	High	5.01 \pm 0.27	2	97.55 - 115.20	106.37
	High	Low	1.25 \pm 0.16		78.43 - 125.12	101.78
	Low	High	3.57 \pm 0.27		81.84 - 94.59	88.22
	Low	Low	0.64 \pm 0.22		71.94 - 153.97	112.86
	Zero standard	High	3.86 \pm 0.06		98.59 - 101.91	100.25
	Zero standard	Low	0.65 \pm 0.12		100.98 - 137.59	119.29
PRL	High	High	16.79 \pm 0.37	3	77.50 - 83.59	80.57
	High	Low	15.02 \pm 0.19		104.27 - 108.48	106.98
	Low	High	10.02 \pm 0.91		59.50 - 81.71	70.64
	Low	Low	6.26 \pm 0.23		78.67 - 89.10	84.72
	Zero standard	High	8.99 \pm 1.17		59.53 - 94.41	79.17
	Zero standard	Low	5.22 \pm 0.43		100.55 - 132.82	114.75

Table 3.5: (a) Intra %CV and (b) Inter % CV for leptin RIA. Mean [leptin] values are given in ng ml^{-1} HE.

a.

	Run1		Run2		Run 3	
	mean	%CV	mean	%CV	mean	%CV
QC	4.70	14.31	3.49	2.08	3.49	10.44
Sample	1.60	29.38	1.35	30.87	0.74	38.25

b.

Sample	mean	%CV
QC	4.06	15.02
1	2.23	22.15
2	2.01	31.58
3	2.46	47.67
4	1.78	26.14
5	1.65	22.69
6	2.51	35.63
7	1.94	27.77
8	1.91	36.75
9	1.05	8.32
10	1.78	30.03

Table 3.6: Inter and intra %CV for cortisol RIA. Mean [cortisol] values are given in nmol l^{-1} .

Sample	Intra-assay								Inter-assay	
	Run1		Run2		Run3		Run4		Overall	
	mean	%CV	mean	%CV	mean	%CV	mean	%CV	mean	%CV
1	55.72	0.54	50.43	3.96	49.63	4.65	53.99	2.73	53.41	6.18
2	30.78	8.02	24.76	6.02	29.03	8.64	30.41	4.60	28.75	9.60
3	95.36	4.95	100.15	5.17	96.62	1.48	97.56	4.15	97.43	2.08
4	118.11	4.08	118.19	1.75	117.98	0.82	126.96	1.99	120.31	3.69
5	30.55	10.08	30.60	8.56	31.31	4.92	36.76	6.90	32.31	9.25

Table 3.7: Inter and intra assay %CV for TH and PRL assays. Mean measured [Ag] values are given in nmol l⁻¹ for [TH] and ng ml⁻¹ for [PRL].

Assay	Sample	Intra-assay						Inter-assay	
		Run1		Run2		Run3		Overall	
		mean	%CV	mean	%CV	mean	%CV	mean	%CV
TT4	QC	193.04	9.77	180.5	8.94	172.85	4.04	182.13	5.6
	1	67.14	17.96	70.31	10.12	73.95	7.63	70.47	4.84
	2	51.78	10.41	48.11	19.45	41.44	11.02	47.12	11.13
	3	41.98	15.38	55.86	4.03	47.92	15.46	48.59	14.33
TT3	QC	5.755	14.31	4.54	10.47	3.93	13.57	4.74	19.65
	1	1.44	14.84	1.84	11.57	1.41	18.64	1.68	12.56
	2	1.11	11.18	1.34	21.95	1.02	12.39	1.16	14.09
	3	1.75	8.67	2.18	1.27	2.33	20.37	2.09	14.21
PRL	QC	11.72	4.41					10.70	11.93
	1	18.97	12.40	-	-	-	-	20.44	8.46
	2	7.69	19.89					4.83	19.40
	3	7.47	1.14					6.99	8.11

3.3.3. Hormone levels

Hormone measurements for those assays that were deemed appropriate for use in grey seal serum are shown in Table 3.8.

Table 3.8: Mean and standard deviation (s.d.) of [Ag] in those grey seal sera used in assay validations

Hormone	Animals	n	mean ± s.d.	units
cortisol	Pups	4	185.93 ± 14.53	nmol l ⁻¹
	Females	3	175.79 ± 41.72	
TT4			135.72 ± 28.37	nmol l ⁻¹
TT3	Pups	7	1.79 ± 0.81	nmol l ⁻¹
TT3:TT4			0.013 ± 0.004	
PRL	Females	6	12.66 ± 9.74	ng ml ⁻¹

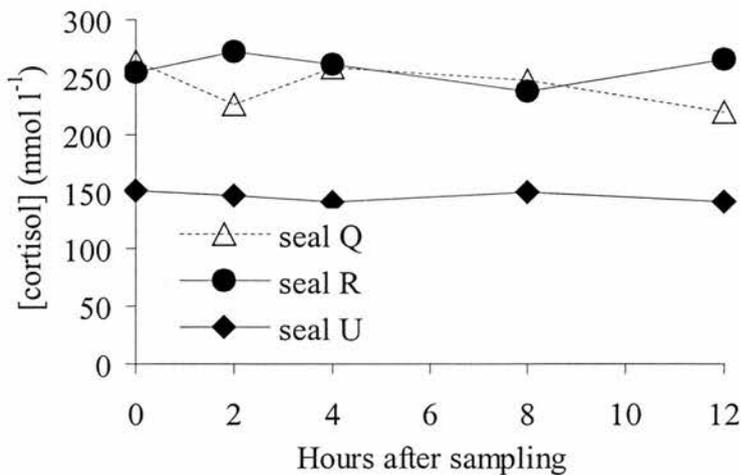
3.3.4. Storage stability of cortisol

[Cortisol] in grey seal serum processed up to 12 hours after sampling are shown in Figure 3.10. The slope of the line of the LME investigating the change in cortisol levels as a function of time taken to process and store the samples was not significantly different from zero (Table 3.9). [Cortisol] is stable in grey seal serum for up to 12 hours after sampling

Table 3.9: Result of LME on the effect of time taken to process sample on [cortisol]. The slope and intercept, standard errors (s.e.), t and p values are given. Number of individuals = 3: number of samples = 15: AIC = 130.06: degrees of freedom = 11.

	Value (nmol l ⁻¹)	s.e.	t	p
Intercept	221.67	35.41	6.25	0.0001
Slope	-1.10	0.81	-1.37	0.1983

Figure 3.10: [Cortisol] in grey seal serum processed and stored up to 12 hours after sampling



3.4. Discussion

3.4.1. Leptin

Accurate measurement of serum leptin in seals is vital to understanding the hormonal control of fat regulation in mammals that routinely undergo cycles of fasting and foraging, which result in dramatic fluctuations in the size of fat reserves.

This is the first confirmation that grey seal serum contains a protein with a similar molecular weight and immunoreactive properties to leptin from another carnivore species. The presence of a protein of the same size as canine leptin (~16kDa), in the equivalent seal serum fractions to those in which radio-labelled human leptin had also eluted, verified that grey seal serum contains a protein of the same molecular weight as other mammalian leptins. This protein is likely to be phocid leptin because its structure is sufficiently similar to canine leptin to allow detection by an anti-canine leptin antibody.

The high $\%(B/B_0)$ values for raw grey seal serum reported here for the RIA indicate very low levels of measurable circulating leptin, similar to those (<5ng/ml) reported in other pinnipeds (Gurin et al, 2001; Ortiz et al, 2001a and b; Arnould et al, 2002; Ortiz et al, 2003a). These low leptin measurements could be a consequence of an inability of the multi-species RIA to detect leptin in raw seal serum, rather than a reflection of true concentrations. Although the anti-human leptin Ab in the multi-species RIA has a wide cross-reactivity with leptin of other species (Hissa et al, 1998; Linco Research Inc., 2000; Nieminen et al, 2000; Nieminen et al, 2002), the present

study calls into question the reliability of leptin measurements in grey seal serum obtained using this kit.

Previous reports have demonstrated that the multi-species leptin RIA responds in the same way to a protein present in northern elephant serum as it does to human leptin (Ortiz et al, 2001a and b). In the current study the ability of the RIA to detect leptin in grey seal serum was poor. Measured leptin levels were very low and close to the detection limit of the kit. The $\%(B/B_0)$ values for grey seal serum lay on the non-linear section of the standard curve where incremental changes in leptin concentration do not produce a large change in assay response. This made visual assessment of parallelism difficult and unreliable, and the more robust test for linearity indicated that the assay measurements were not independent of volume at dilution factors greater than 1.33.

The spike recovery values from the RIA indicated that the accuracy of the assay was acceptable for values between 9 and 25ng/ml. However, these values represent a range of leptin concentrations well above the apparent levels measured here in grey seal serum and elsewhere in other pinniped species (Ortiz et al, 2001a and b; Arnould et al 2002; Ortiz et al, 2003a). The spike recovery values for low leptin concentrations were extremely variable and demonstrated unacceptably low accuracy in the range where serum leptin levels in pinnipeds are expected to lie.

The lack of accuracy of leptin measurements in grey seal serum at the low end of the range of leptin values measured by the RIA was exacerbated by poor precision. Other studies on pinnipeds report much lower inter and intra assay variability using this kit in unextracted serum (Ortiz et al, 2001a and b; Arnould et al, 2002; Ortiz et al, 2003a). However, in some cases (Arnould et al, 2002) assay precision has been calculated from the measurements of the kit QCs (M.J. Morris, *pers comm*), which

does not account for the variability caused by inter-species differences in the Ag or carrier proteins.

The modifications to the kit protocol may have introduced some of the variability seen here. Variation in the human leptin QC measurements was higher than the values reported by the manufacturers for variability in human samples (<15% compared to <9%). If modification of the kit protocol alone was the source of the high assay variability, inter and intra %CV for QC and sample measurements would be expected to be similar. Instead there was a more than two-fold difference in the variability of grey seal serum leptin measurements compared with QC. This is likely due to poor detection of phocid leptin by the kit antibody and/ or the presence of interfering substances in seal serum (O' Fegan, 2000).

Guidelines regarding acceptable levels of error for immunoassays are relatively flexible and depend upon the end use of the assay (O'Fegan, 2000). In clinical assays, the desirable level of analytical imprecision is half or less of the variation in [Ag] between 15 healthy individuals (Ricos and Arbos, 1990), and is usually less than 10% (O' Fegan, 2000). The intra and inter assay %CV for the multi-species RIA in grey seal serum were deemed unacceptable, since important changes in circulating phocid leptin at low concentrations may be masked by these high levels of assay variability.

High assay variability and low accuracy may explain the reported absence in pinnipeds of the relationship between body condition and leptin levels (Ortiz et al, 2001 and b; Arnould et al, 2002; Ortiz et al, 2003a) that is normally found in terrestrial mammals, including carnivore species for which a species-specific immunoassay exists (Ishioka et al, 2002; Sagawa et al, 2002; Shibata et al, 2003). However, the absence of a relationship between body fat content and leptin levels in

the blood of seals may reflect secretion of the hormone by other tissues, including heart, bone marrow and lung, which have been shown to contain both leptin mRNA and protein (Hammond et al, in press). Alternatively, leptin secretion may be uncoupled from fat content in seals because blubber functions as insulation in these animals in addition to its role as the major metabolic fuel depot. Distinguishing between these possibilities requires a more accurate method than the Linco multi-species RIA to detect and measure phocid leptin.

The canine leptin IEMA response to the leptin-containing fractions of grey seal serum was the same as its response to canine leptin. The assay therefore bound phocine leptin when other protein constituents of serum had largely been removed, but matrix effects prevented measurement of leptin in raw serum using this assay.

The inability of two currently available off-the-shelf assays to detect leptin accurately in grey seal serum indicates that these assays should not be used to measure leptin in raw pinniped blood samples. This study does not constitute a thorough examination of all leptin assays available and there may be other Abs that can be used that have not been investigated here. However, if the multi-species and canine-specific assays cannot be used to measure leptin in grey seal serum reliably in their present format, it is unlikely that the performance of assays, using other, species-specific Abs raised against leptin from animals distantly related to seals, will be any better. In the absence of a reliable method to measure circulating levels of phocid leptin, this hormone was not measured in the samples collected from fasting grey seal pups in subsequent studies.

With the availability of the leptin protein sequence it may soon be possible to validate existing off the shelf assays for use in grey seals in a more robust way using phocine leptin. If currently available immunoassays can be shown to detect seal

leptin, but suffer from matrix effects, as demonstrated here, prior extraction may allow use with grey seal serum. However, the development of an immunoassay using an Ab raised against all or part of the phocine leptin protein is now a viable and potentially more accurate alternative.

3.5.2. Cortisol

3.5.2.1. Serum cortisol

The performance characteristics of the cortisol RIA in grey seal serum compared well with those reported by the manufacturers for human serum. On visual inspection, $\%(B/B_0)$ values from sample dilutions were parallel to those from the standard curve and the assay was independent of sample volume to a dilution factor of eight. %R ranged from 82.75-91.64%. In comparison, the manufacturers report that the assay is linear in human samples to 1:20 dilution and that %R is 88-108% (Orion Diagnostica, 2001). The lower %R values in this study corresponded to samples containing low cortisol levels, indicating that cortisol is consistently underestimated in grey seal serum samples that contain low concentrations of cortisol. The difference in performance of this assay in humans and seal samples is likely to be caused by inter-specific variation in serum composition. Constituents in grey seal serum seem to cause increasingly significant matrix effects at lower analyte concentrations in this assay, an effect commonly seen in immunoassays (Ezan and Grassi, 2000; O’Fegan, 2000). Since most cortisol measurements in grey seal serum fall well within the range of values where the kit is linear, the reduced accuracy at low concentrations should not pose a significant problem for the current study.

Measurements obtained using this kit were highly reproducible for the range of cortisol concentrations found here in grey seal serum. Inter and intra %CV reported here (<11% and <10%, respectively) are comparable to the <6% intra %CV and <8% inter %CV reported by the manufacturers in human serum (Orion Diagnostica, 2001). These values are similar to those reported for cortisol immunoassays used elsewhere to detect the hormone in pinnipeds (eg. Ortiz et al, 2001) and are within the range deemed acceptable (Ricos and Arbos, 1990; O’Fegan, 2000). Cortisol levels were directly comparable to those measured in fasting captive grey seals (Nordoy et al, 1990).

The protocol of this cortisol assay is rapid and straightforward and eliminates the need for prior extraction of the samples. It is a convenient assay that provides accurate, reproducible cortisol measurements in grey seal serum and was therefore used throughout this thesis for quantification of circulating cortisol levels.

3.5.2.2. Cortisol storage stability

Cortisol levels in unprocessed grey seal serum maintained in ambient light and temperature were stable for up to 12 hours after obtaining the blood sample. The often substantial delay between blood sampling and storage of the sample under field conditions is therefore not a cause for concern for measurements of this hormone.

3.5.2.3. Salivary cortisol

It was difficult to obtain saliva samples from grey seal pups. The animals resisted restraint, and as a result, it took ~ 15 minutes per animal to obtain saliva samples, compared with ~ two minutes to collect blood from the same animals. This represents a considerable degree of stress. Although the increase in cortisol as a result of that stress would not have had sufficient time to reach the saliva, the degree of disturbance experienced by the animals potentially resulted in long term adjustments to HPA function, especially when repeat samples were taken from the same animal. In addition, there was risk of blood contamination due to damage to the inside of the mouth. As a result of the potential contamination, there was some doubt over the reliability of cortisol measurements in seal saliva, even prior to assay validation.

The animals produced small amounts of extremely viscous saliva, perhaps due to water conservation during fasting, which was often insufficient for duplicate runs in the immunoassay. Salivary cortisol could not be measured using the RIA used here, perhaps due to the high viscosity of the saliva, which prevented accurate transfer of the small volumes required for the assay, and high protein content, which may have substantially interfered with Ab-Ag binding.

The degree of stress caused and the limited usefulness of the saliva obtained defeated the purpose of the use of saliva samples for cortisol measurements as a non-invasive alternative to blood sampling in wild, fasting seals. It is possible that saliva samples could be taken from captive animals trained to tolerate mouth swabbing and that other assays could be tested for their ability to detect salivary cortisol. Until a less stressful method for saliva collection can be devised and an assay developed that

reliably detects cortisol in seal saliva, blood remains the sample-type of choice for determining cortisol levels in wild pinnipeds.

3.5.3. TH and PRL

TH and PRL in grey seal serum have been measured previously using the modified assays described here (Gardiner, 1994; Hall et al, 1998) and the performance of these assays was judged to be adequate for this study.

On visual inspection, absorbance values from sample dilutions were parallel to those of the standard curve. The dilution curve produced was comparable to that found previously using the same modified PRL assay in adult female grey seal serum (Gardiner, 1994). No data were available in the kit protocols to allow comparison with linearity in human samples. Since the sample volumes used in the modified assays were already a quarter of the volumes given in the kit protocols, independence of volume to a dilution factor of four (TT4) and 1.33 (TT3 and PRL) was considered acceptable.

The modified assays did not detect TH and PRL in grey seal serum as accurately as the original format intended for human samples. The manufacturers report intra-assay %CV as <4%, and <5% in TT3 and TT4 kits, respectively, based on 20 repeats of the same sample pool in one assay run. The manufacturers report inter-assay %CVs of <6% in the same assays based on the results from twenty different runs. The higher variation in QC levels found in this study compared to that reported for human serum samples in the kit protocols is likely to be due to a combination of the small number of repeats used here to calculate the results and the modifications to

procedure. Assay variation was higher in serum samples than in QCs, probably due to matrix effects of the grey seal sera. Although the assay variation was higher than recommended levels for clinical assays (Ricos and Arbos, 1990; O’Fegan, 2000), the variation was comparable to that found in the same assays used in other studies. The TH assays showed similar assay precision (Hall et al, 1998) and the PRL assay showed a similar inter-assay %CV and slightly higher intra-assay %CV to those previously reported for grey seal serum (Gardiner, 1994).

The reduced accuracy in TH and PRL assays compared to their performance in humans was generally more apparent in more dilute sera. There was a tendency to overestimate actual TT4 values, especially for lower TT4 concentrations, and a tendency to underestimate actual PRL concentrations. This was likely due to matrix effects of the seal serum (Ezan and Grassi, 2000; O’Fegan, 2000).

Some of the assay inaccuracy could not be ascribed to matrix effects specific to grey seal serum since %R values were similar for spiked zero standards (TT4 = 115-124%; TT3 = 100-120%; PRL = 80-115%) and seal serum. Inaccuracies in the measurements were therefore more likely due to factors inherent in the assays. It is possible that the high %R values for the TH assays and low %R for the PRL assay resulted from modification of the kit protocol to allow use in microtitre plates.

The microtitre plate format of these assays is more convenient and allows the analysis of a greater number of samples than the original format. The assays performed adequately with these modifications and were used to quantify TH and PRL in grey seal serum samples. However, the performance of other available assays for TH and PRL determination in grey seal serum should be assessed.

The modified TH assays described here were used to quantify TH in serum from fasting grey seal pups. TT4 levels were similar to those seen in preweaned

harbour (Haulena et al, 1998), harp and grey seal pups (Engelhardt and Ferguson, 1980; Stokkan et al, 1995). They were higher than levels reported elsewhere for postweaned and adult grey (Hall et al, 199), harbour (Renouf and Brotea, 1991; Renouf and Noseworthy, 1991; Schumacher et al, 1995) and elephant (Ortiz et al, 2001a and b) seals and levels measured in human serum using the same assay (BioChemImmunoSystems c). TT3 concentrations were also slightly higher than levels measured in pinnipeds elsewhere (Renouf and Brotea, 1991; Renouf and Noseworthy, 1991; Hall et al, 1998; Ortiz et al 2001a and b) but comparable with levels in human serum (BioChemImmunoSystems b). The ratio of TT3 to TT4 seen here was therefore lower than in other pinnipeds and in humans. This cannot be explained by inaccuracy caused by the modifications to the assay, since the same procedure was used by Hall et al (1998) to measure [TH] in preweaned and postweaned grey seal pups. The reason for the high TT4 levels, which gave rise to the low TT3: TT4 ratio is unclear. Increased thyroid gland activity during periods of growth and development leads to raised serum TH levels in young animals (BioChemImmunoSystems b and c; Haulena et al, 1998) and may be the cause of the elevated TT4 levels seen here. The high TT4 levels may also be caused by increased concentrations of TH binding proteins in the blood (BioChemImmunoSystems b and c), which were not measured in this study.

Although adult female grey seal PRL levels were similar to those reported in seals elsewhere (Gardiner, 1994; Mellish et al, 1999; Boyd 1991), PRL was not detectable in 91 of 103 grey seal pup serum samples analysed. PRL levels are generally low in young mammals and increase gradually towards puberty as the number of lactotrophs in the pituitary increases and their ability to respond to PRL-releasing factors to synthesise and secrete PRL matures (Becuvillalobos et al, 1992).

The undetectable levels in postweaned grey seals likely reflect very low levels of secretion of the hormone. Since PRL levels are so low in postweaned pups it is unlikely that this hormone plays a significant role in the control of fuel utilisation during the postweaning fast or the signal that initiates departure from the colony. As a result, PRL levels were not measured in subsequent samples from fasting grey seal pups.

3.5.4. Summary

Although the presence of leptin was demonstrated in grey seal serum, levels could not be measured accurately using two currently available immunoassays in their present format. As a result, leptin was not measured in fasting grey seal pup samples. The assays chosen for quantification of cortisol, TH and PRL were appropriate for use in grey seal serum. PRL was not detected in postweaned pup serum and was eliminated as a potential candidate for control of fuel allocation and timing of departure from the colony in these animals. Cortisol could not be measured in grey seal saliva as a result of difficulties in obtaining samples and poor assay performance. Cortisol levels were unaffected by the time taken to process and store the serum samples. The investigation into the role of endocrine signals in fasting grey seal pups was therefore limited to circulating cortisol and TH.

Chapter 4

**The impact of handling regime,
supplementary feeding and natural
fasting on cortisol and thyroid
hormone levels in grey seal pups**

4.1. Introduction

4.1.1. Summary

The signals that regulate fuel allocation during the postweaning fast and inform the decision to go to sea in phocid seal pups have not been characterised, but are likely to incorporate hormonal inputs. Glucocorticoids (GCs) and thyroid hormones (TH) are intimately involved in long-term energy balance in other mammals. In conjunction with other metabolic and endocrine signals, they co-ordinate fuel utilisation and acquisition in response to changes in energy supply and demand. This study explored the potential roles of GCs and TH as signals of changing energy availability during the postweaning fast and as components of the mechanism that prompts grey seal pups to depart from the colony and begin foraging. If GCs and TH are involved in fuel regulation in fasting pups, they should be responsive to changes in energy availability, induced either naturally by fasting or artificially by supplementary feeding. Changes in cortisol and TH levels close to departure may also indicate that these hormones are involved in the mechanism that terminates fasting. In this study the changes in cortisol and TH during the course of the entire postweaning fast, and in response to supplementary feeding, were investigated in wild grey seal pups.

The effects of repeated handling on hormone levels were also investigated. In marine mammals, as in other animals, GC and TH secretion respond dramatically to acute and chronic stress, including that caused by capture and restraint and by prolonged captivity (St Aubin and Geraci, 1988b; Schumacher et al, 1995; Gulland et al, 1999). Some of the discrepancies in reported hormone levels between studies on

fasting seals may therefore result from differences in handling regime. Frequent longitudinal measurements of cortisol obtained from captive grey seal pups during the postweaning fast (Nordoy et al, 1990) may not be an accurate representation of cortisol levels in fasting wild animals. Conversely, infrequent sampling of wild pups (Ortiz et al, 2001b) does not provide detailed information on hormonal changes that may have important consequences for fuel use and behaviour. The current study is the first to investigate directly the impact of repeated handling on hormone levels during the postweaning fast in order to disentangle the effects of handling from those that occur naturally during fasting in wild pups.

4.1.2. Role of GCs in long-term energy balance

GCs maintain circulating glucose levels when metabolic substrate supply is challenged. Most information about the role of GCs in fasting mammals comes from rodents and humans, which do not fast for long periods as a normal part of their life history. In these animal models, GC levels increase as the glucose supply from glycogen stores diminishes under fasting conditions (Bergendahl et al, 1996; Byerley and Heber, 1996; Samuels and McDaniel, 1997; Friedl et al, 2000).

GCs have profound effects on both energy utilisation and food intake (Santana et al, 1995; Tataranni et al, 1996) and are responsive to changes in energy supply (Tannenbaum et al, 1997; Widmaier et al, 1992). Both basal and stress-induced concentrations inhibit glucose uptake by inducing internalisation of membrane glucose transporter proteins in adipocytes and fibroblasts (Sapolsky et al, 2000). GCs enhance the gluconeogenic capacity of the liver through the activation of transcription and stabilisation of the mRNA of critical rate-limiting enzymes and improve substrate

delivery through their permissive effect on the action of catecholamines on cardiovascular tone (Sapolsky et al, 2000). They increase the provision of substrates for gluconeogenesis by facilitating the mobilisation of glycerol and free fatty acids (FFA) from fat (Divertie et al, 1991; Samra et al, 1998; Djurhuus et al, 2002; Djurhuus et al, 2004) and amino acids from protein reserves (Simmons et al, 1984; Legaspi et al, 1985; Darmaun et al, 1988; Tataranni et al, 1996; Weiler et al, 1997; Mantha and Deshaies, 2000). They also sensitise adipocytes to the lipolytic effects of other hormones, including catecholamines and growth hormone (Sapolsky et al, 2000).

GCs can also promote lipogenesis through their actions on key enzymes in the adipose tissue (Samra et al, 1998), causing a greater reliance on protein breakdown to meet energetic needs (Santana et al, 1995, Tataranni et al, 1996; Weiler et al, 1997). The net effect of GCs in the periphery depends on the presence of other hormonal and metabolic signals. For example, insulin often opposes the effects of GCs. The lipolytic and proteolytic effects of GCs are therefore most apparent when insulin levels are low or absent (Divertie et al, 1991; Djurhuus et al, 2002; Djurhuus et al, 2004). In the presence of insulin GCs facilitate the storage of fat as triglycerides (Arvaniti et al, 1998; Samra et al, 1998; Mantha and Deshaies, 2000), reduce lipolysis (Ottosson et al, 2000) and reduce the proportion of fat used in meeting energy requirements (Mantha et al, 1999; Solano and Jacobson, 1999), which results in selective utilisation of protein stores (Santana et al, 1995; Tataranni et al, 1996; Weiler et al, 1997). Increased protein utilisation occurs in semi-starved or fasting humans (Freidl et al, 2000) rats and penguins (Cherel et al, 1988a and b; Robin et al, 1998) in response to natural elevations in basal GC levels when insulin levels are also low and fat stores are diminished.

GCs act centrally, within the hunger centres of the hypothalamus, to stimulate appetite (Debons et al, 1986; Green et al, 1992; Chen and Romsos, 1996; Rohner-Jeanrenaud, 1999). The orexigenic effects of GCs are a result of direct stimulation of the neuropeptide Y (NPY)-producing neurones (Debons et al, 1986; Chen and Romsos, 1996; Rohner-Jeanrenaud, 1999) and suppression of corticotropin releasing hormone (CRH) secretion, which itself inhibits NPY release (Santana et al, 1995; Rohner-Jeanrenaud, 1999).

The absence of GCs results in low voluntary food intake. Lean, underweight, adrenalectomised (ADX) rats exhibit reduced food consumption and lower mass gain in comparison to controls when allowed *ad libitum* access to food (Green et al, 1992). Hyperphagia and weight gain is restored by GC replacement in ADX animals (Green et al, 1992).

Increased basal GC concentrations are associated with an increase in the motivation to seek food. Starved ADX rats do not show an increase in food intake (Green et al, 1992) or NPY concentrations (Ponsalle et al, 1992) unless they are treated with a replacement dose of exogenous corticosterone, which suggests that GCs are important in the central perception of diminished fuel reserves. A natural or artificial elevation in GC levels drives a marked increase in food seeking behaviour in humans (Tataranni et al, 1996) and rodents (Challet et al, 1995; Arvaniti, 1998). Bottlenose dolphins, *Tursiops truncatus*, given an oral dose of the synthetic GC, dexamethasone, show an increase in soliciting for food (Reidarson and McBain, 1999). Elevated GC levels are also thought to prompt fledging and the onset of foraging in many bird species, including kestrels (*Falco sparverius*; Heath, 1997), pied flycatchers (*Ficedula hypoleuca*; Kern et al, 2001) and screech owls (*Otus asio* and *O. kennicotti*; Belthoff and Dufty Jr., 1998). In rats and penguins, running

behaviour, an index of motivation to forage, is stimulated by corticosterone (Cherel et al, 1988a, b and c; Challet et al, 1995; Robin et al, 1998).

The role of GCs in fuel use and feeding behaviour in fast-adapted species has not been the subject of intensive study and is therefore less clearly defined. The involvement of GCs in increased protein utilisation and the onset of behavioural changes associated with the motivation to seek food has been investigated in emperor (*A. forsteri*) and king (*A. patagonica*) penguins, which naturally fast for up to four to six months (Cherel et al, 1988a). These animals show up to a four-fold increase in corticosterone, the major GC in rats and birds, at the transition to phase III of fasting (Cherel et al, 1988 b and c; Robin et al, 1998). This occurs when a low critical threshold body mass is reached and is thought to be indicative of depleted fat reserves (Robin et al, 1998). Raised corticosterone levels have been implicated as the signal that induces both the metabolic shift towards protein use (Cherel et al, 1988b and c; Robin et al, 1998) and the concomitant increase in locomotor activity, thought to reflect the drive to forage (Robin et al, 1998).

The role of cortisol in fasting phocid seal pups remains equivocal. The pattern of change in cortisol in fasting seal pups differs markedly from the general avian and mammalian models, and reported changes in cortisol measurements, as a function of either time spent fasting or absolute or relative changes in body condition, are not consistent within or between phocid species. Northern elephant seal pups show a progressive increase in cortisol levels throughout the first eight weeks of the postweaning fast (Ortiz et al, 2001a and b; Ortiz et al, 2003c). Similarly, lactating Subantarctic fur seal females show an increase in cortisol with declining body condition index and time spent ashore whilst fasting (Guinet et al, 2004). It has been proposed that cortisol helps to maintain high levels of lipolysis for the predominantly

fat-based fasting metabolism, and that it contributes to the eventual signal to depart from the colony in these animals (Ortiz et al, 2001a and b; Guinet et al, 2004).

Captive harp seal pups show no change in cortisol levels over 32 days of fasting (Nordoy et al, 1993). In a study on captive grey seal pups, cortisol remained relatively stable during a 52 day postweaning fast, except for a substantial increase at the end of this period in two of the five study animals (Nordoy et al, 1990). The discrepancies between these findings may reflect methodological differences in handling and blood sampling or hormone assays, and highlight the need to investigate the role of cortisol in fasting phocids in more detail.

4.1.3. Role of TH in long-term energy balance and development

TH (thyroxine (T4) and triiodothyronine (T3)) control both protein synthesis and energy expenditure. While most TH in the circulation is bound to high affinity transport proteins, thyroxine-binding prealbumin, thyroxine binding globulin and albumin, only a small proportion, the free fraction, is available to interact with receptors.

Administration of TH elevates resting metabolic rate (Greco et al, 1998; Jekabsons et al, 1999). TH regulate metabolic rate by their actions on mitochondria, where they stimulate oxygen consumption (Hadley, 1992). They drive an increase in both the number of sodium potassium ATPase proteins, which are responsible for a high proportion of total cellular energy expenditure (Hadley, 1992), and the expression of uncoupling proteins (UCPs) (Guerra et al, 1996; Jekabsons et al, 1999; Branco et al, 1999), which redirect oxidative phosphorylation from ATP synthesis to heat production (Brand et al, 1999). TH also have permissive effects on the action of

catecholamines on sympathetic tone. TH enhance the rate of fat breakdown (Cheikh et al, 1994) both through direct action on hormone-sensitive lipase in adipose tissue and through permissive effects on the lipolytic action of catecholamines on adipocytes (Lafontan and Berlan, 1995).

TH influence feeding and migration behaviour, both directly, and indirectly through their impact on metabolic rate. Raised TH levels drive an increase in food intake as a result of elevated energy expenditure in woodchucks (Concannon et al, 1999), reindeer, (Ryg and Jacobsen, 1982) and rats (Oppenheimer et al, 1991). In birds, TH are associated with many of the behavioural and physiological changes associated with migration (Nair et al, 1994; Rankin, 1991). For example, increased TH cause an increase in nocturnal restlessness prior to migration in redheaded bunting, *Emberiza bruniceps* (Pant and Chandolasaklani, 1993).

There is circumstantial evidence that TH also promote foraging in pinnipeds. An increase in free T₄ (FT₄) is associated with a rise in food intake concomitant with elevated mass and fat loss in captive male harbour seals (Renouf and Noseworthy, 1991). Female harbour seals begin foraging midway through lactation (Boness et al., 1994) at the same time as a rise in FT₄ levels (Haulena et al, 1998).

TH are also key to the regulation of many developmental processes, including skeletal growth (Hadley, 1992; Nilsson et al, 1994; Bland, 2000), nervous system differentiation (Bernal and Nunez, 1995) and erythropoiesis (Dainiak et al, 1978).

The typical mammalian and avian response to food restriction is a decline in TH levels (Moshang et al, 1975; Croxson and Ibbertson, 1977; May, 1978; Spencer et al, 1983; Yen et al, 1994; Byerley and Heber, 1996; Fuglei et al, 2000), and a fall in receptor density (Schussler and Orlando, 1978). This facilitates energy conservation by reducing activity levels and metabolic rate.

In king penguins, TH levels are low and show a progressive decline throughout fasting (Cherel et al, 1988b and c). In contrast, T4 increases and T3 does not change during reduced food intake in West Indian manatees (*Trichechus manatus*; Ortiz et al, 2000). Total T4 (TT4) increases, and the total TT3 (TT3): TT4 ratio decreases during 49 days of fasting in free-ranging northern elephant seal pups (Ortiz et al, 2001a; Ortiz et al, 2003c). The significance of these findings in terms of their effect on fuel use and foraging behaviour remain unclear.

The pattern of change in TH levels seen in northern elephant seal pups has been attributed to alterations in hormone clearance and binding globulins, rather than alterations in thyroid gland activity (Ortiz et al, 2001a). It has been suggested that TH are relatively unimportant in the control of fasting metabolism in these animals (Ortiz et al, 2003a). However, since the impact of thyroid hormones on energy expenditure or fuel use was not investigated directly, and changes in binding globulin concentration, receptor density, clearance rates and thyroid gland activity were not measured, this conclusion appears to be an overinterpretation of the results from these studies. Given the dual regulatory role of TH in metabolism and development in other mammals they may be pivotal in the control of energy expenditure and the timing of departure from the colony in fasting seal pups.

4.1.4. GC and TH response to stress

Acute and chronic stress can cause marked changes in cortisol and TH levels (Dohler et al, 1977; Bianco et al, 1987; Cremaschi et al, 2000; Sapolsky et al, 2000). Repeated, acute stress can lead to chronic alterations in the feedback systems that regulate the secretion of these hormones (Jensen et al, 1996; Meerlo et al, 1999).

Measurements of GCs and TH are therefore susceptible to variability introduced by the frequency and duration of handling stress.

Measurement of baseline cortisol concentration is typically problematic because the procedure for obtaining blood samples is itself an acute stressor that stimulates cortisol production. GCs mediate the response to stress (Sapolsky et al, 2000) and circulating levels rise dramatically within a few minutes of exposure to an acute stressor (Dohler et al, 1977). Cortisol increases over 5–35 minutes of handling in suckling southern elephant seal pups (Engelhard et al, 2002) and during the first ten minutes of handling in captive harbour seals (Gardiner and Hall, 1997).

The speed and magnitude of the cortisol response to a specific stressor can be influenced by prior experience (Jensen et al, 1996; Meerlo et al, 1999). In adult rats, the neuroendocrine and behavioural response to stress can be permanently altered by early postnatal handling (Meerlo et al, 1999). Handled animals often have a blunted adrenaline and corticosterone response to acute stress (Gulland et al, 1999; Meerlo et al, 1999; St. Aubin and Dierauf, 2001). This is thought to reflect lowered anxiety as a consequence of habituation to handling. Engelhard and co workers (2002) investigated the effect of handling frequency on cortisol levels in southern elephant seal females and pups during lactation. Pups demonstrated no change in the cortisol response to restraint stress, irrespective of the number of times they had been handled previously. However, females that had been handled and chemically immobilised three to four times previously showed an attenuated cortisol response to the handling procedure compared to those that had been handled on up to two previous occasions (Engelhard et al, 2002).

Prolonged or repeated stimulation of the adrenals can be detrimental to health and ultimately to survival (Jensen et al, 1996; St. Aubin and Dierauf, 2001). Feeding

behaviour, immunocompetence and reproductive status can be adversely affected by the cortisol-mediated effects of chronic or repeated acute stress (St. Aubin and Dierauf, 2001). Chronic overproduction of ACTH can lead to downregulation of glucocorticoid receptor (GR) density and result in adrenal insensitivity to the hormone. This reduces the ability of the animal to respond to subsequent periods of stress and may compromise survival because animals that do not secrete cortisol in response to a novel noxious stimulus may suffer from vascular collapse, inflammatory overshoot and tissue damage (Hadley, 1992). Alternatively the hypothalamo-pituitary-adrenal (HPA) axis may become sensitised by repeated acute or chronic stress, producing a heightened cortisol response (Jensen et al, 1996; St. Aubin and Dierauf, 2001).

T3 and T4 are not directly involved in the acute stress response and do not change rapidly within a handling episode. However, in response to acute stress, rats show an increase in TSH within five minutes and T3 within an hour, followed by a decline within six hours (Dohler et al, 1977; Bianco et al, 1987). T4 remains unaltered over the same time period (Dohler et al, 1977; Bianco et al, 1987). TH are also influenced by stress in marine mammals. In juvenile beluga whales, *Delphinapteras leucas*, concentrations of T3 decline markedly within 24 hours, and T4 levels fall within two to four days of capture (St Aubin and Geraci, 1988a). The effects of stress on TH levels are mediated by the action of GCs. In rats, T3 production is decreased as a direct result of stress-induced GC levels (Bianco et al, 1987). An elevation in cortisol levels, caused by either ACTH or handling stress, produces a reduction in T3 within six to twelve hours in belugas (St. Aubin and Geraci, 1988a).

Chronic stress causes a reduction in TH in terrestrial mammals (Cremaschi et al, 2000). Captivity may cause chronic stress and is linked to profound changes in TH

in marine mammals. Ten weeks of captivity is associated with continued suppression of TH concentrations after the initial response to capture in belugas, compared to levels measured in free-ranging conspecifics (St. Aubin and Geraci, 1988a). Captive manatees have lower TT3 and higher FT3 and TT3 than their wild counterparts (Ortiz et al, 2000). TH are also lower in harbour seals held in captivity for nine months compared with those held for three months or less (Schumacher et al, 1995). Although these differences may be a consequence of reduced food intake, dietary differences and/ or contaminant burden, they may be driven by the stress of captivity itself (St. Aubin and Geraci, 1988a; Schumacher et al, 1995). Repeated handling is another form of chronic stress that may influence thyroid axis function in a similar way. This possibility has not previously been explored in fasting seal pups.

A degree of stress is inherent in the handling procedures used to obtain the blood samples required for hormone analysis. If regular handling causes chronic disruption to the mechanisms that control secretion of cortisol and TH in seal pups, the information obtained from frequent blood sampling of the same individuals may not represent accurately the changes that occur naturally during the postweaning fast. In addition, the potential effects of repeated acute stress on hormone levels make it difficult to compare hormonal data from studies that utilise different handling regimes.

4.1.5. Experimental aims

Cortisol and TH levels and the cortisol response to acute stress were compared between fasting grey seal pups that experienced different sampling regimes, to separate those hormonal changes that occur naturally during fasting from the chronic

impact of handling stress. From a welfare perspective it was useful to establish whether repeated blood sampling at high frequency had a long-term detrimental effect on the ability of pups to respond to stress, to inform the planning of future sampling regimes. It was also necessary to determine whether hormone levels are influenced by frequency of handling to establish whether studies using different handling regimes are comparable.

The response of cortisol and TH to supplementary feeding was investigated to determine directly whether these hormones could act as endocrine signals of changing energy supply.

Changes in the levels of these hormones were measured in relation to fast duration, mass and body condition to investigate whether they may be involved in either regulation of fuel use or timing of departure from the colony. Blood samples were obtained as close to departure from the colony as possible because levels of hormones involved in the cue that prompts departure from the colony were expected to change towards the end of the postweaning fast.

4.2. Materials and methods

4.2.1. Sample collection

The study was carried out on the Isle of May during October to December, 2001. Details of general handling and sampling procedures are given in chapter 2.

Twenty eight grey seal pups of known age and weaning date were captured and penned. Seven animals were assigned to each of four different handling regimes; UNKNOWN, LOW, HIGH and FED. Twenty-one of these animals had been involved in another investigation prior to weaning and had been handled during the nursing period. Their mothers had been handled, not only during the 2001 breeding season, but also during several previous breeding seasons. These pups were assigned to the LOW, HIGH or FED groups. The remaining seven animals comprised the UNKNOWN group because they had not been handled prior to capture and penning.

Animals were assigned to LOW, HIGH and FED groups based on their sex and weaning mass to ensure that, as far as possible, there was no bias in the range of masses and the mix of sexes represented in each group. This was not possible for the UNKNOWN group because their mass and sex was not known until they were captured for the study. Summary information for the animals used in each group is given in chapter 2.

Blood samples were taken, mass and length measured and condition index calculated for each animal throughout the postweaning fast at intervals depending on experimental group. Blood samples and measurements were taken every three days until departure from the colony from both FED and HIGH animals to provide detailed information on hormone changes, whilst allowing the animals to recover for two days between handling episodes. On the first five days postweaning, after blood sampling

and measurement was completed, FED animals were given 1 - 1.5kg per day whole herring. Samples and measurements were taken from LOW animals three times during the fast whilst penned: at first capture postweaning (early), midway through the study period, at approximately 13 days postweaning (mid), and on release from the pen (late). These occasions are referred to as *timepoints*. The UNKNOWN group was also sampled at these three *timepoints*. They were included as a comparison to ensure that pups that had been handled previously were typical of pups on the colony as a whole, in terms of their hormone levels.

In all four treatment groups a second blood sample (*S2*) was taken five minutes after the first (*S1*) at each of the three *timepoints*, to assess changes in the acute cortisol response to handling stress within and between treatment groups over time.

All pups were marked and released from the pen when they reached 30kg or 70% of their estimated weaning mass, whichever happened first. Animals still present on the colony three days after release from the pen were recaptured to obtain a blood sample as close to departure as possible.

4.2.2. Blood sample analysis

Blood samples were centrifuged and frozen, as described in chapter 2, within ten hours of obtaining the sample. Cortisol and TH concentrations were measured within six months using the immunoassays described in chapter 3.

4.2.3. Statistical analysis

Time postweaning was expressed in one of three ways. Firstly, the three *timepoints* allowed comparison of cortisol concentration ([cortisol]), magnitude of the change in [cortisol] between *S1* and *S2* (Δ [cortisol]), and the rate of Δ [cortisol], TT4 concentration ([TT4]) and TT3 concentration ([TT3]), between all four handling regimes. Secondly, days postweaning were used to investigate more detailed changes in [cortisol] and [TH] and were placed into three-day time bins (*day*) to reflect the sampling regime of the HIGH and FED groups. Time was also expressed as a proportion of fast duration to identify consistent changes in [cortisol] and [TH] in relation to the overall duration of the fast, irrespective of its absolute duration. Proportion of fast duration was divided into time bins (*prop*) of tenths of total fast duration for those animals for which departure date was known. One male (51146) from the FED group was therefore excluded.

Kruskal-Wallis tests were used to determine whether there were differences between groups in the timing (days postweaning) of the early, mid and late *timepoints* and time taken to obtain *S1* and *S2* in each case.

Since *timepoints* were unequally spaced throughout the fasting period, too few animals from the LOW and the UNKNOWN groups were present in each *day* and *prop* bin to allow statistical comparison across all four groups. Rather than lose resolution by increasing bin size to include all four groups, LOW and UNKNOWN animals were excluded from this part of the analysis. The number of animals from each of the FED and HIGH groups varied between *prop* (Table 4.1). Only those *day* and *prop* bins that contained three or more animals from each of the FED and HIGH groups were included in each analysis, which eliminated *day* 28 and 31, and *prop* 0.51-0.6.

Table 4.1: Number of animals in FED and HIGH groups in each *prop* category

<i>Prop</i>	FED	HIGH
0-0.1	5	7
0.11-0.2	5	6
0.21-0.3	7	5
0.31-0.4	4	5
0.41-0.5	7	7
0.51-0.6	2	6
0.61-0.7	7	6
0.71-0.8	4	7
0.81-0.9	5	6
0.91-1	4	7

Time postweaning (*timepoint*, *day* or *prop*), group, sex, time of day (*hour*), time taken to obtain the blood sample (*sample time*), cumulative handling time (*CHT*), mass and condition index (*condition*) were included as fixed effects in saturated linear mixed models (LMEs) to explain postweaning changes in [cortisol], [TH], TT3:TT4, Δ [cortisol]) and rate of Δ [cortisol]. Model selection was performed as described in chapter 2.

4.2. Results

Details of all LMEs are given in appendix 2

4.3.1. Timing of samples

There were no significant differences between groups in the number of days after weaning of each *timepoint* (Table 4.2.).

Table 4.2: Timing (days postweaning) of early, mid and late *timepoints* for each group (Kruskal –Wallis: p values adjusted for ties)

<i>Timepoint</i>	Group	median	range	H ₍₃₎	p
early	FED	2	0-4	5.01	0.179
	HIGH	1	1-3		
	LOW	3	1-5		
	UNKNOWN	2	1-3		
mid	FED	13	13-14	5.78	0.123
	HIGH	13	13		
	LOW	13	12-13		
	UNKNOWN	13	4-13		
late	FED	25	16-24	5.18	0.159
	HIGH	21	18-25		
	LOW	21.5	17-26		
	UNKNOWN	22	8-23		

It took significantly longer to obtain *S2* from the FED group than the UNKNOWN animals at the early *timepoint* (Table 4.3.). There were no other differences between groups in time taken to obtain *S1* or *S2*.

Table 4.3. Time taken (minutes) to obtain *S1* and *S2* at early, mid and late *timepoint*s. Bold font indicates a significant difference between group (Kruskal –Wallis: p values adjusted for ties)

<i>Timepoint</i>	Group	<i>S1</i>				<i>S2</i>			
		median	range	H ₍₃₎	p	median	range	H ₍₃₎	p
early	FED	1	1-5	3.70	0.296	6	6-10	8.20	0.042
	HIGH	2	1-3			7	6-8		
	LOW	2	1-3			7	6-7		
	UNKNOWN	1	0-6			6	5-6		
mid	FED	1	1-5	1.30	0.729	6	6-15	2.95	0.400
	HIGH	1	1-2			6	6-7		
	LOW	1	1-2			7	6-7		
	UNKNOWN	1	1-2			6	6-7		
late	FED	2	1-3	2.80	0.423	8	6-10	3.34	0.342
	HIGH	1	1-2			6	6-11		
	LOW	1.5	1-4			7	6-9		
	UNKNOWN	2	1-4			7	6-8		

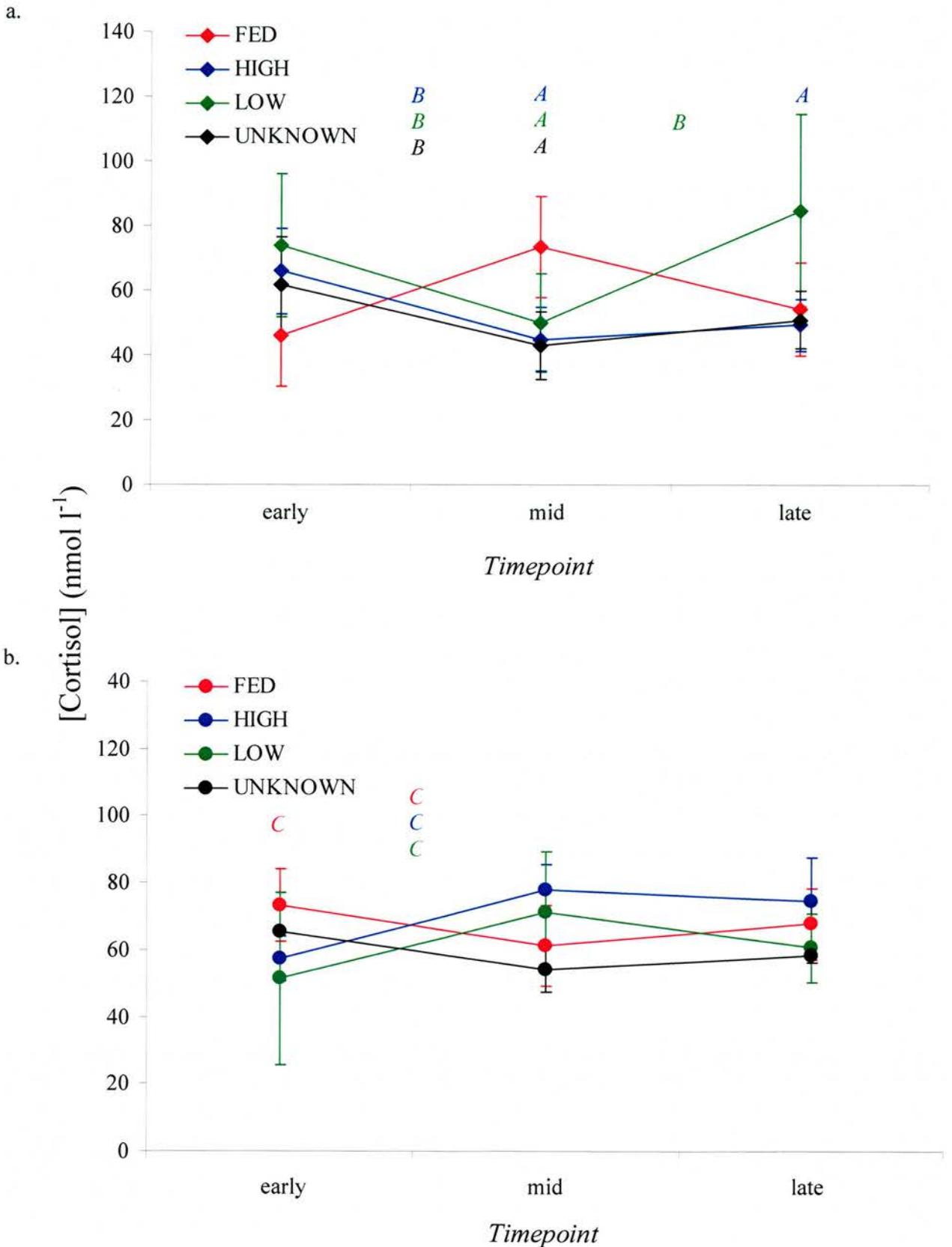
4.3.2. Change in hormone concentrations with *timepoint*

4.3.2.1. [Cortisol] at *S1*

The pattern of change in [cortisol] at *S1* between *timepoint* is shown in Figure 4.1. There was a significant interaction between the effects of group, *timepoint* and sex on [cortisol] at *S1* (Table A2.1, appendix 2).

There was a significant negative linear relationship between [cortisol] and mass shown in Figure 4.2. All other explanatory variables were rejected during the model selection process. It was necessary to weight the model to take account of the difference in variance in [cortisol] between *timepoint*.

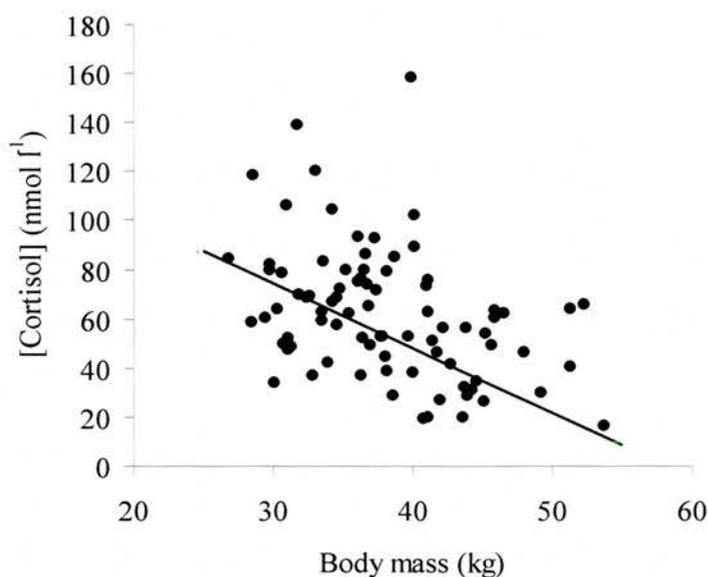
Figure 4.1: Change in *SI* [cortisol] (\pm s.e.) between *timepoints* in each group in a. females and b. males. *A* denotes a significant difference ($p < 0.05$) from the EARLY *timepoint*. *B* indicates that the change in [cortisol] between *timepoint* is significantly different from the FED group. *C* (shown only in graph b.) indicates a sex difference. All letters are colour coded by group.



There were no significant differences in [cortisol] between groups within each *timepoint* and within each sex. FED males had significantly higher [cortisol] than FED females early in the fast. There were no other differences between males and females within group and *timepoint*. At the early *timepoint*, [cortisol] was slightly higher in males than females in the FED group, whereas the opposite was true in the LOW group.

[Cortisol] changed significantly between *timepoints* in females but not in males. The pattern of change in [cortisol] between *timepoints* within females was significantly different between the FED group and the other three groups. Whilst females from HIGH, LOW and UNKNOWN groups showed a significant reduction in [cortisol] from early to mid fast, females in the FED group showed no significant change in [cortisol]. [Cortisol] was significantly lower late compared with early in HIGH females, but not in females from the other three groups. There was also a significant difference between FED and LOW females in the change in [cortisol] from mid to late.

Figure 4.2: Relationship between [cortisol] and body mass, given the effects of group, *timepoint*, and sex. LME: [cortisol] = -2.63 (mass) +153.26; $T_{(37)} = 4.75$, $p < 0.0001$).



4.3.2.2. Stress induced [cortisol]

The changes that occurred between *timepoints* in both Δ [cortisol] and rate of Δ [cortisol] are shown in Figure 4.4 and table A2.2 (appendix 2). There was no sex difference in the values or patterns of change in each case. Δ [cortisol] was significantly lower at the late compared to the early *timepoint*. The rate of Δ [cortisol] was significantly lower at the mid and late *timepoints* than at the early *timepoint*. There was also a significant negative relationship between mass and rate of Δ [cortisol], shown in Figure 4.3.

Figure 4.3: Relationship between rate Δ [cortisol] and mass, given the relationship with *timepoint*. LME: rate Δ [cortisol] = -0.202 (mass) + 14.74 ; $T_{(51)} = 2.02$; $p = 0.0489$).

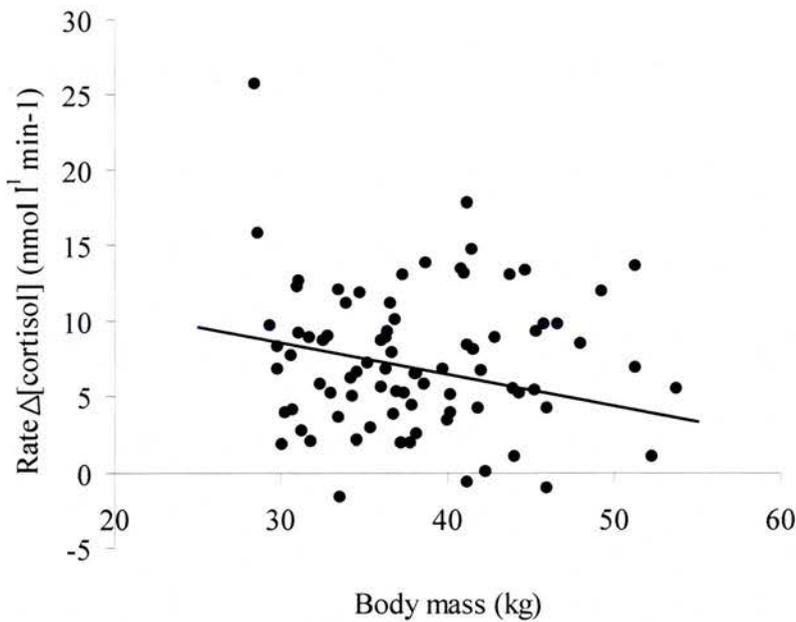
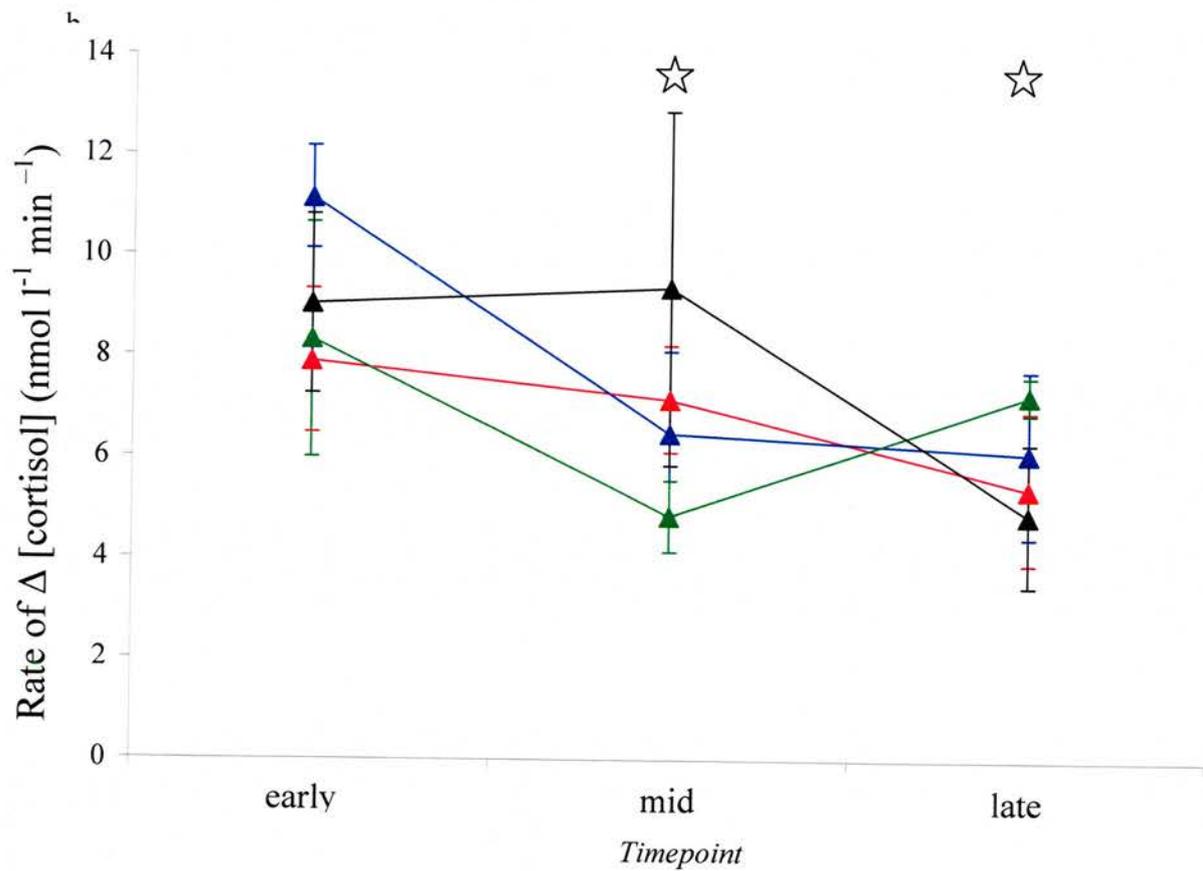
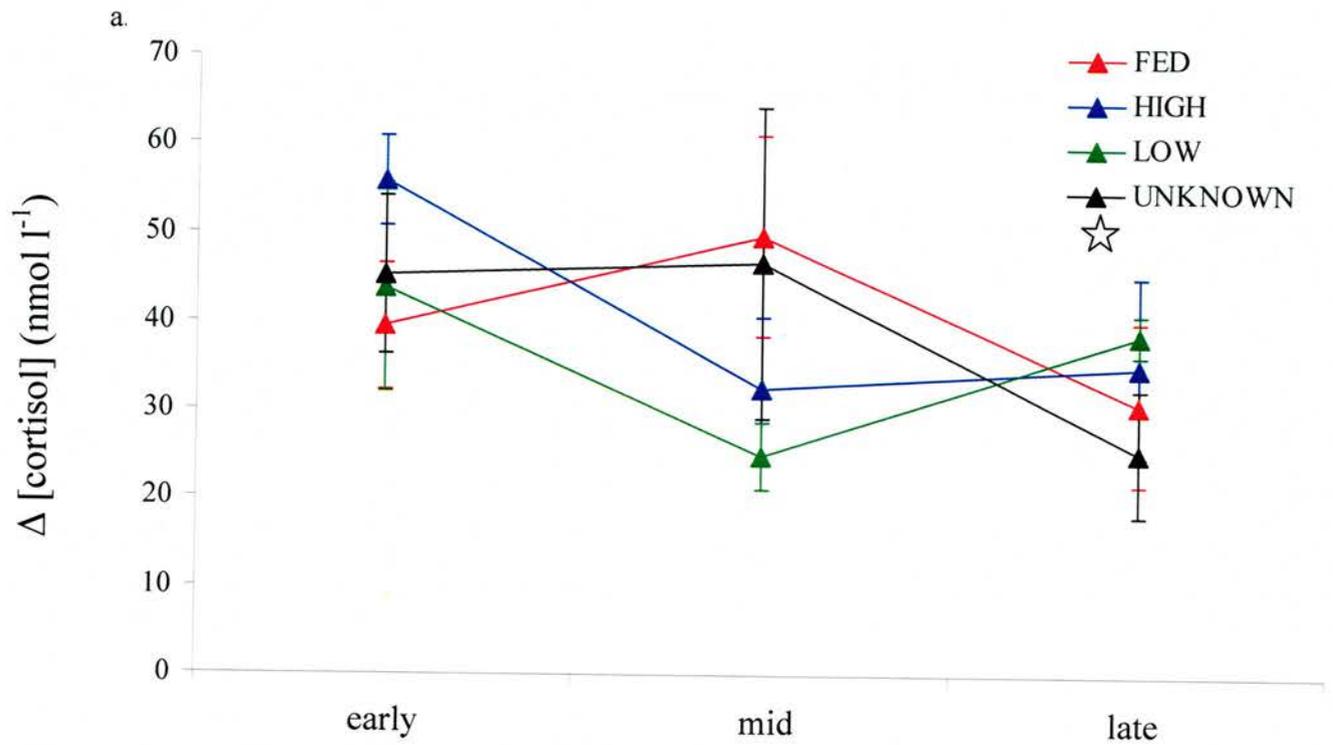


Figure 4.4: Change in a. Δ [cortisol] and b. rate of Δ [cortisol] with *timepoint* in each group. ☆ denotes a significant difference ($p < 0.05$) from the EARLY *timepoint*

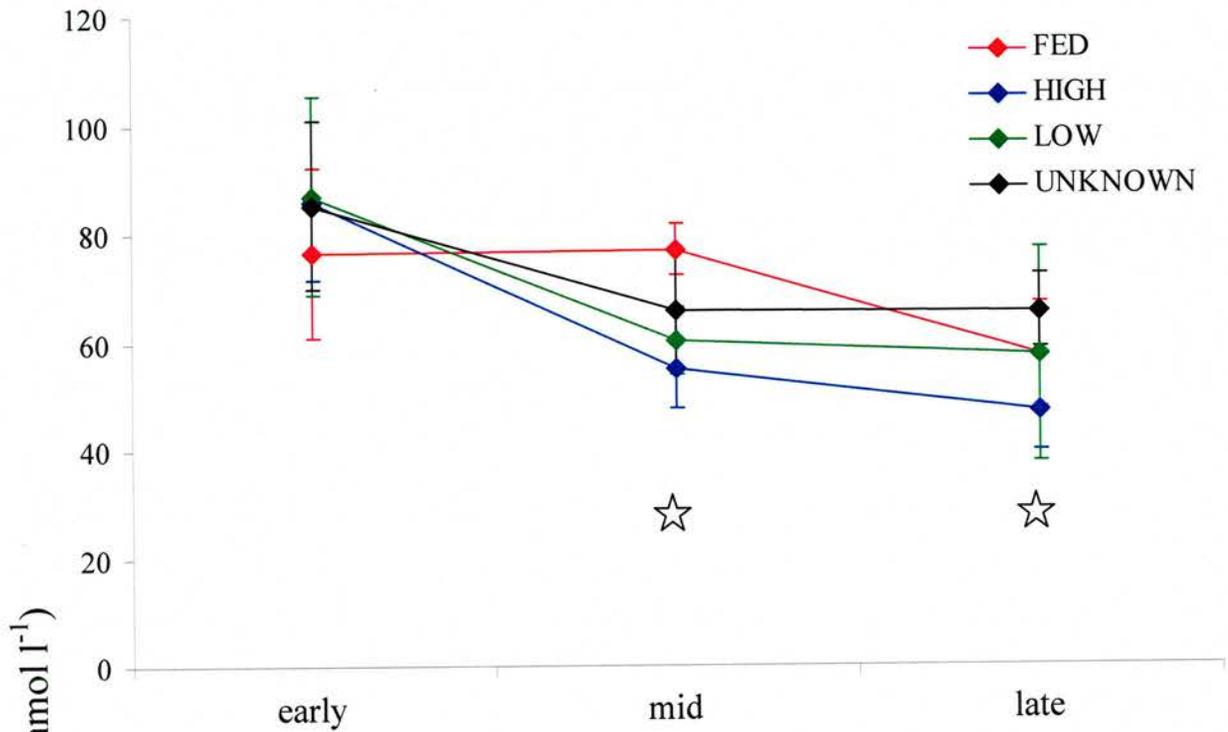


4.3.2.3. [TH]

[TT4] and [TT3] are shown as a function of *timepoint* in Figures 4.5 and 4.6. In all groups [TT4] was significantly higher at the early *timepoint* than at mid or late *timepoint*, but there was no significant difference in [TT4] between mid and late (Table A2.3, appendix 2). There was no change in [TT3] as a function of *timepoint*. Both [TT4] (LME; $T_{(26)} = 2.41$; $p = 0.0233$) and [TT3] (LME; $T = 3.63$, $p = 0.0012$) were significantly lower in males than in females in all four groups. There was no relationship between TT3:TT4 and any of the explanatory variables.

Figure 4.5: Change in [TT4] with *timepoint* in a. females and b. males. ☆ denotes a significant difference ($p < 0.05$) from the EARLY *timepoint*

a.



b.

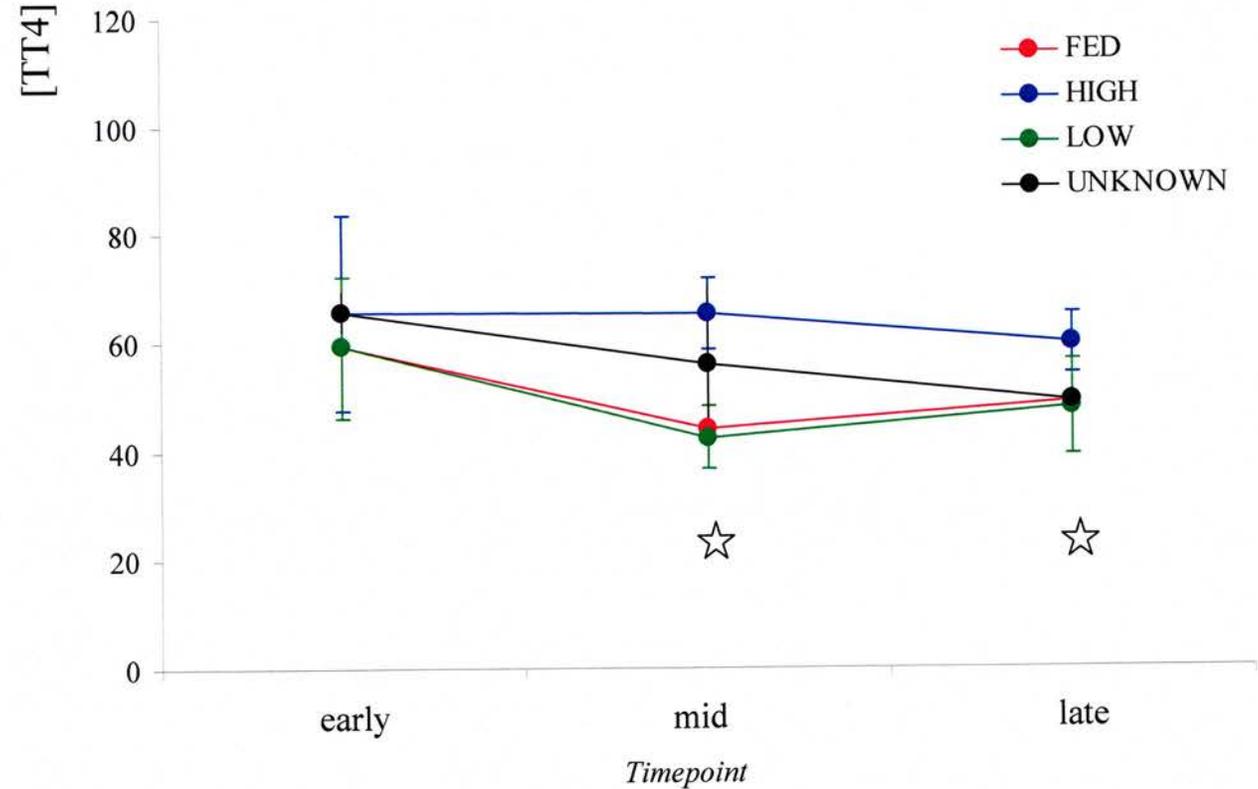
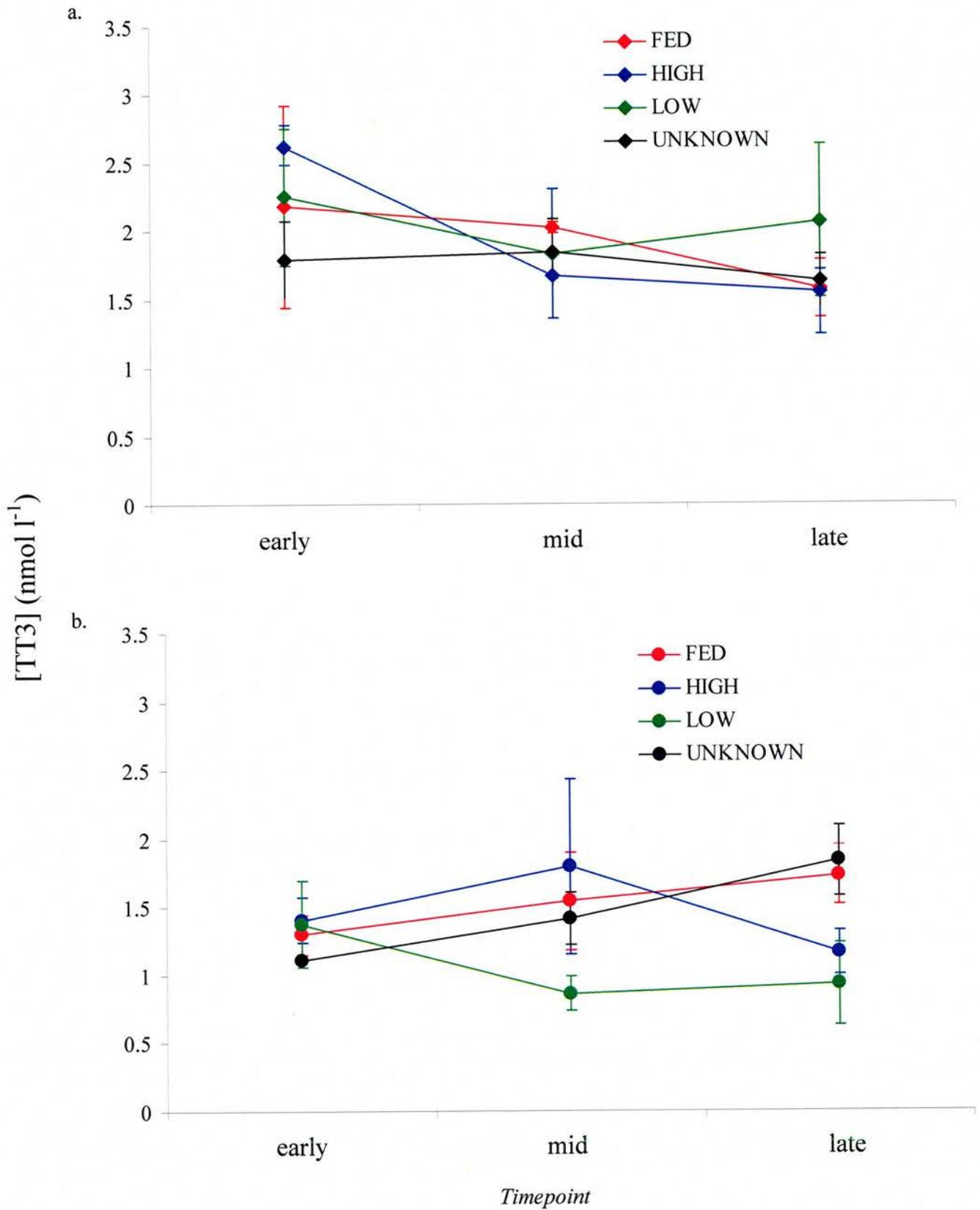


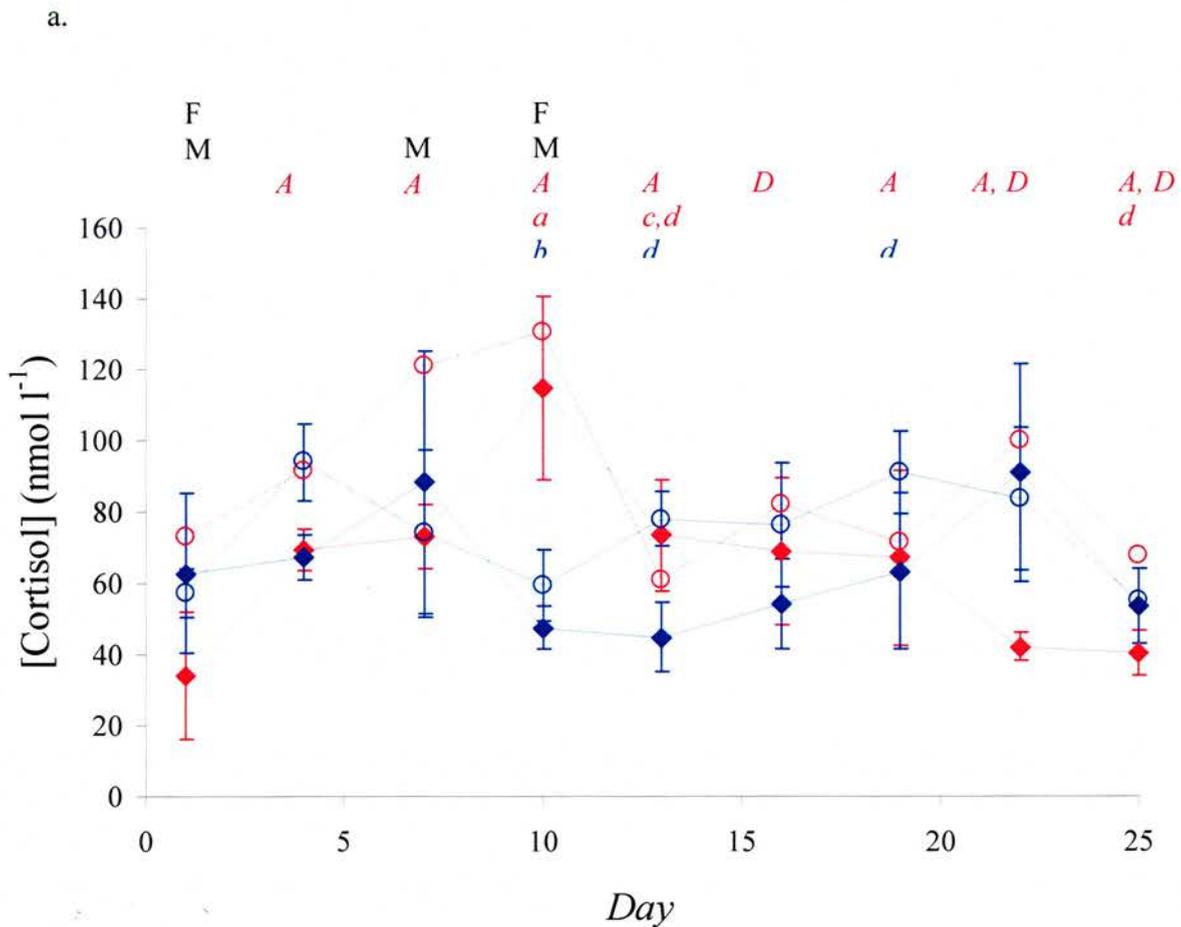
Figure 4.6: Change in [TT3] with *timepoint* in a. females and b. males

4.3.3. Change in hormone concentrations with *day* and *prop*

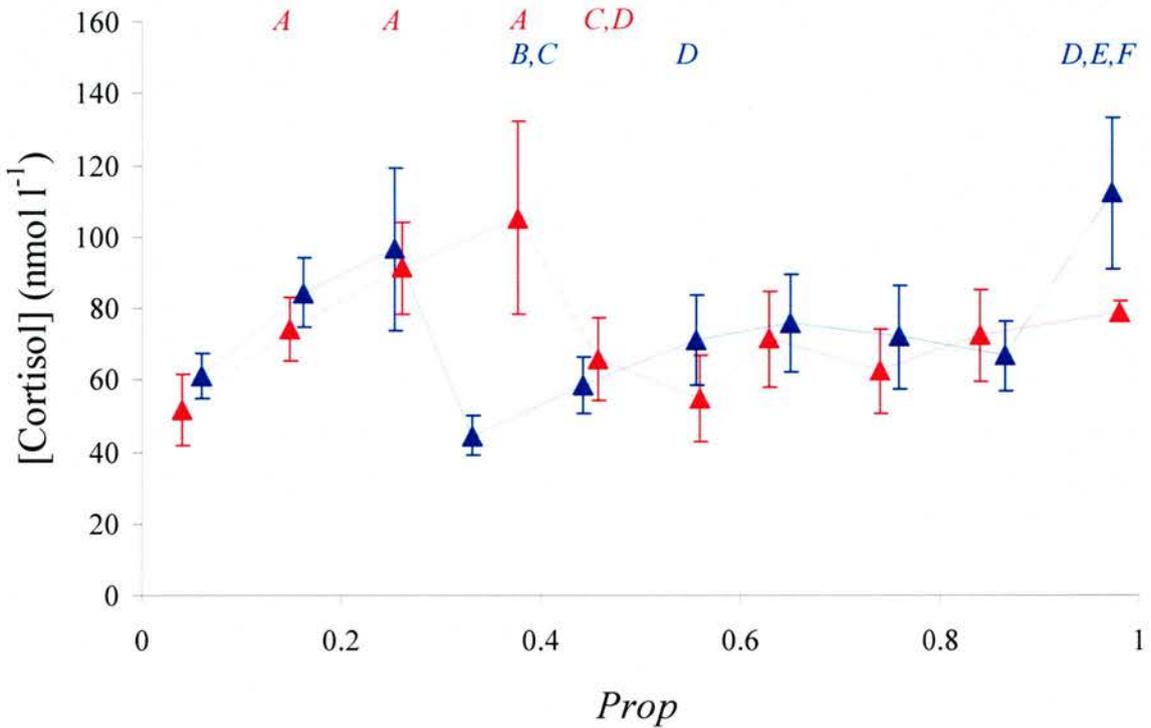
4.3.3.1. [Cortisol]

The pattern of change in [cortisol] with *day* and *prop* in males and females from FED and HIGH groups was complex and is shown in Figure 4.7.

Figure 4.7: Change in [cortisol] (\pm s.e.) with a. *day* (F and M indicate a significant difference ($p < 0.05$) within day between groups for females and males respectively. *A-d* indicate a significant difference from *day* 1, 4, 7 and 10 respectively. Capital letters represent females and small case letters represent males and are colour coded by group). b. *prop* (*A-F* indicate a significant difference from *prop* 0.1, -0.7 and are colour coded by group). FED animals ($n=7$) are shown in red and HIGH animals ($n=7$) in blue. Diamonds represent females ($n=3$) and circles represent males ($n=4$). Data for males and females are presented together in b because there was no sex difference.



b.



There were significant differences in [cortisol] within each *day*. At the start of the fast (*day 1*), there was no significant difference in [cortisol] in males from the two groups, whereas females from the HIGH group had higher [cortisol] than those from the FED group. [Cortisol] was significantly higher in the FED group than the HIGH group in males on *day 7* and in animals of both sexes on *day 10*. Within the FED group, [cortisol] was significantly higher in males than in females on *day 1* and *22*, whereas [cortisol] was not significantly different between the two sexes in the HIGH group. An interaction between group and sex was significant only on *day 1*. There were no other differences between the two sexes within each group within any other *day*.

Significant differences between groups in the change in [cortisol] as a function of *day* were largely driven by an early elevation and subsequent drop in [cortisol] in the FED group that did not occur in HIGH animals. [Cortisol] did not change significantly as a function of *day* in the females from the HIGH group. In HIGH males [cortisol] was significantly lower on *day* 10 than at *day* 4, 13 and 19. In contrast, within the FED group, both males and females showed a gradual, significant increase in [cortisol] from *day* 1 to *day* 10. [Cortisol] decreased from highest levels at *day* 10 to *day* 13 and did not change significantly thereafter. The pattern of the change in [cortisol] was different between sexes within the FED group between *day* 1 and 13 and between *day* 13 and 22. Males showed a slower and smaller increase in [cortisol], followed by a more marked reduction from *day* 1 to 13. Males had higher [cortisol] at *day* 22 than at *day* 13, whereas females had lower [cortisol] at *day* 22 than at *day* 13. From *day* 13 onwards [cortisol] was similar in both groups and showed no substantial changes as a function of *day*.

[Cortisol] also increased significantly as a function of both *sample time* (LME: $T_{(68)} = 3.181$; $p = 0.0022$) and *condition* (LME: $T_{(68)} = 2.132$; $p = 0.0366$), and decreased as a function of mass (LME: $T_{(68)} = 2.372$; $p = 0.0205$).

[Cortisol] changed significantly as a function of *prop* and the pattern of change was significantly different between the two groups, but not between sexes.

[Cortisol] was not significantly different between FED and HIGH animals during the first tenth of the fast. [Cortisol] was significantly higher in FED animals compared to HIGH animals 0.31-0.4 through the fast. There were no other differences between groups within *prop*.

[Cortisol] did not change as a function of *prop* in the HIGH group until a significant reduction occurred 0.31-0.4 through the fast. These lower levels persisted

for most of the remainder of the fasting period, except that [cortisol] was significantly higher in the final tenth of the fast than 0.31-0.7 through the fast.

The change in [cortisol] between 0.31-0.4 through the fast and all other *prop* bins was significantly different between the two groups. The pattern of change in [cortisol] in the FED group was similar to that seen when *day* was used as the time variable. [Cortisol] increased significantly over the first four tenths of the fast, then decreased halfway through the fast and remained at reduced levels for the remainder of the fasting period. As a result, [cortisol] was highest 0.31-0.4 through the fast in the FED group, when it was lowest in the HIGH group. A late increase in [cortisol] did not occur in the FED group, and the pattern of change in [cortisol] and absolute levels of the hormone during the final stages of fasting were not significantly different between the two groups.

There was a significant positive relationship between [cortisol] and *sample time* (LME: $T_{(75)} = 4.61$, $p < 0.0001$) and a trend towards higher [cortisol] in males than in females in both FED and HIGH animals (LME: $T_{(10)} = 2.20$, $p = 0.0523$).

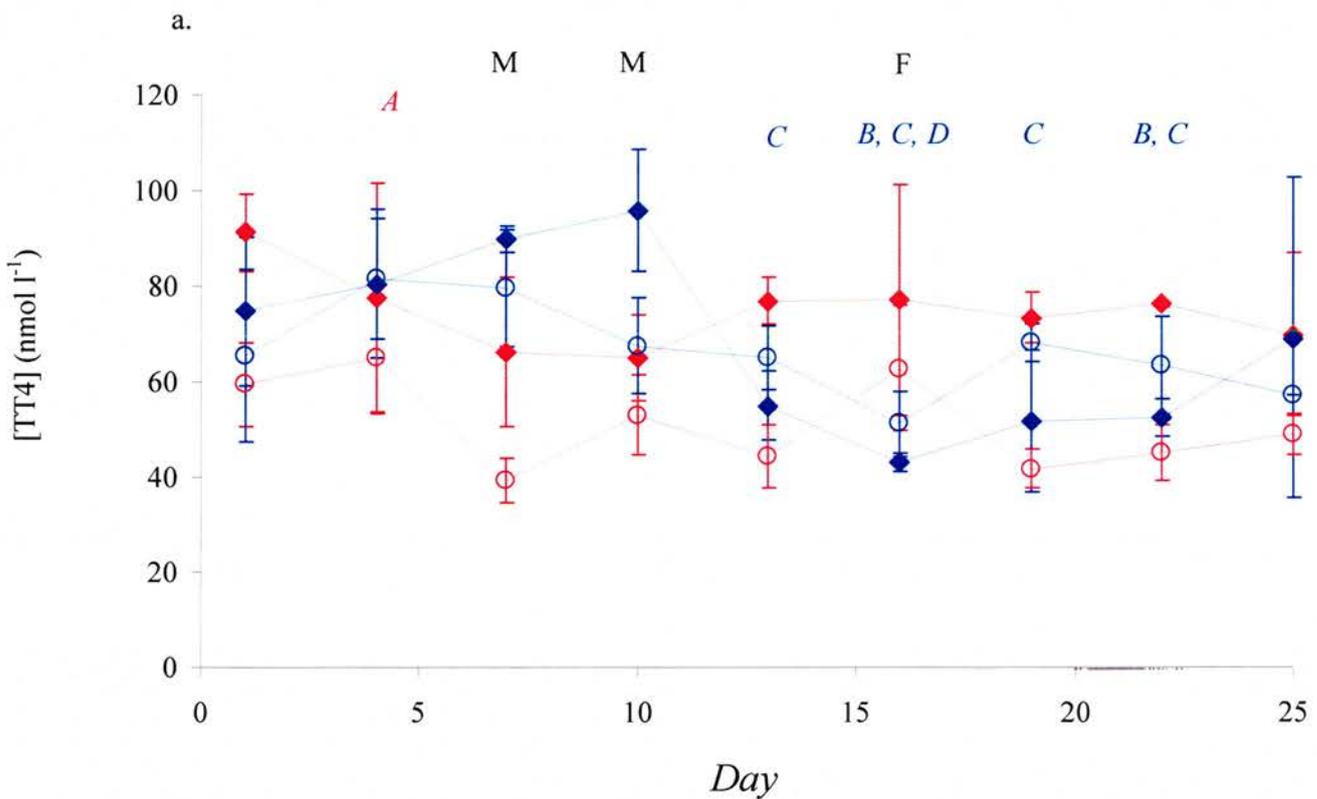
4.3.3.2. [TT4]

[TT4] is shown as a function of *day* in Figure 4.8. [TT4] changed significantly as a function of *day* and the pattern of change was significantly different between the two groups.

There was no difference in [TT4] between HIGH and FED animals of either sex on *day* 1. FED females had higher [TT4] than HIGH females on *day* 16. HIGH males had higher [TT4] than the FED males on *day* 7 and 10. FED females had significantly lower [TT4] than FED males (LME: $T_{(10)} = 2.61$; $p = 0.0260$), but there

was no significant difference in [TT4] between the sexes in the HIGH group (LME: $T_{(10)} = 0.19$; $p = 0.8549$). Although a sex by group interaction term was included in the model, the difference in the [TT4] response of males and females to treatment was not significant (LME: $T_{(10)} = 0.39$; $p = 0.0750$).

Figure 4.8: Relationship between [TT4] and *day* (mean values \pm s.e.) Closed symbols represent females (n=3) and open symbols represent males (n=4). FED animals are shown in red and HIGH animals in blue. F and M indicate significant ($p < 0.05$) differences between groups within day within females and males respectively. A–C represent differences from *day* 1, 4 and 7 respectively and are colour-coded by group

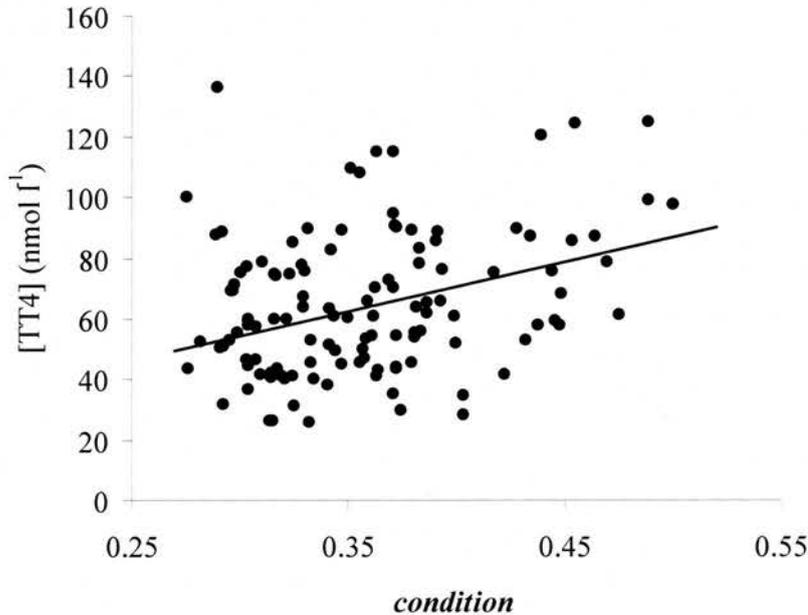


There was a significant difference in the change in [TT4] as a function of *day* between FED and HIGH animals. Within the FED group, the only significant change in [TT4] was a significant decrease between *day* 1 and 7. In contrast, [TT4] decreased in the HIGH group from *day* 4, 7 and 10 to lower levels on *day* 13, 16, 19 and 22.

[TT4] did not change as a function of *prop*. There was a significant positive relationship between [TT4] and *condition*, shown in Figure 4.9.

Figure 4.9: Relationship between [TT4] and *condition*.

LME: [TT4] = 162.19 (*condition*) + 5.76; $T_{(91)} = 2.52$, $p = 0.0133$, AIC = 64.421.



4.3.3.3. [TT3] and TT3:TT4

[TT3] did not change as a function of time postweaning (*day* or *prop*). [TT3] increased significantly with *sample time* when either *day* (LME: $T_{(102)} = 2.05$, $p = 0.0429$; AIC = 132.89) or *prop* (LME: $T_{(91)} = 2.09$; $p = 0.0399$; AIC = 113.70) was used as the time variable in the model. When *prop* was used as the time variable, [TT3] was significantly lower in males than in females (LME: $T_{(11)} = 2.72$; $p = 0.0198$; AIC = 113.70). TT3:TT4 did not change significantly as a function of any of the explanatory variables.

4.4. Discussion

4.4.1. The effect of handling frequency on cortisol and TH

Handling frequency and cumulative handling time did not alter either baseline or stress induced [cortisol], [TH] or TT3:TT4 in grey seal pups. At the onset of the postweaning fast, levels of these hormones were similar in animals that had been handled twice and those that had not been handled at all whilst suckling. Both cortisol and TH measurements from animals that had been handled during nursing were therefore assumed to be representative of pups on the colony as a whole.

There were also no differences in cortisol and TH measurements early, mid and late in the fast between pups handled every three days and those handled only three times over the course of the postweaning fast. Cortisol and TH measurements obtained from pups handled every three days were therefore assumed to be typical of all weaned pups on the colony and were considered to be an accurate reflection of natural changes that occur during the postweaning fast.

These findings corroborate and expand upon work by Engelhard et al (2002), which found no effect of previous handling (three to five times) during suckling on absolute cortisol levels or rate of cortisol increase at late lactation in southern elephant seal pups. The current study indicates that repeated handling during both suckling and fasting has no long-term impact on either cortisol secretion or TH dynamics in grey seal pups.

The fasting cortisol levels seen here were similar to those from other captive studies in grey seal pups, in which the animals were handled frequently (Nordoy et al, 1990; Nordoy et al, 1993). The current results were markedly different from the

increase in cortisol seen in fasting free-ranging elephant seal pups handled only twice in eight weeks (Ortiz et al, 2003c). This disparity may relate to captivity, despite the large enclosure used here, or species differences in hormonal changes.

The handling regimes used here were of much lower intensity than those that cause long-lasting changes to basal or stress-induced cortisol and TH levels in other animals. Permanent alterations to HPA function in rats result from prolonged (21 days) and intensive (daily for 15 minutes per day) postnatal handling schedules (Meerlo et al, 1999). The cortisol response to acute handling stress is attenuated in southern elephant seal females after three to five 45-minute handling bouts involving chemical immobilisation, over a 21-day period during lactation (Engelhard et al, 2002). Long-term changes in TH levels in belugas occur as a consequence of prolonged chase and capture followed by extended captivity (St. Aubin and Geraci, 1988). In contrast, in the current study, pups from known females were sampled twice during nursing, ten days apart, and were handled twice on each day for a total of ~20 minutes, to give an average cumulative handling time of 47 ± 12 minutes by the end of lactation. Postweaning handling bouts lasted a maximum of 15 minutes and were separated in time by three to ten days. The enclosure in which the animals were penned was large and it seems unlikely that they were aware of being confined. Only those animals that were fitted with an SRDL (chapter 7) experienced mild chemical immobilisation for a maximum of twenty minutes. Compared to other studies, the lower handling frequency, reduced contact time per handling episode and minimal use of anaesthesia in this study likely avoided chronic stress-induced changes to those endocrine axes under examination.

It is possible that the HPA and HPT axes were affected in the longer-term by repeated handling in ways that were not addressed in this study. For example, ACTH,

the hormone released in response to stress that stimulates corticosteroid production, has a particularly strong effect on aldosterone secretion in captive ringed seals (*Phoca hispida*; St Aubin and Geraci, 1986) and rehabilitated harbour seals (Gulland et al, 1999). Repeated stimulation of the adrenals by ACTH can cause the zona glomerulosa cells to downregulate receptor density and thereby decrease responsiveness of aldosterone production to further episodes of ACTH release (St Aubin and Geraci, 1986). This effect may not be apparent in the cortisol response to the same stimulation. A hyponatremic ringed seal exhibited a normal cortisol secretion response to ACTH, but failed to secrete aldosterone (St Aubin and Geraci, 1986). Repeated handling of pups in the current study may therefore have altered aldosterone secretion and influenced fluid balance without significantly impacting on cortisol production.

Stress caused by placing horses in a novel environment abolishes the circadian rhythm in cortisol levels by preventing the normal daily trough (Irvine and Alexander, 1994). The grey seal pups in this study may have experienced a similar effect of stress on circadian rhythmicity in hormone levels. However, since blood samples were taken during a four-hour interval in the morning, to minimise the effects of time of day on hormone measurements, the impact of stress on diurnal rhythms could not be explored.

In horses, chronic stress, induced by exposure to a novel environment and social instability, causes an increase in free cortisol levels, due to a decline in cortisol binding globulin (CBG) (Alexander and Irvine, 1998). Whilst bioavailability of the hormone is substantially increased, no change in total circulating levels of cortisol is seen. Total levels of TH are often influenced by variation in the concentration of binding proteins in the plasma, whereas free TH, the fraction able to interact with

receptors, are unaffected by such changes and track thyroid gland activity more closely (Hadley, 1992). Changes in free hormone levels, driven by either fasting or by repeated handling stress, could have occurred in pups in the present study, but would not have been detected since the assay used here measured total hormone levels.

4.4.2. Acute cortisol and TT3 response to stress

The increase in cortisol in response to physical restraint in grey seal pups compared well with changes in cortisol seen during handling in other phocid species (Gardiner and Hall, 1997; Engelhard et al, 2002). In all treatment groups, Δ [cortisol] was lower late compared to early in the fast, and the rate of Δ [cortisol] was lower mid and late compared to early in the fast.

A decrease in initial cortisol levels and the adrenal response to ACTH with time spent in captivity in rehabilitated harbour seals was attributed to adaptation to handling (Gulland et al 1999). It is possible that the blunted cortisol response in the later *timepoints* in the current study was due to habituation to handling, irrespective of handling frequency. To investigate this possibility more thoroughly requires samples at *S1* and *S2* from pups handled only once, at either the mid or late *timepoint* for comparison to pups handled more frequently, using a similar sampling regime to that employed by Engelhard and colleagues (2002). The response to exogenous ACTH could be used as an alternative approach to detect differences in adrenal function between animals from different handling regimes, or stages in the postweaning fast.

The absence of a “dose” effect of handling regime (ie, pups handled more frequently did not show a more marked attenuation in the cortisol response than pups

handled only three times) suggests that the change in responsiveness was a natural developmental or fasting-related alteration in cortisol secretion.

Developmental changes in HPA activity could be the cause of the decline in acute cortisol responsiveness to acute stress observed throughout fasting in postweaned grey seal pups. However, young mammals often show adrenal hyporesponsiveness at birth, and the response to stress increases within the first weeks of life (Sapolsky and Meaney, 1986). Engelhard et al (2002) reported a higher rate of increase in cortisol concentration at mid and late lactation compared to early lactation in southern elephant seal pups.

There is often an increase in HPA responsiveness to stress during food limitation or starvation (Chang et al, 2002). However, in humans, the stress-induced increase in cortisol is greater in volunteers given a glucose preload, indicating that adrenal stress responsiveness is dependent on a ready energy supply (Kirschbaum et al, 1997). Fasting imposes an additional constraint on fuel availability in developing animals and may modify their response to stress. Repeated mobilisation of limited fuel reserves in response to minor or non-novel stressors could be detrimental to survival in fasting animals and a moderate reduction in responsiveness of the adrenals to repeated stress may be adaptive in these circumstances.

Heightened adrenal responsiveness early in the fast may reflect a role of cortisol in the moult of the lanugo, which begins around weaning and lasts for several days. Cortisol is associated with hair loss in terrestrial mammals and is thought to play a role in moult in pinnipeds (Ashwell-Erickson et al, 1986; Riviere et al, 1977). High levels of cortisol are seen in moulting harbour (Riviere et al, 1977; Ashwell-Erikson et al, 1986) and largha seals (*Phoca largha*; Ashwell-Erikson et al, 1986) and during the moult of the lanugo in harp seals (Engelhardt and Ferguson, 1980).

Endocrinological preparation for moulting has been suggested as one explanation for increasing cortisol responsiveness throughout the suckling period in southern elephant seal pups (Engelhard et al, 2002). In the current study the early sample was taken during or immediately after the moult of the lanugo. The apparent reduction in adrenal responsiveness at mid and late *timepoints* may reflect the downregulation of the HPA axis after the role of cortisol in the moult was complete.

Repeated handling may also have altered the acute, stress-induced pattern of cortisol secretion in ways that were not apparent in this study. For example, chronic changes in the cortisol response to repeated handling and chemical immobilisation in southern elephant seal females were observed between ten and forty minutes after anaesthetic administration (Engelhard et al, 2002). The need to immobilise female southern elephant seals before blood sampling precluded the measurement of cortisol levels within ten minutes after anaesthesia (Engelhard et al, 2002). The current study, however, was designed to identify alterations to the stress response within five to ten minutes of the onset of handling and did not attempt to detect later changes in the acute cortisol response. This shorter time period was appropriate because dramatic changes in cortisol concentration occur within the first ten minutes of handling in seals (Gardiner and Hall, 1997; Engelhard et al, 2002), blood samples were typically obtained within this time period and handling rarely lasted longer than ten minutes in unanaesthetised pups. Differences in the rate of increase in cortisol between rats from different handling regimes were apparent over the first ten minutes after initial exposure to stress (Meerlo et al, 1999). Any chronic alterations in adrenal responsiveness to acute stress are also likely to be observed during the first ten minutes after exposure to a stressor in seals.

T3 increased as a function of sample time in grey seal pups. An elevation in T3 occurs in rats within an hour of exposure to acute disturbance stress, due to either TSH or catecholamine release (Dohler et al, 1977). This demonstrates the need to obtain blood samples as quickly as possible after initial contact with study animals to measure baseline concentrations of hormones, even though those hormones may not be directly involved in the acute response to stress.

4.4.3. Effect of supplementary feeding on cortisol levels

Artificial alteration of energy availability, by supplementary feeding at the start of the normal fasting period, significantly changed the pattern of cortisol secretion. In the FED animals, cortisol levels rose progressively over the first ten days postweaning then decreased suddenly and dramatically. These changes did not occur in the HIGH group. It is unlikely that the elevation in cortisol in the FED group was a result of more intensive handling experienced by this group. The sustained elevation in cortisol is likely to have been a response to feeding itself. In humans, cortisol levels are normally elevated for four hours or more after food intake (Kasckow et al, 2001) but return to pre-feeding levels well within 24 hours. The animals in this study had fasted for 24 hours before each blood sample was taken and the elevation in cortisol persisted for several days after the cessation of supplementary feeding. The elevation in cortisol in the FED group therefore appeared to be a chronic, rather than an acute effect of feeding. This could relate to changes in the gastrointestinal tract required to process food. Alternatively, cortisol secretion may be responsive to changes in energy availability in the longer term in these animals. The possibility that cortisol levels are linked with changes in energy availability needs further attention. The use of fat

infusions rather than feeding could be used to eliminate the possible confounding effects on cortisol secretion of signals from the gut. There are no changes in cortisol concentrations associated with the natural transition from suckling to fasting in either grey seals (Hall, 1998) or elephant seal pups (Ortiz et al, 2003a). However, fuel availability is unlikely to change markedly between these two states, because pups have a ready, abundant supply of fat both whilst suckling and early in the postweaning fast, either from milk or from their own extensive blubber reserves.

Artificially elevated FFA or a high-fat diet, which also increases circulating FFA, activate the HPA axis in rats (Widmaier et al, 1992; Tannenbaum et al, 1997). Increased dietary fat drives persistent changes in corticosterone levels in rats. After seven days of high (20%) fat feeding, rats show two to three fold higher basal levels of corticosterone and a heightened responsiveness to restraint stress compared to controls (Tannenbaum et al, 1997). An increase in FFA levels are correlated with a concomitant rise in cortisol levels in fasting northern elephant seal pups (Ortiz et al, 2003c), but it is unclear whether cortisol drives the change in FFA or vice versa. The higher levels of cortisol in FED animals in this study may have been a consequence of elevated FFA levels as a result of feeding.

In other animals the elevation in GC levels and insulin secretion caused by feeding promotes fat deposition (Santana et al, 1995). In the current study, the elevation in cortisol caused by supplementary feeding may reflect the assimilation of the additional fat into blubber reserves.

Since circulating cortisol levels in fasting grey seal pups were responsive to a change in energy availability, they could act as a signal of fuel reserve dynamics during the postweaning fast.

4.4.4. Postweaning changes in cortisol

Low, stable cortisol levels were characteristic of most of the fasting period, which is consistent with findings from captive grey and harp seal pups (Nordoy et al, 1990; Nordoy et al 1993). When time was expressed as either *timepoint* or *prop*, however, cortisol levels tended to be higher at the onset of fasting than later in the fast. This contrasts with the progressive increase in cortisol observed throughout the postweaning fast in northern elephant seal pups (Ortiz et al, 2001a and b). The difference in the observed pattern of change in cortisol may result from methodological differences between studies. It is unlikely that there is a fundamental difference in the action of cortisol in closely related species. The difference in the pattern of change in cortisol between phocids may result from differences in metabolic or developmental requirements during the fast, perhaps due to disparity in size, fast duration, and life history traits.

Cortisol levels may provide information about the size of fuel reserves. In addition to the increase observed in response to artificial alterations in energy supply in FED animals, there was a negative relationship between mass and cortisol levels in all four groups when the change in cortisol as a function of the duration of fasting was taken into account. This relationship is also evident in suckling northern elephant seal pups (Ortiz et al, 2003a). In fasting, lactating Subantarctic fur seals there is a negative relationship between cortisol levels and body condition index (Guinet et al, 2004). In the grey seal pups in this study (chapter 6) and elsewhere (Reilly, 1991; Iverson et al, 1993), mass is positively correlated with percentage body fat and is a good indicator of total energy reserves. Cortisol levels were therefore inversely related to the size of energy reserves and may provide an important endocrine signal of fuel availability.

In the model that examined the changes in cortisol with *day* there was a positive relationship between cortisol and *condition*, a rough measure of total body energy reserves that is different from the condition index used by Guinet et al (2004). Although cortisol was generally lower in heavier animals, pups in better *condition* (possessing proportionally greater fat reserves) had higher cortisol levels at any given mass. In captive redknots, *Calidris canutus*, corticosterone levels are highest when body fat stores are maximal prior to the normal migratory period, and are thought to form part of a preparatory mechanism for mobilisation of fuel reserves for a long flight (Piersma et al, 2000). Higher cortisol levels in pups in better condition and at the start of the fast could reflect a greater degree of fuel mobilisation when fat reserves are larger for animals of any given size. It is possible that higher cortisol levels in pups with larger energy reserves form part of the mechanism responsible for the greater proportional contribution of fat to energetic needs in fatter animals, described in chapter 6. Together the effects of *condition* and mass indicate that a complicated relationship exists between cortisol levels and energy availability.

The changes in cortisol concentrations may be related to a change in energy expenditure. GCs increase energy expenditure in humans (Tataranni et al, 1996). A reduction in the rate of mass loss in the early stages of fasting in grey seal pups (chapter 6; Nordoy et al, 1990) is driven by a decline in metabolic rate (Nordoy et al, 1990), but the mechanism for the fall in energy consumption is unknown. GCs influence mass changes through their impact on energy expenditure (Strack et al, 1995). It is possible that the decline in cortisol as a function of time since weaning causes a reduction in overall energy expenditure that leads to the observed decrease in mass loss rate (chapter 6; Nordoy et al, 1990).

GCs help to regulate the balance between proteolysis and lipolysis in other mammals. The net effect of cortisol on fat and protein breakdown depends on the hormonal and metabolic context. In the presence of insulin, cortisol causes a reduction in lipolysis (Ottoson et al, 2000) and increases fat deposition (Mantha and Deshaies, 2000) at the expense of protein reserves (Santana et al, 1995; Weiler et al, 1997). In the absence of changes in the levels of other hormones, such as glucagon, insulin and growth hormone, cortisol causes a dramatic increase in lipolysis (Divertie et al, 1991; Djurhuus et al, 2002; Djurhuus et al, 2004). Cortisol also drives changes in the levels of and sensitivity to many hormones that are also involved in energy balance, including insulin, leptin and growth hormone (Dallman et al, 1993; Elimam et al, 1998; Lerario et al, 2001), thereby indirectly affecting the rate of utilisation of different energy components. The fasting related changes in cortisol levels seen here could therefore relate to the utilisation of different reserves at different times to achieve the necessary balance between fat and protein utilisation.

In fasting elephant seal pups the increase in FFA concurrent with a rise in FFA has been attributed to the lipolytic effects of cortisol (Ortiz et al, 2003c). No differences in FFA were observed in grey seal pups early, middle or late in the fast (Walton and Bennett, submitted). The absence of a change in FFA may be related to the lack of an increase in cortisol during the postweaning fast in the current study, and elsewhere in captive phocid pups (Nordoy et al, 1990; Nordoy et al, 1993). This may reflect a difference between species in fatty acid disposal during fasting.

GCs often have lipogenic effects on adipose tissue (Santana et al, 1995; Mantha and Deshaies, 2000; Ottoson et al, 2000). Cortisol levels increase during suckling in northern elephant seal pups (Ortiz et al, 2001a and b; Ortiz et al, 2003a) and were elevated in this study in response to feeding, even after feeding had ended.

This increase may facilitate rapid fat deposition. The higher cortisol levels early in the postweaning fast in the unfed animals in this study may result from intensive fat assimilation during suckling.

There is some evidence that acute hypercortisolaemia can cause an increase in the availability of FFA in the blood by activating intravascular lipoprotein lipase, whilst reducing the activity of hormone-sensitive lipase in the adipose tissue (Samra et al, 1998). This seems to maintain fatty acid availability for metabolism derived from the circulating pool of triacylglycerols, but prevent the depletion of fat stored in adipose tissue. The elevation in cortisol in northern elephant seal pups could be a response to an increasing need to spare fat as the fast progresses, to prevent significant depletion of blubber reserves whilst maintaining high circulating levels of FFA to meet energy requirements. Cortisol levels in grey seal pups are also likely to reflect the balance between fat utilisation and the requirement to spare blubber for insulation and fuel when they go to sea. However, the absence of an increase in cortisol in this species, and other smaller phocids, may occur if the need to spare fat is less crucial in these animals.

In ADX or food-restricted rats, GC treatment acts to maintain fat stores at the expense of protein reserves (Santana et al, 1995; Mantha and Deshaies, 2000). Similarly, GC treatment in piglets leads to increased protein catabolism and fat sparing (Weiler et al, 1997). It has been suggested that an increase in cortisol in female Subantarctic fur seals contributes to protein mobilisation for milk production during the four-day periods spent ashore fasting whilst suckling (Guinet et al, 2004). Higher cortisol levels in this study may reflect periods of greater reliance on protein catabolism.

The difference in the pattern of change in cortisol between males and females may be a result of sex differences in the prioritisation of development of particular tissue types and allocation of fat and protein reserves for metabolism and growth. This is seen in otariid pups and lean southern elephant seals pups (Arnould et al, 1996; Arnould et al, 2001; Donohue et al, 2002; Beauplet et al, 2003; Biuw, 2003). A similar difference in requirements of male and female grey seal pups may be responsible for the sex difference in the change in cortisol during the postweaning fast. There is little evidence for a sex difference in fuel allocation in the current study, which did not include very lean individuals (chapter 6).

Cortisol clearly has several potential roles in fuel use in grey seal pups but its role in energy disposal could not be addressed directly in this study. The size of protein and fat reserves and the contribution of these two components to energy expenditure in these animals was based on two body composition estimates close to weaning and departure (chapter 6). This prevented meaningful comparison with hormone measurements, which were taken at three to ten day intervals. To determine directly whether GCs can alter fuel usage patterns, the effect of a cortisol analogue, dexamethasone, on the rate of mass loss and body composition changes was investigated in chapter 6.

Higher baseline, as well as stress-induced, cortisol levels at the onset of fasting may relate to the moult of the lanugo, as discussed previously. Timing of the moult of the lanugo with respect to weaning varies between individuals. This variability may explain why higher levels of cortisol are observed at the onset of fasting in some individuals and not others. In addition, if the early change in cortisol levels is related to the time elapsed since weaning or moulting, variability in the timing of the first sample with respect to these events could contribute to the differences seen in the

initial changes in cortisol between pups. Inaccuracies in assigning weaning date may also have led to slight time-shifts in the observed patterns of change. The absence of significant changes in cortisol in all groups may be due to a lack of statistical power as a result of small sample size combined with relatively large inter-individual differences in hormone levels and the timing of changes.

In northern elephant seal pups and lactating Subantarctic fur seal females, increasing cortisol levels during fasting have been implicated in the cue to depart from the colony (Ortiz et al, 2001 a and b; Guinet et al, 2004). In this study cortisol levels increased in HIGH animals towards the end of the fast, but did not change in FED pups. Similarly, cortisol was not higher at the late *timepoint* in LOW or UNKNOWN animals, even when the late sample was taken immediately prior to departure. Moreover, the elevation in cortisol levels did not occur in all animals in the HIGH group, and cortisol levels in the final sample were not different between HIGH and FED animals. The crucial feature of these results is that most animals left the colony without exhibiting an increase in cortisol at the end of the fast. The slight increase in cortisol in some HIGH pups at the end of fasting in this study may reflect the depletion of fuel reserves to levels lower than normally experienced by healthy pups fasting in the wild, due to an artificially extended fast. Together these results suggest that, under normal circumstances, a sustained elevation in cortisol is not required to trigger departure from the colony in fasting grey seal pups. The effect of GCs on the timing of departure was investigated directly in chapter 6 using dexamethasone.

4.4.5. Effect of supplementary feeding on TH levels

Supplementary feeding altered the pattern of change in TH after weaning in grey seal pups. TT4 was higher during the first ten days after weaning than later in the fast in the HIGH group, but these changes did not occur in FED animals. Instead, a small reduction in TT4 occurred in the FED group seven days after weaning. This difference was most likely a consequence of food intake. Rat pups fed a high fat diet convert a greater proportion of T4 to T3 than starved or glucose fed rats and therefore show a higher disappearance rate of T4 from the blood (Nathanielsz, 1970). The presence of elevated FFA in the circulation after feeding may displace T4 from its carrier proteins and result in its increased bioavailability and utilisation (Hollander et al, 1967). A higher rate of T4 utilisation in FED compared to HIGH animals may explain the absence of higher TT4 during the first ten days after weaning in FED pups. However, the absence of a concomitant increase in TT3 in FED animals at this time suggests that the difference in TT4 levels between groups is more likely due to reduced T4 secretion and compensatory changes in peripheral utilisation to maintain TT3 at a constant level.

Since changes in TT4 in the HIGH group occurred when time was expressed as *day*, but there was no relationship between TT4 and *prop* the observed pattern of change was likely related to the time elapsed since the animals weaned. However, since FED animals did not experience a fall in TT4 after the cessation of supplementary feeding, these natural changes in TT4 in the HIGH group seem unlikely to be related specifically to time since the animals last fed.

The thyroid axis appears to be responsive to artificially induced changes in energy availability in grey seal pups and changes in TH could therefore provide information about fuel supply in these animals.

4.4.6. Postweaning changes in TH levels

All treatment groups showed a reduction in TT4 from early to mid fast, but no change in TT3. This suggests that TH secretion fell, whereas TT3 production from the circulating TT4 pool remained unchanged. The changes in TT4 levels in the absence of concurrent changes in TT3 were not large enough to alter the TT3: TT4 ratio. Measurement of free as well as total levels of TH is needed to determine whether changes in TH availability occur in peripheral tissues and mirror those seen in total circulating concentrations.

The reasons for the changes in TT4 levels and their biological significance are unclear. In migrating birds, blockade of T4 conversion to T3 does not prevent all the actions of the hormone, providing evidence that T4 can act independently of T3 (Pant and Chandola-Saklani, 1993). T3 and T4 may also have different roles in fasting seal pups, which may explain the difference in the pattern of change in the two hormones.

Since there was no difference in TT4 between different handling regimes at equivalent *timepoints* during the fast, it is likely that the reduction in TT4 was a consequence of developmental processes or a response to fasting. The pattern of change in TH in the current study was different to that reported in fasting elephant seal pups. TT4 increased, and the TT3: TT4 ratio decreased during 49 days of fasting in free-ranging northern elephant seal pups (Ortiz et al, 2001 a and b). In this study TT4 was higher during the first ten days postweaning than later in the fast in the

HIGH group and a small reduction in TT4 occurred in FED animals seven days after weaning. These changes did not result in an altered TT3: TT4 ratio. The disparity in results may be due to methodological differences between the two studies or differences in fuel allocation and development between species.

The decline in TT4 levels is similar to that seen in TT3 in non-fast adapted species in response to food deprivation (Croxson and Ibbertson, 1977; Spencer et al, 1983; Blake et al, 1991). Since TT3 is the metabolically more active form of TH, the changes in TT4 seen here may not have had a significant impact on fasting metabolism. Alternatively, if TT4 is able to act independently of TT3, the reductions in TT4 may have contributed to energy conservation during fasting. The reduction in TT4 in conjunction with declining *condition* suggests that T4 levels change in response to fat content, rather than as a function of time spent fasting *per se*. Changes in TT4 could provide an endocrine link between fuel supply and utilisation in these animals. The involvement of TH, especially free levels of the hormones, in fuel allocation in fasting seal pups warrants further investigation.

A reduction in TT3 was not observed in this study as in other fasting marine mammals (Ortiz et al, 2000; Ortiz et al, 2001 a and b). Although TT4 secretion changed in response to fasting, TT3 levels seem to be maintained at a constant level, perhaps due to changes in peripheral deiodination and clearance of T4. It is possible that this is due to a conflict between the need to conserve fuel for an extended fast and the requirement for active development and high levels of lipolysis to sustain fat-based metabolism. The way that seal pups are able to deal with this dichotomy is unknown. Rat liver cells are able to modify T3 receptor density in response to their own metabolic requirements, irrespective of the metabolic status of the whole animal (Schussler and Orlando, 1978). It is possible that T3 receptor density changes in grey

seal pups in individual cells, while serum levels remain stable. This provides a plausible mechanism for pups to undergo development in specific tissues and organs and to conserve energy in others when required.

Higher levels of TT4 at the onset of fasting followed by a rapid decline may occur as a result of TH changes during moult of the lanugo. In other mammals, T4 stimulates hair growth but the precise role of TH in moulting pinnipeds is unclear. One study on harbour seals that measured TH at weekly intervals found that the elevation in TH around the time of the moult was not significantly greater than the substantial variations seen at other times of the year, and moulting was not consistently associated with increased TH (Renouf and Brotea, 1991). However, in harbour and largha seals, T4 levels are reduced at the same time as the period of cessation of hair growth, when the animals moult, and increase again when hair growth resumes (Riviere et al, 1977; Ashwell-Erickson et al, 1986). T3 and T4 are also high in moulting adult harp seals, and T4 levels remain high after moulting (John et al, 1987). An elevated T3: T4 ratio is thought to mark the onset of the moult in these animals. However, TT3: TT4 was consistently low in the animals in this study compared to moulting animals (John et al, 1987; Renouf and Brotea, 1991; Renouf and Nosewothy, 1991). In captive grey seals, high T4 is consistently associated with the end of the moult and is thought to play a role in maintenance of rapid hair growth (Boily, 1996). The high levels of TH at the end of the moult in captive grey seals may act to maintain heat production while the insulative capacity of the hair is reduced (Boily, 1996). If TH are involved in hair growth or thermoregulation during the shedding of the lanugo, the observed drop in TT4 over the course of fasting in this study may be a consequence of declining TH secretion after their role in the moult was complete.

The postweaning fast is a period of intensive development for phocid seal pups in preparation for the initiation of diving and foraging behaviour. Skeletal growth and development of blood and muscle oxygen storage capacity occurs during this time (Pattersonbuckendahl et al, 1994; Thorson and Le Boeuf, 1994; Noren et al, 2003b). Since TH levels underpin the regulation of these and other developmental processes in other animals, it is likely that the pattern of TH change observed here in grey seal pups reflects the high rate of development of this early period of their life history. The variation within and between groups in the timing of TH changes may reflect variability in the timing of developmental events in different animals. Males exhibited lower TT4 and TT3 than females, which may reflect sex differences in developmental and metabolic requirements.

Although high TH levels are associated with the onset of foraging in harbour seals (Renouf and Noseworthy, 1991; Boness et al, 1994; Haulena et al, 1998), the absence of a change in [TH] at the end of fasting in grey seal pups suggests that they are unlikely to form part of a direct cue to depart from the colony. It is possible that changes in TH levels inform the decision to leave. For example, a reduction in TT4 may occur after completion of essential development processes and departure may be prevented until this endocrine change occurs.

4.4.7. Summary

The handling regimes used during this study, either during suckling or fasting, did not impact significantly on cortisol secretion or TH levels. The changes in these hormones observed in animals handled every three days were therefore representative of those that occur naturally during fasting.

Cortisol and TT4 levels change from weaning to midway through the fast. The precise role of these changes and the significance of the contrast with those seen in northern elephant seal pups remain unclear, but are likely related to fuel use and development. Since cortisol and TT4 are responsive to both natural and artificially induced changes in fuel availability they are both potentially involved in fuel allocation during fasting and could act as signals of fuel reserve dynamics during fasting. Neither hormone is likely to be involved in the cue to leave the breeding colony in healthy pups since there were no changes consistently associated with the timing of departure. This does not eliminate the possibility that cortisol plays a role in the initiation of foraging behaviour in pinnipeds under other circumstances such as lactation or in starveling pups.

Chapter 5

The impact of dexamethasone administration on cortisol and thyroid hormone levels and leucocyte number in grey seal pups

5.1. Introduction

5.1.1. Summary

Glucocorticoids (GCs) may be involved in the regulation of fuel use and the timing of departure from the breeding colony in fasting phocid seal pups. To clarify their role in this mechanism, it is necessary to manipulate GC levels directly. This requires the selection of an appropriate drug and dosage regime to imitate the elevation in GC levels induced by fasting in other animals (Bergendahl et al, 1996; Cherel et al, 1988a, b and c; Robin et al, 1998). This study describes the preliminary test of a suitable dose of the synthetic GC, dexamethasone, in young captive grey seals and wild, fasting pups. The ability of dexamethasone to downregulate cortisol production was investigated as an indication that it had bound to GC receptors (GR). The magnitude and timecourse of its effect on endogenous cortisol levels was used to determine its duration of action. Its effects on thyroid hormone (TH) and white blood cell (WBC) levels in the circulation were also monitored to investigate the impact of this dose on the thyroid axis and immune function. This is the first experimental use of an artificial GC in pinnipeds intended for investigation of the effects of GCs on fuel utilisation and behaviour.

5.1.2. Role of GCs in energy utilisation and acquisition

The general role of GCs is to provide substrates for metabolism in the face of changing energy demands and availability. They mobilise protein reserves (Simmons et al, 1984; Legaspi et al, 1985; Darmaun et al, 1988; Santana et al, 1995), and/or enhance lipolysis (Samra et al, 1998; Sapolsky et al, 2000; Djurhuus et al, 2002;

Djurhuus et al, 2004), depending on other hormonal and metabolic signals, and increase hepatic gluconeogenesis. GCs can also promote conservation and storage of fat if insulin is present (Santana et al, 1995; Solano and Jacobson, 1999; Mantha and Deshaies, 2000). The central actions of GCs increase hunger (Strack et al, 1995; Tataranni et al, 1996) by suppressing secretion of CRH, an anorexigenic neuropeptide, and enhancing production of the appetite-stimulant, NPY, in the hypothalamus (Debons et al, 1986; Ponsalle et al, 1992; Hsiao-Ling and Romsos, 1996).

GCs increase abruptly and dramatically at the onset of phase III of fasting, when fat reserves reach a low critical threshold (Cherel et al, 1992; Friedl et al, 2000; Robin et al, 1998; Groscolas and Robin, 2001). This directs fuel utilisation towards an increased reliance on protein catabolism, and promote food-seeking behaviour (Koubi et al, 1991; Challet et al, 1995; Robin et al, 1998; Groscolas and Robin, 2001).

In fasting seals, reported changes in cortisol are not consistent between species. A progressive increase in cortisol during eight weeks of the postweaning fast was observed in wild northern elephant seal pups (Ortiz et al, 2001 a and b), whereas cortisol levels did not change in fasting harp (Nordoy et al, 1993) and grey seal pups in captivity (Nordoy et al, 1990). In the previous chapter in this thesis, there were some postweaning changes in cortisol but the timing of these changes was not consistent between individuals. Disparities between studies may result from methodological differences in sample collection, handling techniques, assay procedure or data analysis, or from species differences in metabolic or developmental requirements.

Although hormone measurements provide information about potential endocrine signals during fasting, changes in circulating hormone levels do not provide

direct evidence of mechanistic involvement in fasting metabolism and departure from the colony. Clearly direct manipulation of GC levels is required to investigate the role of cortisol in fasting seals.

5.1.3. Suitability of dexamethasone

The fasting induced elevation in baseline GC levels in rats, penguins and humans begins when adiposity reaches a low critical level and persists until re-feeding (Cherel et al, 1988a, b and c; Challet et al, 1995; Robin et al, 1998; Friedl et al, 2000; Groscolas and Robin, 2001). To mimic this sustained elevation using exogenous cortisol would require constant infusion of the drug because it is metabolised rapidly and has a half- life of 40 ± 11 minutes in the blood (Kraan et al, 1997). This is not a viable option in a study on wild animals. A further problem with the use of cortisol itself is the potential effects on fluid balance due to its high affinity for mineralocorticoid receptors (MR), which mediate the osmoregulatory effects of the adrenal steroids.

Many of the physiological and behavioural effects of cortisol can be reproduced using synthetic GCs. This study required a GC that would provide stimulation of GR over a period of a few days to mimic a fast-induced rise in GC levels, whilst avoiding a negative impact on either long-term adrenal function or osmoregulation.

The route and frequency of drug administration was also a major consideration in selecting an appropriate GC for this study. Frequent handling of wild animals in field conditions is a potential source of stress and increases infection risk in the study animals, especially when venipuncture, catheterisation or surgical techniques are

required to administer drugs (eg repeated injection, osmotic minipumps). Although food and water are often used to deliver GC analogues to experimental animals (eg. Weiler et al, 1997; Reidarson and McBain, 1999) this is not possible in fasting grey seal pups.

Dexamethasone is a pharmaceutical agent widely used as an anti-inflammatory and an appetite stimulant (Bishop, 2000). It is also used experimentally to reproduce the wide range of effects of cortisol *in vivo* (eg. Green et al, 1992; Santana et al, 1995; Tataranni et al, 1996; Weber et al, 2002) and *in vitro* (Musiani et al, 1998; Ong et al, 1992), and has a number of properties that meet the requirements for the current study. It can be administered intramuscularly as well as subcutaneously (eg. Cole et al, 2000) and orally (eg. Weiler et al, 1997; Reidarson and McBain, 1999). It has 30 times the anti-inflammatory and gluconeogenic potency of cortisol (Bishop, 2000). Unlike the native hormone, dexamethasone does not bind to corticosteroid-binding globulin (CBG), such that most dexamethasone in the circulation is available for binding to GR (Siiteri et al, 1982). It has a longer duration of action than cortisol because it has a higher affinity for GR, is metabolised more slowly and has a longer half-life in the blood (Dhabhar and McEwen, 1999). The duration of action varies widely depending on the solubility of the preparation used (Bishop, 2000). In captive dolphins, a single dose of dexamethasone administered orally is active for 48 hours (Reidarson and McBain, 1999). Together these properties allow dexamethasone to be administered at a low dose and low frequency to produce a sustained elevation in GC concentration.

Dexamethasone has a lower affinity with MR than the native hormone and many other synthetic GCs (Miller et al, 1992) and therefore has low mineralocorticoid activity, which reduces the risk of side effects on fluid balance.

Side effects of dexamethasone treatment include elevated insulin levels, reduced ACTH and cortisol concentrations, increased appetite, and alteration in the composition of WBC types in the circulation (Reidarson and McBain, 1999). The immunosuppressive effects of GCs can result in the worsening of pre-existing infections (Bishop, 2000). Side effects of a single oral dose of dexamethasone in dolphins persist only for the duration of the main effects of the drug (Reidarson and McBain, 1999). Suppression of the HPA axis tends to occur only with prolonged GC treatment (Bishop, 2000).

Dexamethasone has been used therapeutically in captive California sea lions (*Zalophus californianus*) and elephant seals (Stoskopf et al, 2001), but has not been utilised previously in experimental procedures on wild or captive seals.

5.1.4. GC effects on thyroid hormones

Thyroid stimulating hormone (TSH) secretion is suppressed by the elevation in cortisol level seen during fasting, administration of exogenous cortisol to mimic fast-induced levels, and chronically high endogenous levels of cortisol seen in Cushings syndrome. This reduces TH levels (Samuels and McDaniel, 1997). In rats, T3 production is decreased as a direct result of stress-induced elevation of GCs (Bianco et al, 1987), and in response to dexamethasone administration in rats and humans (Burr et al, 1976; Chopra et al, 1975). The effects of endogenous cortisol levels and dexamethasone administration on TH levels in seals are unknown. This requires investigation, since cortisol could mediate physiological and behavioural changes during fasting through an impact on TH levels.

5.1.5. Experimental aims

The aim of this study was to establish a dose of dexamethasone sufficient to provide sustained activation of GR (several days), as indicated by suppression of cortisol levels, in grey seal pups without long term effects on immune and adrenal function. TH levels were also examined because GC-induced changes in fuel use or behaviour could be mediated through changes in TH. The effect of a single intramuscular dose of dexamethasone on cortisol, TH, and WBC number and composition were monitored in two captive pups-of-the-year and over the course of the postweaning fast in a larger study involving wild, fasting grey seal pups.

5.2. Materials and methods

5.2.1. Captive study

Two ten-month old grey seal pups, one male and one female, were used to investigate the effect of a single intramuscular dose of dexamethasone on cortisol and TH levels and WBC counts. This experiment was conducted in the captive facility at the Sea Mammal Research Unit (SMRU). Details of the handling procedures are given in chapter 2.

The pups were held together for the duration of the experiment and had access to an unheated seawater pool (5m diameter) and a dry area. Both pups had previously been trained to station on a specific focus shape with fish as a food reward. In this experiment the focus shapes were used to move the animals to a small dry area, where there was no access to a pool, for the duration of manual restraint and blood sampling. Animals were given 2 kg (male) or 2.5kg (female) herring per day as a reward. Before sampling, the pups were only given the amount of herring needed to move them to the dry area each time. The remainder of their daily food allowance was given after the sample had been taken, or, on day one, not until after the twelve hour sample had been taken. The pups were left in the dry area for twenty minutes prior to restraint and sampling to dissociate the response to the focus shapes and feeding from the experience of being handled. They were allowed to return to the pool area immediately after each sampling period.

The experiment was conducted using both pups simultaneously. A blood sample was taken at 09:00 on day one (0 hour) followed by an intramuscular injection of injectable terramycin, to provide antibiotic cover. The animals were then injected on the opposite side of the body with either 0.025ml/kg dexadreson™ (Intervet,

Bucks, UK; 2mg/ml dexamethasone sodium phosphate), to give a dose of 0.05mg kg⁻¹ dexamethasone, or the equivalent volume of sterile saline solution (Aquapharm). The animals were blood sampled every four hours up to 12 hours after the injection and then at 24, 48 and 72 hours. The saline trial was performed first, followed immediately by the dexamethasone trial.

5.2.2. Field-based study

The field-based study was carried out on the Isle of May from October to December 2002. Thirty grey seal pups of known age and weaning date were penned after weaning. Each animal was assigned to one of three treatment groups (CONTROL, SALINE or DEX) based on its estimated mass at weaning and sex, such that, as far as possible, each group contained a range of animals of different sizes and a similar number of males and females. One pup from the CONTROL group developed an infection in the right axilla and was excluded from the study. Summary information for the animals used in the study is given in chapter 2.

Blood samples were taken every three days from weaning until departure of the animal or the end of the study period (18/12/02). At 10-12 days postweaning animals were given intramuscular terramycin, and either no additional injection (CONTROL), 0.025ml/kg sterile saline (SALINE) or 0.025ml kg⁻¹ dexadreson™ (DEX), as described for the captive study. A blood sample was taken 24 hours later, to allow post-injection measurement of cortisol levels, and pups were then released from the pen and allowed to range freely for the remainder of the fast. Date of departure was established for each animal as described in chapter 2.

5.2.3. Blood sample analysis

5.2.3.1. Hormone levels

After an aliquot of whole blood was removed for WBC counts, blood samples were centrifuged and frozen as described in chapter 2. Cortisol and TH concentrations were measured using the immunoassays described in chapter 3. Blood samples were analysed within ten days from the captive study, and six months from the field-based study.

5.2.3.2. Total WBC counts

Total and differential WBC counts were performed on all samples from captive animals, and on those samples taken immediately prior to treatment, and one and four days after treatment in the wild pups. WBC counts in the wild pups were restricted to the sample prior to injection and the two subsequent samples, to avoid potentially confounding effects of developmental changes in cell type composition (Hall, 1998).

WBC counts and smears were performed as quickly as possible after each sample had been taken. In the captive experiments this was almost immediately, whereas in the wild study the counts were performed up to ten hours after the sample had been taken.

The plasma was mixed by gentle inversion and a 10 μ l aliquot of the whole blood was transferred to 100 μ l of Barr's fluid. The red blood cells were allowed to lyse before 20 μ l of the mixture was transferred to each side of a Neubacher

haemocytometer. All blue stained cells in the large square were counted at x 40 magnification under a light microscope and multiplied by 0.11 to give the number ($\times 10^6$) of cells per ml of blood.

5.2.3.3. Differential WBC counts

Blood film smears were performed as described in Kerr (Kerr, 1989). Briefly, a clean glass microscope slide was polished and a drop of blood was applied close to the end of it. Using another slide as a spreader, the drop was teased out to form a straight line along the edge of the spreader. The leading edge of the blood was spread down the slide in a continuous movement to produce a monolayer of cells.

Slides were air dried and then stained with Kynvett-Gordon stain as follows: Slides were placed in glass slide holder and submerged in neat Kynvett-Gordon stain for 1.5 minutes, drained and blotted. They were then rinsed in distilled water and transferred to a 50:50 Kynvett-Gordon stain: water solution, pH6.8, for ten minutes, drained and blotted. They were rinsed in distilled water for two minutes, then allowed to dry 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 their appearance and staining characteristics: neutrophils, eosinophils, monocytes and lymphocytes. To prevent double counting the slide was moved in a systematic way from left to right as follows: down to the lower edge of the smear; across for two fields of view; and then up to the upper edge of the smear; repeated until 200 cells had been counted. The number of each cell type per ml of blood was then calculated from overall WBC number as follows:

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

5.2.4. Statistical analysis

The small sample size precluded statistical analysis on results from the captive facility experiment. Statistical analyses for the field-based study were performed as described in chapter 2.

5.2.4.1. Hormone levels

Linear mixed effects models (LMEs) were used to investigate the effect of dexamethasone on the change in cortisol and TH levels and TT3: TT4 with days postweaning. As in chapter 4, days postweaning were divided into three-day time bins (*day*). Those bins that contained two or less animals in any one group were excluded from analysis. Bins included in cortisol and TH analyses incorporated *day* 1-20 and 1-17 respectively and therefore allowed comparison of the three groups up to ten days after treatment.

Fixed effects included in the saturated models were time of day (*hour*), time taken to obtain the blood sample (*sample time*), cumulative handling time (*CHT*), mass and *condition* and interactions between group and *day*, group and sex, and sex and *day*. Individual was included as a random effect. Model selection is described in chapter 2.

5.2.5.2. WBC counts

LMEs that included a random term for individual were used to investigate whether time, at day 0, day 1 and day 4 after injection (*time* 0, 1 and 4 respectively), and treatment group influenced total and differential WBC counts.

5.3. Results

Details of all LMEs are provided in appendix 3.

5.3.1. Cortisol response

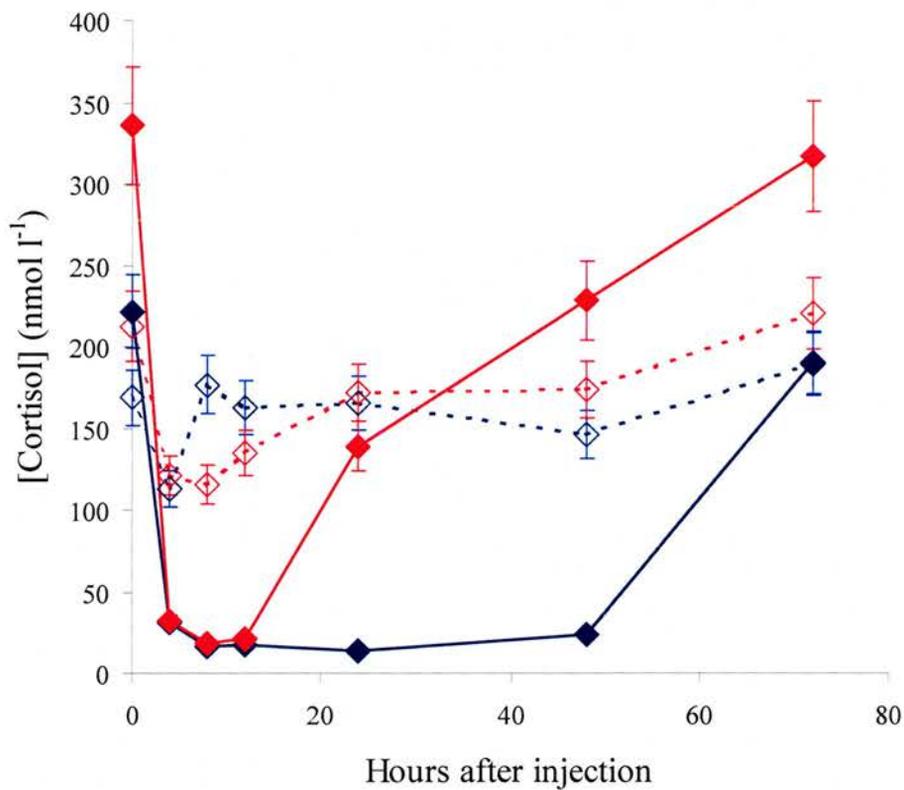
5.3.1.1. Captive study

Changes in cortisol concentration ([cortisol]) during the saline and dexamethasone treatments in both animals are shown in Figure 5.1. Four hours after saline injection, [cortisol] had fallen in both animals to 67% and 58% initial values. [Cortisol] then increased to levels comparable to preinjection values, by eight hours after treatment in the male, and 24 hours after treatment in the female. [Cortisol] was noticeably higher in the male than the female eight hours after treatment, but was similar in the two animals at all other times.

At the start of the dexamethasone trial, [cortisol] was 31% and 58% higher, in the male and female respectively, compared to values at zero hours in the saline trial. Both animals showed a dramatic reduction in [cortisol] in response to dexamethasone by four hours postinjection, compared with the concentration at both pre-treatment (0 hours in the dexamethasone trial) and the equivalent time point (4 hours) in the saline trial. Levels were reduced to 14% of pre-treatment, and 28% of 4-hour saline values in the male, and 10% of pre-treatment and 27% of the 4-hour saline value in the female. [Cortisol] was similar in both animals four hours after the dexamethasone injection (31.1 nmol l⁻¹ in the male and 32.6 nmol l⁻¹ in the female). A further decrease in [cortisol] occurred in both animals by eight hours postinjection. In the male, [cortisol] remained low until 48 hours after injection, then increased to levels

indistinguishable from pre-treatment levels and values seen at 72 hours postinjection in the saline trial. In the female, [cortisol] increased between 12 and 24 hours after dexamethasone treatment to levels similar to those seen at the equivalent time in the saline trial. [Cortisol] continued to increase above values seen at 48 and 72 hours in the saline trial, and reached levels comparable with those at the start of the dexamethasone trial by the 72 hour sample.

Figure 5.1: Change in [cortisol] over 72 hours after saline (open symbols, dashed lines) and dexamethasone (closed symbols, solid lines) injection in two captive animals. Red symbols represent the female, blue symbols represent the male. Error bars represent maximum recorded assay variation of 10.08% (chapter 3).



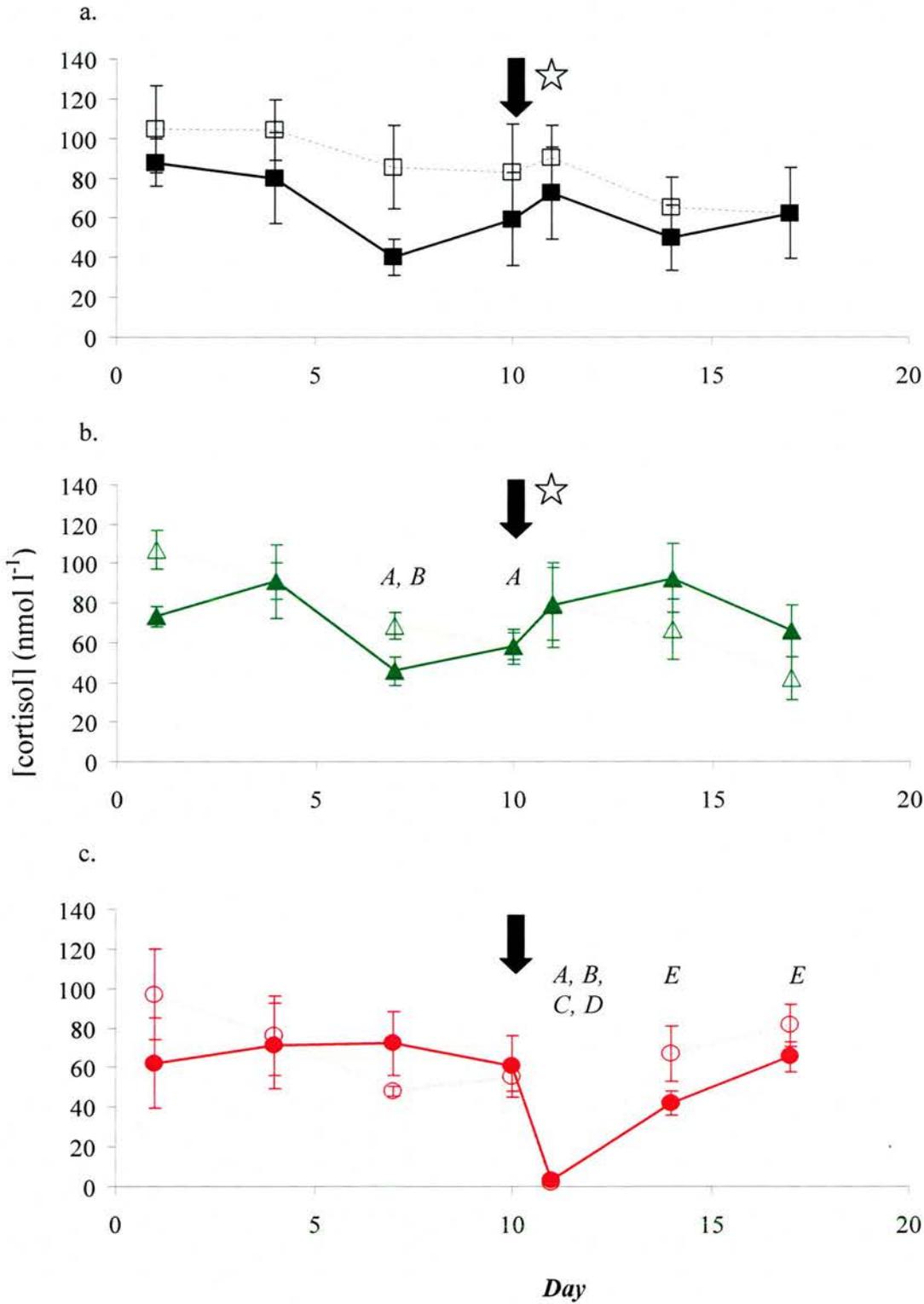
5.3.1.2. Field-based study

Changes in [cortisol] as a function of *day* for all three groups are shown in Figure 5.2. Variability in [cortisol] between individuals was high. The LME that best described the changes in [cortisol] included fixed terms for an interaction between *day* and group. All other explanatory variables were rejected during the model selection process.

At the start of the fast [cortisol] was similar in all three groups. SALINE animals showed a significant reduction in [cortisol] from higher initial levels on *days* 1 and 4 to lower levels on *days* 7 and 10. A similar, non-significant pattern was observed in the CONTROL animals. There were no further changes in [cortisol] with *day* in either the CONTROL or SALINE animals, and levels were similar in both groups on all *days*.

DEX animals showed a highly significant drop in [cortisol] 24 hours after dexamethasone injection (*day* 11) that did not occur in the other two groups, such that [cortisol] was significantly lower in the DEX group on *day* 11, than in SALINE and CONTROL animals. Levels recovered to preinjection values by *day* 14 and were not significantly different from the other two groups for the remainder of the fast. On *day* 20, [cortisol] was higher than on *days* 10 and 14 in the DEX group.

Figure 5.2: Change in mean [cortisol] \pm s.e.e. as a function of *day* in a. CONTROL b. SALINE and c. DEX groups. Closed symbols represent females and open symbols represent males. Arrow indicates timing of treatment. \star denotes a significant difference ($p < 0.05$) from the DEX group within *day*. A-E indicate significant differences within groups from *day* 1, 4, 7, 10 and 11, respectively



5.3.2. TH response

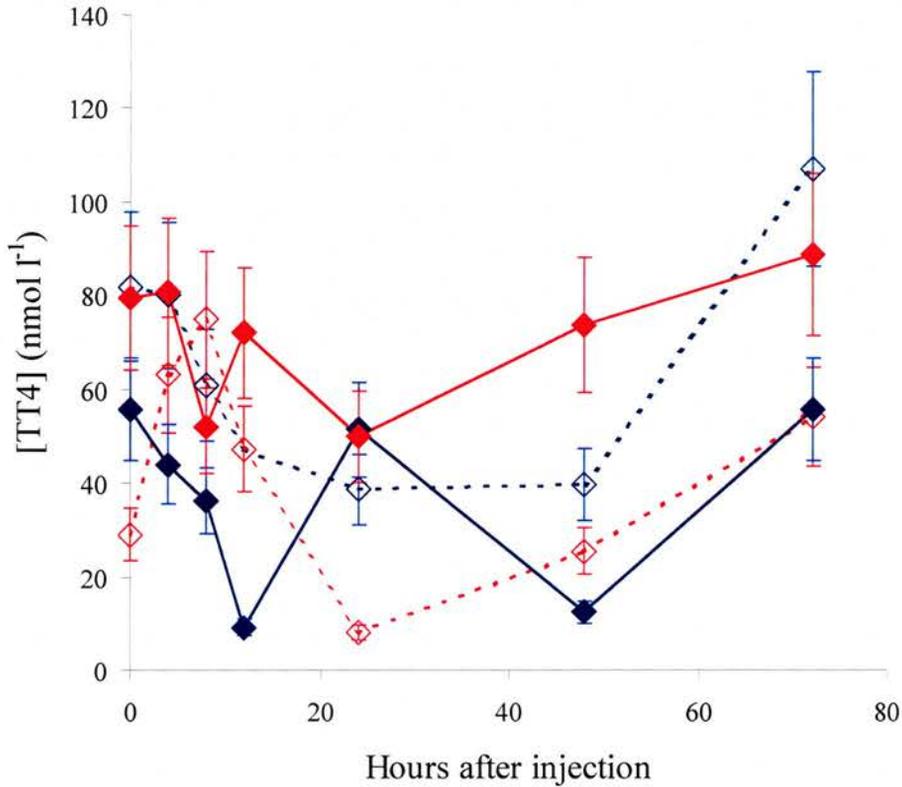
5.3.2.1. Captive study

Changes in TT4 concentration ([TT4]) during the saline and dexamethasone trials in both animals are shown in Figure 5.3. [TT4] was extremely variable in both animals during both trials.

The male pup showed a decline in [TT4] over the first 24 hours after saline injection to 47% of initial values. In the female pup [TT4] reached highest levels, at 250% of the initial value, eight hours after injection, then fell to 28% of initial values by 24 hours after injection. In both the male and female, [TT4] then remained low until a substantial increase to 130-190% of the initial values between 48 and 72 hours after injection.

[TT4] was 32% lower in the male and 174% higher in the female at the start of the dexamethasone trial than at the start of the saline trial. Both pups showed a reduction in [TT4] after dexamethasone treatment to 16% and 65% of initial dexamethasone trial values. In the male pup the reduction occurred by 12 hours after injection and was of a similar magnitude (42nmol l^{-1}) to the reduction seen after saline treatment. In the female, the decline was less pronounced (27nmol l^{-1}) and occurred by eight hours after injection. [TT4] fluctuated in the male pup for the remainder of the trial and reached preinjection levels by 72 hours after dexamethasone treatment. The female showed less pronounced fluctuations and [TT4] increased steadily to preinjection levels over the final 48 hours of the trial.

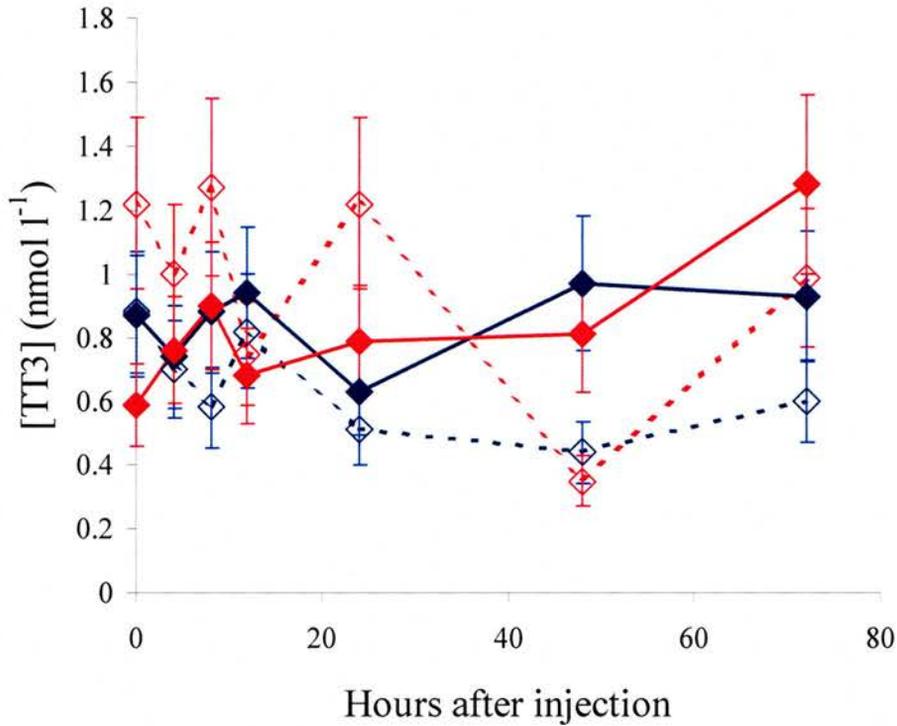
Figure 5.3: Change in [TT4] over 72 hours after saline (open symbols, dashed lines) and dexamethasone (closed symbols, solid lines) injection in two captive animals. Red symbols represent the female, blue symbols represent the male. Error bars represent maximum recorded assay variation of 19.45% (chapter 3).



Changes in TT3 concentration ([TT3]) during the saline and dexamethasone treatment in both animals are shown in Figure 5.4. In the male, [TT3] did not change substantially over time and the changes were similar between trials. Levels were higher at 48 and 72 hours after injection in the dexamethasone trial than in the saline trial.

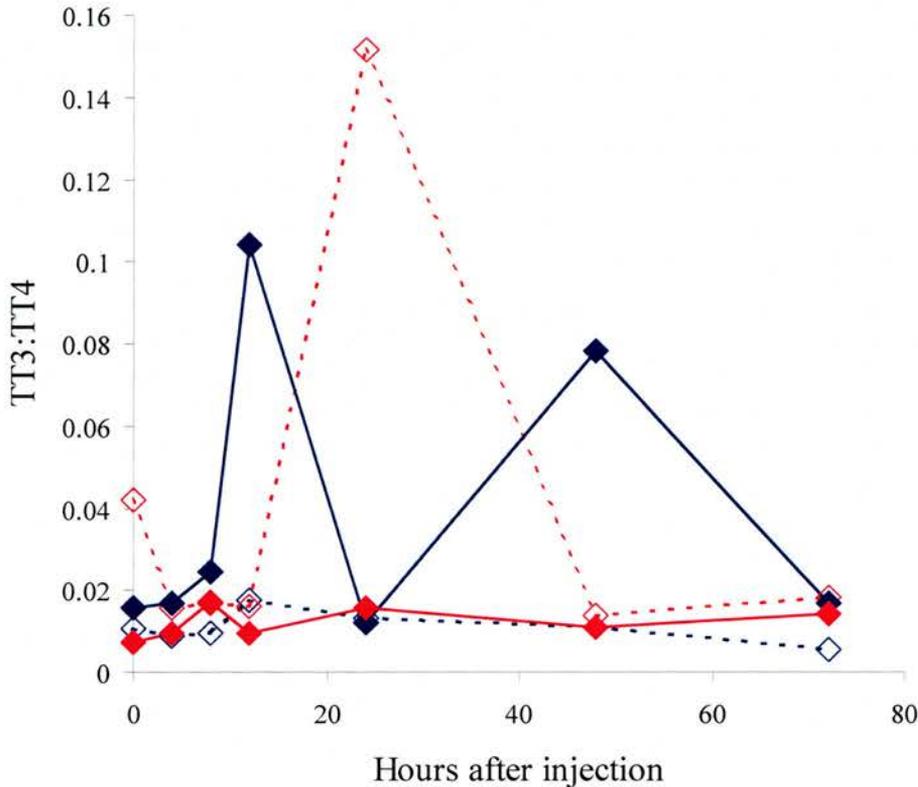
[TT3] fluctuated widely in the female pup after saline injection. After dexamethasone treatment, values increased steadily over the first eight hours, fell slightly by twelve hours after injection then increased progressively to reach levels comparable with those seen at the start of the saline trial.

Figure 5.4: Change in [TT3] over 72 hours after saline (open symbols, dashed lines) and dexamethasone (closed symbols, solid lines) injection in two captive animals. Red symbols represent the female, blue symbols represent the male. Error bars represent maximum recorded assay variation of 21.95% (chapter 3).



Changes in TT3: TT4 during the saline and dexamethasone treatment in both animals are shown in Figure 5.5. The ratio was generally between 0.01 and 0.02 for both animals. It did not change substantially in the male during the saline trial and showed two large increases, at 12 and 48 hours after dexamethasone injection, which corresponded to reductions in [TT4] when [TT3] remained unaltered. The ratio was initially relatively high in the female pup and increased 24 hours after saline injection, when [TT3] was high and [TT4] was low, but did not change during the dexamethasone trial.

Figure 5.5: Change in TT3:TT4 ratio over 72 hours after saline (open symbols, dashed lines) and dexamethasone (closed symbols, solid lines) injection in two captive animals. Red symbols represent the female, blue symbols represent the male.



5.3.2.2. Field-based study

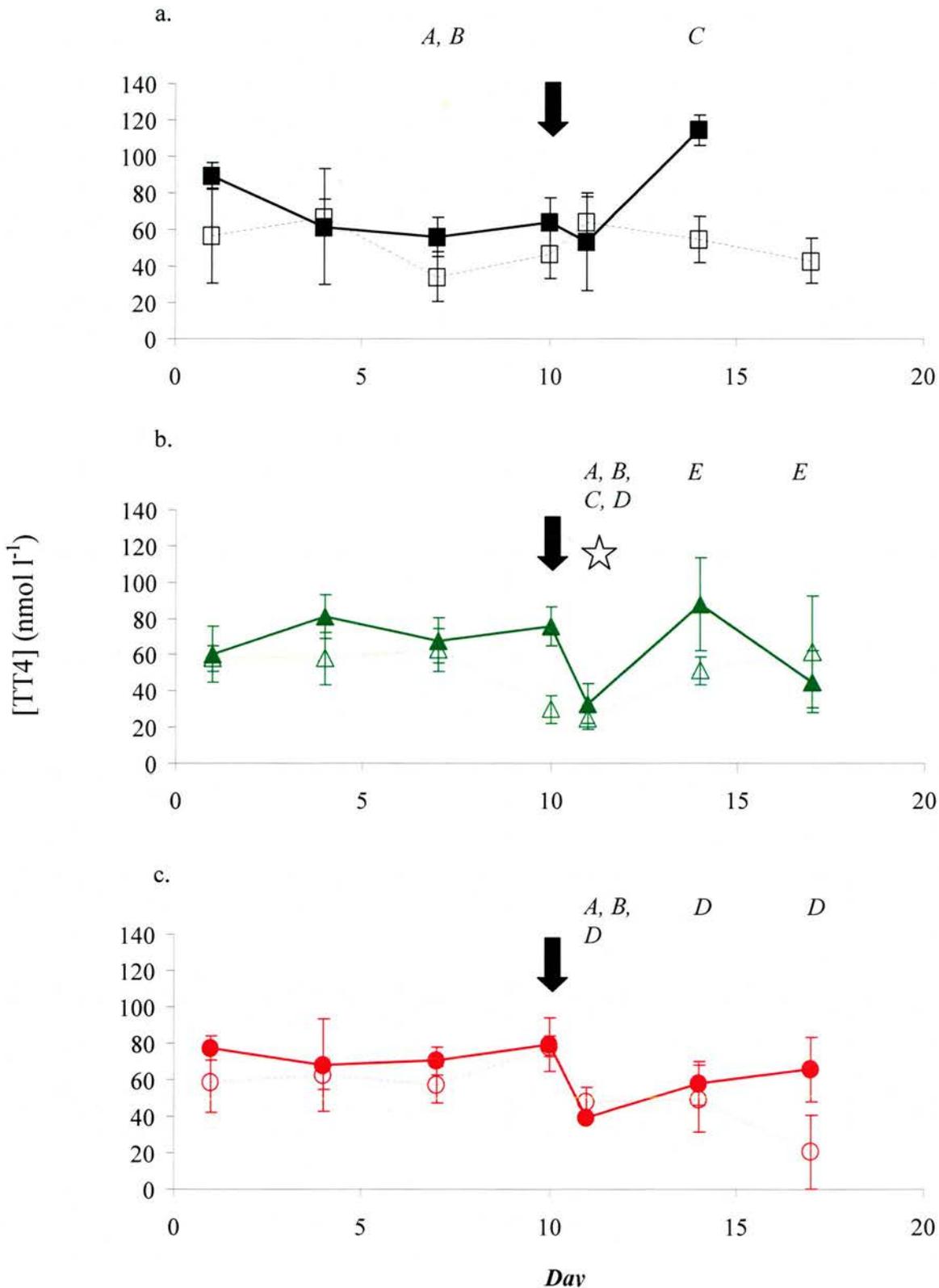
Changes in [TT4] as a function of *day* are shown in Figure 5.6. The LME that best described the changes in [TT4] included fixed terms for sex and an interaction between *day* and group. All other explanatory variables were rejected during the model selection process.

Significant changes occurred in [TT4] with days postweaning in all groups and are summarised below. The pattern of change in [TT4] was different in the CONTROL group compared to that seen in DEX and SALINE animals. CONTROL pups showed a significant reduction in [TT4] from *day* 1 and 4 to *day* 7. In contrast,

[TT4] did not change significantly for the first ten days and declined on *day* 11 (24 hours after treatment) in SALINE and DEX animals. The magnitude of the decline in both groups was similar and the pattern of change was not significantly different between these two treatments. CONTROL pups showed significantly higher [TT4] than SALINE, but not DEX animals, 24 hours after treatment. There were no significant differences in [TT4] between groups on any other day.

The pattern of change in [TT4] after treatment showed some differences between groups. [TT4] in the DEX animals did not change significantly from *day* 11 to 17. The SALINE group showed a significant increase in [TT4] between *day* 11 and 14 before levels stabilised again. In CONTROL pups, [TT4] increased significantly from lowest levels on *day* 7 to *day* 14, but did not change significantly between *day* 10 and 17. Males had significantly lower [TT4] than females ($t = 2.10$, $df = 25$, $p = 0.0459$).

Figure 5.6: Change in mean [TT4] \pm s.e. with *day* in a. CONTROL b. SALINE and c. DEX groups. Closed symbols represent females and open symbols represent males. Arrow indicates timing of treatment. \star denotes a significant difference ($p < 0.05$) from the CONTROL group within *day*. A-E indicate significant differences within groups from *day* 1, 4, 7, 10 and 11, respectively

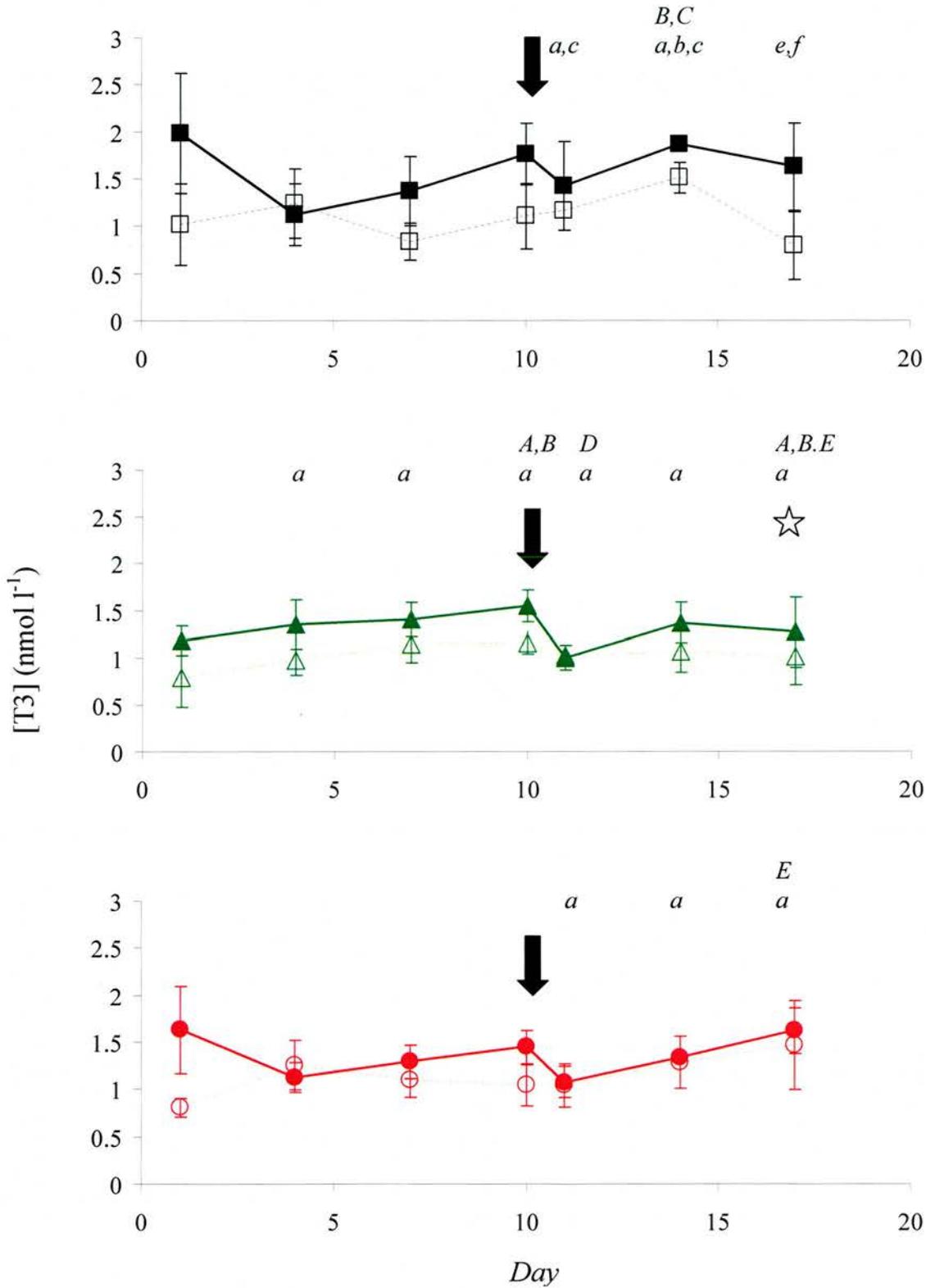


Changes in [TT3] as a function of *day* are shown in Figure 5.7. The LME that best described the changes in [TT3] included fixed terms for *CHT*, and interactions between *day* and group, and *day* and sex. All other explanatory variables were rejected during the model selection process. At the start of the fast [TT3] was similar in all three groups. The only group difference on any *day* was significantly lower levels in CONTROL compared to SALINE animals on *day* 17 ($t = 2.63$, $df = 25$, $p = 0.0145$).

Significant changes occurred in [TT3] with *day* in all groups, and were different between males and females. Most between group differences were between CONTROL and SALINE animals. There were no significant differences in the change in [TT3] with *day* between SALINE and DEX groups. The differences between males and females in the change in [TT3] with *day* arose as a result of lower [TT3] in the males at *day* 1 ($t = 3.50$, $df = 25$, $p = 0.0018$) and *day* 10 ($t = 2.21$, $df = 25$, $p = 0.0362$).

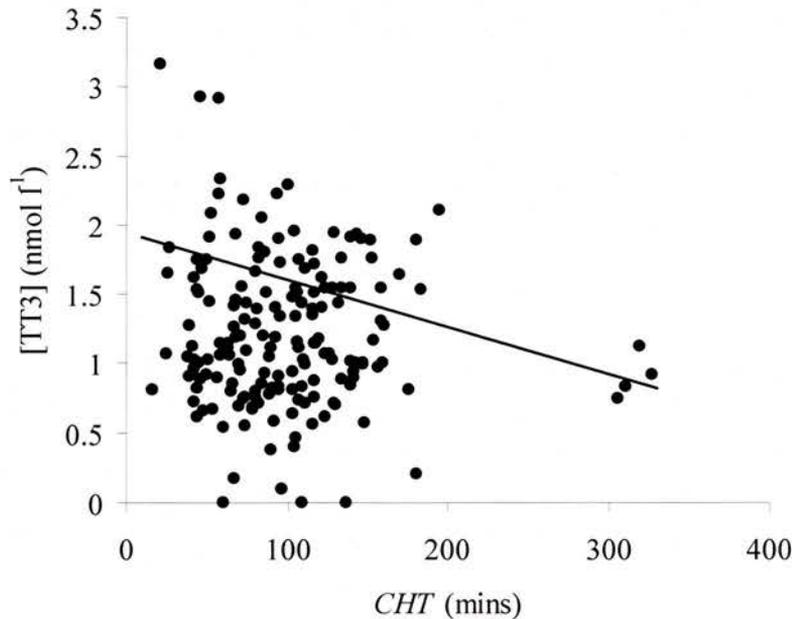
[TT3] was higher on *day* 14 than on *day* 4 and 7 in CONTROL females, but there were no other significant differences between *day* in these animals. SALINE females showed substantial variability in [TT3]. There was an increase from *day* 1 and 4 to higher levels on *day* 10, followed by a significant reduction by *day* 11. Levels then increased again and were significantly higher on *day* 17 than on *day* 1, 4 and 11. Females in the DEX group showed no significant changes in [TT3], except for an increase between *day* 11 and 17. The males in the CONTROL group showed lower [TT3] on *day* 1-10 and 17 than on *day* 11 and 14. In the SALINE and DEX males, [TT3] was relatively stable but was significantly lower on *day* 1 than on all other *days*.

Figure 5.7: Change in mean [TT3] \pm s.e. with *day* in a. CONTROL b. SALINE and c. DEX groups. Closed symbols represent females and open symbols represent males. Arrow indicates timing of treatment. \star denotes a significant difference from CONTROL group within *day*. A-f denote differences within group between days. capital letters are females, small case letters are males.



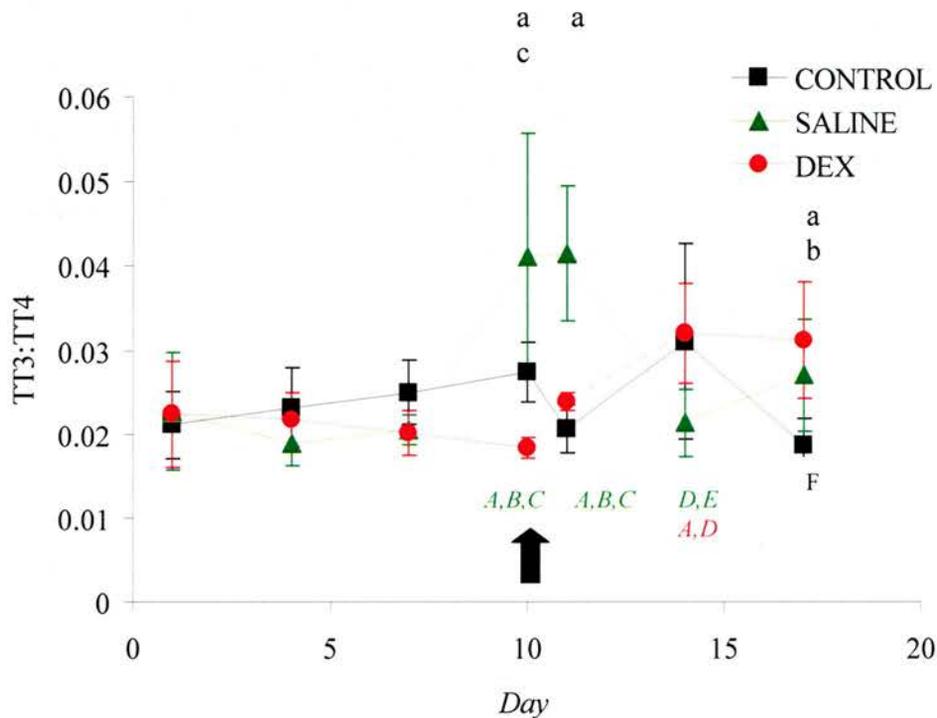
In all groups, [TT3] decreased significantly as a function of *CHT*, as shown in Figure 5.8.

Figure 5.8: Relationship between [TT3] and *CHT*, given the effects of *day* and sex and *day* and group on [TT3]. LME: $[TT3] = -0.003 (CHT) + 1.95$; $T = 2.76$, $df = 116$, $p = 0.0067$).



Changes in TT3: TT4 as a function of *day* are shown in Figure 5.9. The LME that best described the changes in TT3: TT4 included fixed terms for *CHT*, and an interaction between *day* and group. TT3: TT4 showed little change during the fast and was similar in all three groups except on *day* 10 and 11, when an increase occurred in the SALINE group, but not in the other animals. DEX and CONTROL groups had higher TT3: TT4 on *day* 14 than on *day* 1 and 10 (DEX) or *day* 17 (CONTROL). There was a trend towards a negative relationship between TT3: TT4 and *CHT* (LME: $T = 1.96$, $df = 119$, $p = 0.0526$).

Figure 5.9: Change in mean TT3: TT4 \pm s.e. with *day*. Arrow indicates timing of treatment. a, b, and c denote significant differences ($p < 0.05$) within *day* between CONTROL and SALINE, CONTROL and DEX, and SALINE and DEX, respectively. A-F indicate significant differences within groups from *day* 1, 4, 7, 10, 11 and 14, respectively, and are colour coded by group.



5.3.3. WBC response

5.3.3.1. Captive study

Changes in the number of total WBC and individual cell types during the saline and dexamethasone trials are shown in Figure 5.10.

Average WBC number (12.84×10^6 cells ml^{-1}) and composition (63.8% neutrophils, 22.8 % lymphocytes, 9.5 % monocytes and 3.9 % eosinophils) were similar at the start of both treatment periods.

Overall WBC number increased dramatically in both pups after both saline and dexamethasone treatment. During the saline trial, the male pup showed a 21% increase in WBC number from zero hours to highest levels at 48 hours, after an initial drop in WBC number at four hours postinjection. The female showed a 50% increase in WBC number over the same period of time.

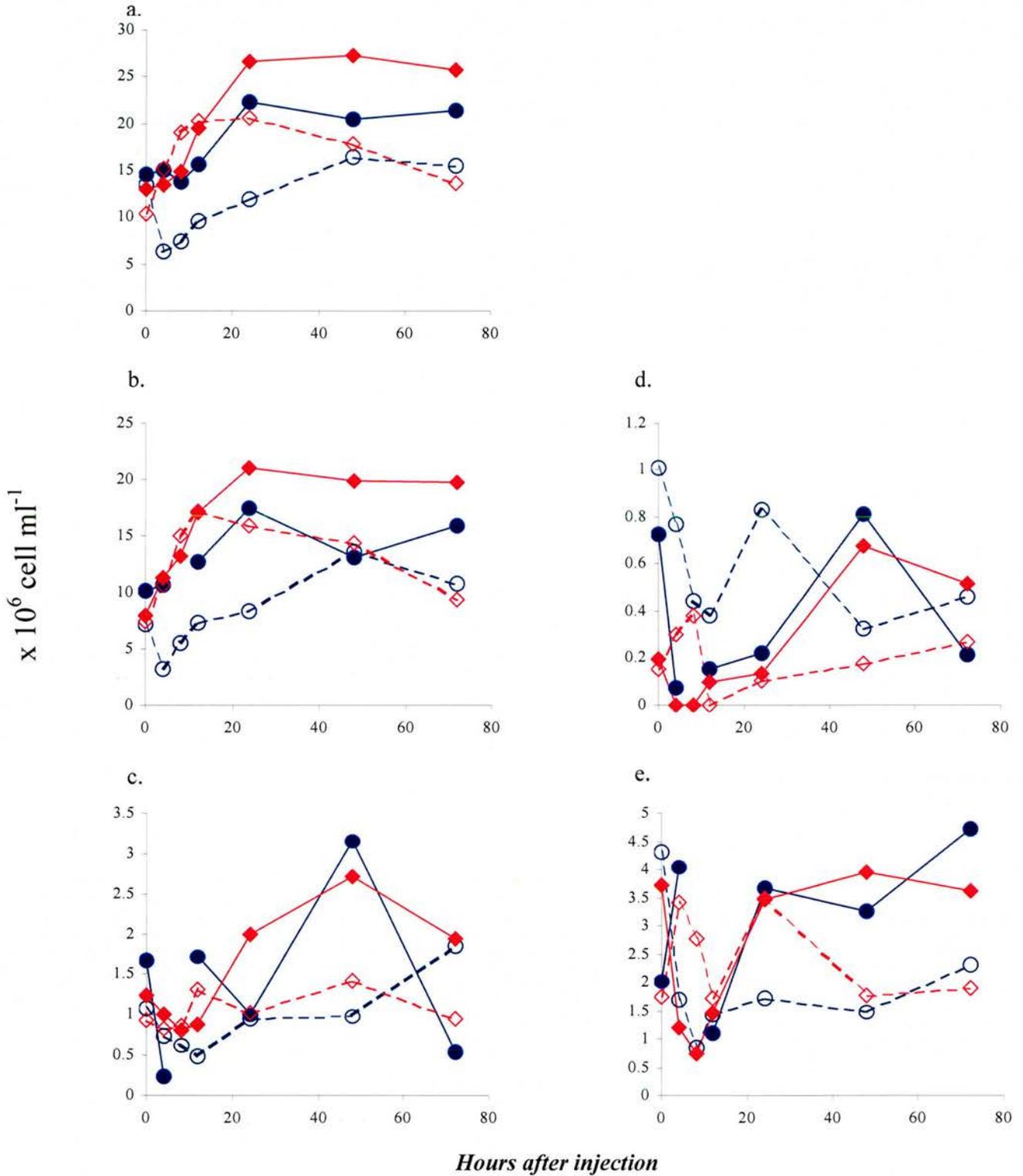
The increase in WBC number was greater after dexamethasone injection in both animals. The male pup exhibited a 53% increase in WBC number, and the female showed a 108% increase from zero hour of the dexamethasone trial to maximum values at 48 hours postinjection.

In each trial, neutrophil number increased dramatically within the first 12 – 24 hours after injection in both animals. Highest neutrophil number in the saline trial (male: 190% initial value; female: 230% initial value) occurred between 12 and 48 hours after injection.

At the start of the dexamethasone trial, neutrophil number was 7% higher in the male and 26% higher in the female than at the start of the saline trial. Peak numbers in the dexamethasone trial (male: 172% initial dexamethasone value and female: 266% initial dexamethasone value) occurred at 24 hours after injection. High neutrophil numbers persisted in both animals at least until the end of the sampling period. In both animals, maximum neutrophil number was higher in absolute terms but similar in relative terms during the dexamethasone compared to the saline trial

Monocyte number did not appear to change substantially during the saline trial in the female pup. The male pup exhibited a 56% decline in monocyte number during the first twelve hours of sampling, followed by a progressive increase to highest levels 72 hours after injection.

Figure 5.10. Change in a. total WBC, b. neutrophils c. monocytes d. eosinophils e. lymphocytes after saline (open symbols, dashed lines) or dexamethasone (closed symbols, solid lines) injection in two captive animals. Red symbols represent the female and blue symbols represent the male.



The pattern of change in monocyte number after dexamethasone treatment was more consistent between the pups. Both animals showed an initial decline in numbers in the first eight hours after injection followed by a large increase to a maximum of double initial levels by 48 hours after treatment.

Eosinophil numbers were high in the male pup compared to numbers seen in the female pup. Eosinophils accounted for up to 12% of the total WBC count in the male pup, compared to a maximum of 2.5% in the female.

There was no consistent pattern in eosinophil number between animals during the saline trial. During the first twelve hours after saline injection the male pup showed a 62% drop in eosinophil number. The female showed an increase in eosinophil number followed by a drop to undetectable levels over the same period. The female pup then showed a progressive increase up to 72 hours after treatment, while numbers in the male pup fluctuated widely.

The two animals showed a similar pattern of change in eosinophil number in response to dexamethasone. Eosinophil numbers were dramatically reduced by four hours after injection to 10% (male) and 0% (female) of values at the start of the dexamethasone trial. Eosinophil number then increased steadily to maximum levels (112% (male) and 347% (female) of values at the start of the dexamethasone trial) at 48 hours after injection, followed by a downturn in both animals.

Lymphocyte number varied widely within both pups at the start of each trial. In each trial both pups exhibited a large reduction in lymphocyte levels between four and twelve hours after injection. The timing of this reduction was not consistent between animals or trials. Lymphocyte number recovered by 24 hours after injection in the female in each trial, and in the dexamethasone trial in the male. Numbers did not recover to initial values in the male in the saline trial. In the saline trial, the female

showed a reduction in lymphocyte number by 48 hours and numbers then remained low. In contrast, lymphocyte number remained high in both animals from 24 hours after dexamethasone injection to the end of sampling.

5.3.3.2. Field-based study

Changes in total and differential WBC numbers within and between groups at *time* 0, 1 and 4 are shown in Figure 5.9.

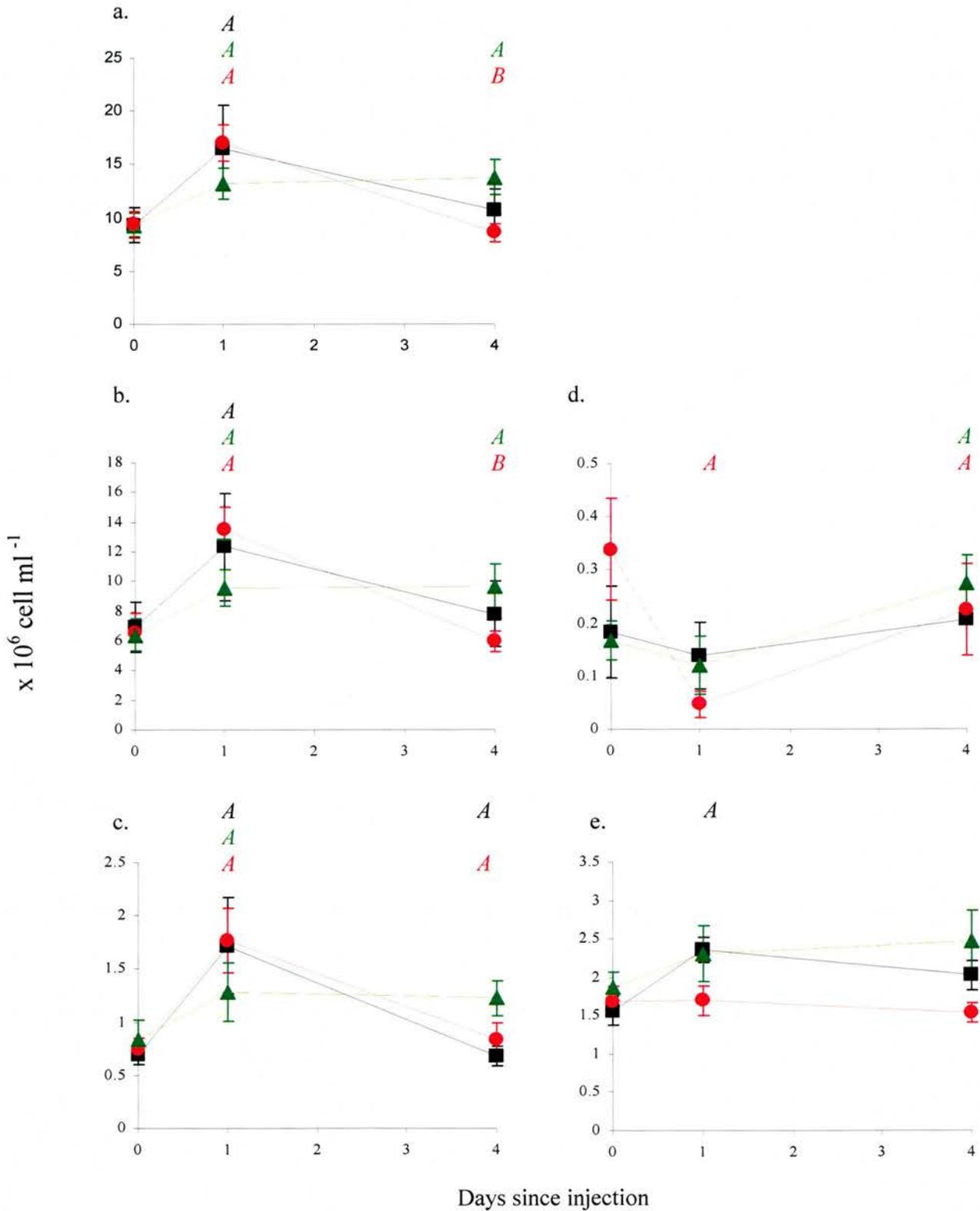
Total WBC, neutrophil and monocyte numbers showed a similar pattern of change. They were not significantly different between the three treatment groups at any *time* ($p > 0.05$). All three groups showed a significant elevation in all these counts from *time* 0 to *time* 1 ($p < 0.05$). In the CONTROL animals, total WBCs and neutrophils began to decline to an intermediate level by *time* 4, but remained elevated above *time* 0 numbers in the SALINE group. DEX animals showed a significant reduction in both counts from *time* 1 to *time* 4, to levels that were not significantly different from those seen at *time* 0. In both the CONTROL and DEX treated animals, monocyte number returned to preinjection levels by *time* 4. Monocyte number remained significantly higher than preinjection levels at *time* 4 in SALINE treated animals. The pattern of change in total WBC, neutrophil and monocyte number from *time* 1 to 4 was significantly different between SALINE and DEX animals.

Eosinophil number was not significantly different between the three treatment groups at *time* 0 or 4, or between the three *times* in the CONTROL group ($p > 0.05$). There was a significant reduction in eosinophil number from *time* 0 to *time* 1 in the DEX group ($t = 3.98$, $df = 39$, $p = 0.0003$) that did not occur in the other two groups. This resulted in a significant difference in the change in eosinophil number between

time 0 and *time 1* between DEX animals and the CONTROL ($t = 2.24$, $df = 39$, $p=0.0309$) and SALINE groups ($t = 2.18$, $df = 39$, $p = 0.0356$). SALINE and DEX pups showed a significant increase in eosinophils from *time 1* to *time 4* ($p < 0.05$).

Lymphocyte number was not significantly different between groups and did not change significantly between *times*, except for an increase in the CONTROL group from *time 0* to *time 1*.

Figure 5.11. Effect of group on a. total WBC, b. neutrophils c. monocytes d. eosinophils e. lymphocytes in wild pups. CONTROL, SALINE and DEX groups are represented by black, green and red symbols, respectively. *A* and *B* indicate a significant difference ($p < 0.05$) within groups from *time 0* and *time 4* respectively and are colour-coded by group



5.4. Discussion

5.4.1. Cortisol response to dexamethasone

There was a rapid, dramatic and sustained reduction in cortisol levels in response to dexamethasone in young grey seals that did not occur in saline treated or control animals. This indicates that the drug interacted with GR to suppress endogenous cortisol secretion by feedback inhibition of the HPA axis, as in other mammals (Froin et al, 1998; Reynolds et al, 1998; Reidarson and McBain, 1999; Barton et al, 2002). Since dexamethasone was able to suppress cortisol secretion through interaction with GR it was assumed that it had reached its GR targets in all parts of the body and that other GR-mediated effects of the drug would therefore also be observed.

The time course and magnitude of the effect of dexamethasone on circulating cortisol levels in both the captive and wild animals in the present study was comparable with that seen in other mammals in response to a dose of a similar size. In bottlenose dolphins, a single oral dose of 0.11 mg kg^{-1} dexamethasone caused complete suppression of circulating ACTH and cortisol within 24 hours, and values returned to normal baseline levels within 48 hours (Reidarson and McBain, 1999). Fifteen to nineteen hours after a $40 \mu\text{g kg}^{-1}$ dose of dexamethasone, healthy horses showed a significant reduction in circulating cortisol to 11% of initial levels (Froin et al, 1998). A 0.25mg dose of dexamethasone reduced cortisol in healthy human subjects to 30% initial levels by 10.5 hours after administration of the drug (Reynolds et al, 1998). In another study on human volunteers, oral administration of a single dose of 0.25, 0.5 or 1mg dexamethasone significantly reduced circulating cortisol

levels by 9-11 hours later in a dose dependent manner (Barton et al, 2002). In the captive pups in this study, $50\mu\text{g kg}^{-1}$ ($\sim 1\text{mg}$ total dose) dexamethasone reduced cortisol levels relative to pre-injection levels by 86% - 90% within four hours of treatment and levels remained suppressed for 48-72 hours. Maximal reduction occurred between eight and 24 hours after injection. This dose had a similar impact on cortisol levels in the wild pups, in which cortisol concentrations were suppressed 24 hours after dexamethasone treatment, and returned to preinjection levels within four days.

In vivo studies have demonstrated that dexamethasone acts on the HPA axis at the level of the pituitary to reduce corticosterone production in the rat (Miller et al, 1992; Cole et al, 2002). A functional analysis demonstrated that dexamethasone blocks the stimulatory action of a dose of CRH on corticosterone production by preventing ACTH stimulation, but does not prevent corticosterone secretion in response to exogenous ACTH (Cole et al, 2002). Although ACTH levels were not assessed in the current study, it is likely that dexamethasone suppressed cortisol levels in grey seal pups through inhibition of ACTH production by the pituitary.

The duration of action (48-72 hours) and the magnitude of the effect of $50\mu\text{g kg}^{-1}$ dexamethasone made it a suitable choice for investigating the effects of sustained high GC levels on fuel use and timing of departure in wild grey seal pups.

The similarity of cortisol levels between all three treatment groups from four to ten days after treatment indicates that the single intramuscular dose of dexamethasone did not have a long-term suppressive effect on the HPA axis. To confirm this assumption experimentally it would have been necessary to perform an ACTH challenge test on these animals. Administration of ACTH should elicit cortisol

production despite the presence of high GC levels, if the adrenal glands are functioning normally.

Dexamethasone caused a massive and persistent drop in cortisol levels because it is a potent, high affinity GR agonist. However, there was also a reduction in cortisol levels after saline treatment in the captive facility experiment. This was most apparent in the female. There was no evidence of a decline in cortisol levels after saline injection in wild pups, perhaps due to less frequent sampling than in the captive study.

Acute stress caused by handling may have contributed to some of the variation seen in cortisol levels in both studies. Stress, including that caused by handling, dramatically increases circulating cortisol concentrations within minutes of the onset of the stimulus (Döhler et al, 1977; Gardiner and Hall, 1997; Engelhard et al, 2002) and as a result, the time taken to obtain each blood sample inevitably introduces variation into cortisol measurements. However, variability due to differences in the time taken to obtain the sample is unlikely to have been the cause of the large observed changes in cortisol because blood samples were obtained rapidly after first physical contact with the animal to minimise the effect of handling on cortisol measurements. Of the 28 samples taken, 25 were obtained within two minutes of the onset of physical restraint, and all samples were obtained within five minutes of handling the animals.

The adrenocortical system is activated in response to psychological stress (Alpers et al, 2003), which may include anticipation of handling. The HPA axis is also stimulated by movement and exercise (Kanaley and Hartman, 2002). The captive animals were allowed to move around freely within the confines of the pool and dry area between sampling, but were isolated in the dry area for twenty minutes before blood samples were taken. They may have been aware that this indicated imminent

restraint and blood sampling. The wild pups were also free to move within a large pen. The presence of researchers in the pen caused alarm in some cases, when the animals were alert. In both studies it was impossible to quantify the degree of anticipation of handling and the amount of movement that had occurred prior to handling. Some of the observed variability in cortisol concentration in both the wild and captive studies may have resulted from differences in activity and anxiety during the course of the experiment, especially during the first twelve hours of each captive trial when handling and movement were more frequent. Exercise and psychological stress cannot be responsible for the reduction in cortisol levels seen in the female over the course of the first twelve hours of the captive saline trial.

The pattern of change in cortisol levels after saline injection in the captive pups mirrored that seen after dexamethasone treatment, but was shorter in duration and less dramatic. This may be the standard response to a GC pulse in grey seals. An elevation in cortisol levels is expected within minutes of exposure to a stressor, to peak within two hours and then return to baseline within five hours, in the absence of repeated stimulation (St. Aubin and Dierauf, 2001). However, the consequences of repeated handling during that time-frame are unclear. It is possible that the early changes in cortisol levels were a consequence of a modest downregulation of the adrenocortical system caused by frequent exposure to high endogenous cortisol levels during repeated handling. The surge in endogenous cortisol that likely occurred in response to handling stress during the saline treatment may have been followed by an overshoot, whereby cortisol declined to levels lower than preinjection values by four hours after handling. Repeated handling every four hours may have caused persistently lower cortisol measurements at each sampling time over the first twelve hours of the saline trial in the female. The twelve-hour interval between the 12 and

24-hour samples is likely to have provided sufficient time for cortisol to return to pre-handling levels. Cortisol has a lower affinity for GR and a much shorter half-life in the blood than dexamethasone (Bishop, 2000), which likely explains the less pronounced response to the endogenous hormone.

In the captive study, cortisol levels may have changed in response to food intake. Pups were fed a few fish each time they were moved to the dry area and then allowed to relax for twenty minutes to dissociate training from the handling experience. Several of the blood samples taken during the first day of each trial contained chylomicrons, indicative of ongoing digestion of recent meals, which may have triggered changes in cortisol levels. Cortisol levels increase in rats (Tannenbaum et al, 1997), dogs (Sander and Thomas, 1988) and humans (Ishizuka et al, 1983) within 30 minutes of food intake and levels remain elevated above baseline for over four hours (Kasckow et al, 2001). The acute cortisol response to food intake in seals has not been documented. An increase in cortisol after food intake may explain the higher levels of cortisol observed in the captive seals here, blood sampled twenty minutes after feeding, compared with both the wild pups in this study and fasting captive grey seal pups elsewhere (Nordoy et al, 1990). However, because food intake drives an increase in cortisol, it cannot be responsible for the initial reduction in cortisol levels seen within the first twelve hours of the saline trial.

Increases in cortisol as a result of food intake can be obscured by diurnal changes in cortisol levels that occur irrespective of meal timing (Wang et al, 1999b). Captive harbour seals have a diurnal rhythm in cortisol concentration (Gardiner and Hall, 1997). Levels are high during the hours of darkness and the morning, begin to decline around 13:00 and reach their lowest levels in the late afternoon. If grey seals show a similar change it is possible that the decline in cortisol over the first twelve

hours post-injection was due to a fall in cortisol secretion from high levels in the morning, when the first sample was taken, to low levels in the afternoon and evening. No effect of time of day on cortisol was apparent in the wild pups because sampling was restricted to a four-hour period in the morning.

As discussed in chapter 4, higher levels of cortisol at the onset of fasting may be related to the moult of the lanugo, or to the time elapsed since the animal last fed. Some of the observed differences in the change in cortisol between animals and groups could have resulted from individual variability in the timing of weaning, moulting and other developmental events. Inaccuracies in assigning weaning date may also have led to slight time-shifts in the observed patterns of change.

Cortisol levels and the pattern of change with days postweaning reported here for wild fasting grey seal pups compare well with those seen in captive fasting harp and grey seal pups (Nordoy et al, 1990; Nordoy et al, 1993). They are also consistent with findings from the wild, fasting study pups from 2001, in which cortisol levels tended to be higher early in the fast and declined 4/10 through the fast (chapter 4). In the current study, all groups showed a gradual decline in cortisol by day seven to ten postweaning from initial levels at the start of fasting. This change was significant in the SALINE animals, but not in the other two groups, which may be a consequence of the small sample size combined with high inter-individual variability in cortisol levels. The pattern of change was, again, markedly different from that seen in wild, fasting northern elephant seal pups (Ortiz et al, 2001a and b), which may be due to methodological differences between the two studies, or species differences in fuel use or cues to leave the breeding colony. The role of GCs in fasting energy expenditure and departure from the colony is explored further in chapter 6.

5.4.2. TH response to dexamethasone

Dexamethasone treatment did not drive a change in [TT4] or [TT3]. A fall in [TT4] occurred after both saline and dexamethasone treatment in the captive male seal and in the wild pups. No change occurred after either treatment in [TT4] in the captive female or in [TT3] in either of the captive pups. Erratic changes in TT3: TT4 were observed in both pups and were not associated with the type of treatment the animals had received. Post treatment changes in [TT3] and TT3: TT4 were not consistent between groups in the wild study.

In rats and humans, pharmacological doses of dexamethasone reduce [T3] and elevate levels of reverse T3 (rT3), the inactive form of T3, within 24 hours of administration for up to 48-72 hours after the withdrawal of treatment (Chopra et al, 1975; Burr et al, 1976). The GC-mediated reduction in T3 occurs both as a result of suppression of TH secretion at the level of the hypothalamus by reducing TRH and peripherally by inhibiting monodeiodination of T4 to T3 (Burr et al, 1976).

Dexamethasone did not alter TH levels and therefore did not suppress TH secretion or alter monodeiodination in grey seal pups. It is possible that this is a consequence of the comparatively low dose used in this study. Dexamethasone is largely excluded from the brain by the multidrug resistance 1a P-glycoprotein (De Kloet et al, 1998; Sapolsky et al, 2000). At high doses (2-12mg total dose) dexamethasone seems to saturate this mechanism and gain access to the central nervous system where it can act on TRH secretion (Burr et al, 1976). The lower dose of $50\mu\text{g kg}^{-1}$ (total dose $\sim 1\text{mg}$) used here may have been unable to overcome the blood brain barrier to alter TRH release in the hypothalamus (Cole et al, 2000). It also seems to have been low enough to avoid a peripheral effect on the monodeiodination

of T4 to T3. This indicates that any physiological effects of the dose of dexamethasone used here were not mediated through changes in TH levels.

It is unlikely that the fall in [TT4] after saline and dexamethasone treatment and resultant changes in TT3:TT4 in both the wild and captive studies were driven by altered GC levels, either as a result of dexamethasone or stress. In the wild study, CONTROL animals showed a different pattern of change in [TH] than the other two groups, and the change in TT3: TT4 was altered only in SALINE animals, despite an almost identical handling regime. Dexamethasone does not consistently affect [TT4] concentrations in either rats or humans (Chopra et al, 1975; Burr et al, 1976). After eight hours of immobilisation stress, rats show no change in [TT4], but a dramatic fall in [TT3] (Bianco et al, 1987). Captive beluga whales show a decline in [TT3] but not in [TT4] in response to exogenous ACTH (St. Aubin and Geraci, 1986). A reduction in [TT3] rather than [TT4] would therefore be more likely in response to acute stress, but was not observed in this study.

The changes in TH in the first twelve hours of the captive study may have resulted from a circadian rhythm. TH show diurnal rhythms in most mammalian species (Jordan et al, 1980; Lucke et al, 1977) and tend to be higher in the day than at night, peaking around midday. No obvious diurnal rhythm in TH was found in harp, hooded (*Cystophora cristata*) and grey seals 0-14 days old, despite a daily rhythm in melatonin, the hormone responsible for the entrainment of physiology and behaviour to the light-dark cycle (Stokkan et al, 1995). This may have been a consequence of the lag in the development of daily hormone cycles commonly observed in young animals. The postweaned pups in the current study may also have been too young to show appreciable diurnal changes in TH. Again, any fluctuations in TH caused by time of day were minimised by the sampling regime in the wild study.

In the wild study, there was a large reduction in [TT4] in the absence of a notable change in [TT3] in all animals. This occurred at eleven days postweaning in DEX and SALINE animals and earlier in CONTROL pups. This is consistent with the change in TH observed in the fasting pups from the previous study (chapter 4), in which [TT3] did not change but [TT4] was lower from 13 days postweaning onwards. In the SALINE pups the reduction in [TT4] was large enough to cause an increase in the TT3: TT4 ratio. This suggests that TH secretion fell, whereas T3 production from the circulating T4 pool remained unchanged. The significance of these changes are unclear, but the reproducibility of this pattern of change between years, despite slight shifts in the timing of the reduction in [TT4], indicates that it is likely to be physiologically important during the postweaning fast. As discussed in chapter 4, TH changes may reflect the balance required in these actively growing, fasting animals, between the regulation of developmental events, such as moulting, and the control of energy expenditure and fuel allocation. The variation within and between groups in the timing of TH changes in both the captive and wild studies may reflect variability in the timing of developmental events in different animals.

Males had lower [TT4] than females and generally lower [TT3]. This is also consistent with the findings from the previous study (chapter 4). The changes in [TT3] with days postweaning were different between males and females, possibly due to differences in the prioritisation of development of particular tissue types and allocation of fat and protein reserves for metabolism and growth. The CONTROL group contained three females and six males, compared to an even sex ratio in the other groups and this difference in sex structure may have caused the observed disparity difference in the timing of changes in TH between groups.

5.4.3. WBC response to dexamethasone

Total WBC counts, neutrophil and eosinophil numbers at the start of the captive saline and dexamethasone trials were comparable with levels reported by Hall (1998) for wild yearling grey seals. In the wild study there were no differences between groups in total WBC counts or any of the individual cell types at the pre-injection sample. These values were similar to levels found previously in other wild, postweaned grey seal pups. During both captive trials and in some cases in the wild pups, total WBC, neutrophil and monocyte numbers reached levels higher than those generally seen in postweaned and yearling grey seals. In both experiments, eosinophil and lymphocyte numbers fell within the range reported for grey seals of similar ages (Hall, 1998).

WBC numbers increased dramatically during both trials of the captive experiment and reached a maximum of 27.17×10^6 cells ml^{-1} . A large increase in WBC number was also observed 24 hours after treatment in the wild study pups. These increases were largely driven by elevated neutrophil number, and, to a lesser extent, by changes in monocyte number.

A dramatic increase in neutrophil number is a typical mammalian response to adrenaline and GCs, either released during stress (Kerr, 1989; St Aubin and Geraci, 1988b) or administered experimentally or therapeutically (Kerr, 1989; Reidarson and McBain, 1999). Neutrophilia was observed after a dexamethasone injection in bottlenose dolphins, however, that study did not account for the stress of handling (Reidarson and McBain, 1999). Belugas show a dramatic increase in neutrophil numbers in response to capture and three days to nine weeks in captivity (St. Aubin and Geraci, 1988b). In the current study, handling and blood sampling alone was

sufficient to cause pronounced neutrophilia, and dexamethasone treatment did not cause a further elevation in neutrophil numbers.

Large changes in monocyte number occurred under all experimental conditions. In the captive pups, a decline in monocytes occurred during the first twelve hours of both trials, perhaps reflecting diurnal rhythmicity in the number of this cell type. An extremely large increase in numbers occurred in both animals by 48 hours after dexamethasone treatment, which did not occur after saline treatment. This was not due to an effect of dexamethasone treatment because monocyte number increased in the wild pups in all three groups by 24 hours after treatment.

The elevated WBC count due to neutrophilia and an increase in monocyte number can largely be attributed to an acute inflammatory response to both to venipuncture and the stress of handling. The handling and blood sampling procedure used in this study likely caused acute stress, even in animals used to human contact, and hence exposure to high levels of adrenaline and GCs. The action of these hormones on vasculature results in the flushing of neutrophils out of inactive capillary beds into the circulation (Kerr, 1989). Neutrophilia in response to steroids is also a consequence of reduced migration out of the blood vessels and increased mobilisation from the site of production in the bone marrow (Kerr, 1989). Neutrophils are phagocytic cells that migrate towards sites of tissue damage in response to proinflammatory mediators, such as cytokines (Kerr, 1989). Monocytes are the precursors of tissue macrophages and monocytosis can be caused by chronic inflammation. It is possible that the increases in neutrophil and monocyte number seen here in both the captive and wild study pups occurred as a result of localised inflammation at the site of venipuncture. In both experiments, repeated sampling from the same area of the epidural vein may have damaged the blood vessel wall and

resulted in local inflammation. This situation may have been more pronounced in the captive pups, which were subjected to a much higher sampling frequency.

In the captive study, the absolute magnitude of the increase in WBCs was greater after the dexamethasone injection than after the saline injection in both animals, but the percentage increase from levels at the start of each trial was similar. Since the two trials ran consecutively, the higher levels in the dexamethasone trial were likely due to an already heightened stress-mediated inflammatory response.

There was a significant difference between the SALINE and DEX groups in the recovery of total WBC, neutrophil and monocyte numbers after treatment. DEX animals showed a more rapid return to preinjection neutrophil number than the other groups. Neutrophil and monocyte number remained elevated in the SALINE group. This difference may be a result of the potent anti-inflammatory properties of the artificial GC. Dexamethasone may have counteracted the inflammatory effects of handling and venipuncture more rapidly and effectively than the body's own natural mechanisms in the other two groups.

Dexamethasone treatment caused a dramatic, but short-lived decline in eosinophil number. The response was less clear in the captive seals than in the wild pups because numbers varied considerably within and between the two animals. A marked reduction in eosinophils is a typical mammalian response to GCs (Kerr, 1989). Dolphins showed a pronounced, but short-lived eosinopenia in response to a single dose of dexamethasone that persisted for 48 hours (Reidarson and McBain, 1999). GCs neutralise histamine and prevent mast cells from degranulating, which reduces movement of eosinophils into the circulation (Kerr, 1989).

Lymphocyte number changed erratically in the captive trials and remained largely unchanged in the wild pups, except for an increase in the CONTROL group

over the first 24 hours after treatment. High GC levels, during chronic stress, Cushing's disease and steroid therapy, commonly cause lymphopenia.

Dexamethasone inhibits the proliferation of cultured lymphocytes from brown bears (*Ursus arctos*; Musiani et al, 1998), mice and humans, and causes lymphopenia within 24 hours of administration in dolphins (Reidarson and McBain, 1999). A consistent reduction in lymphocyte number in response to either dexamethasone, or acute stress was not seen in this study.

High numbers of lymphocytes are sometimes seen in the circulation after acute stress as a result of flushing of capillary beds. The changes in lymphocyte number in captive grey seal pups and the wild CONTROL animals may have occurred as a result of changes in tissue perfusion. High numbers during the captive study could have been indicative of a latent infection, since lymphocytes are sometimes elevated during viral infections (Kerr, 1989).

The dose of dexamethasone may not have been large enough or administered for long enough to elicit the large and persistent changes in neutrophil and lymphocyte number commonly observed in response to GC therapy in other animals (Kerr, 1989). The animals showed no sign of ill health during the experiment and we were confident that dexamethasone treatment did not render animals more susceptible to infections due to compromised immune function during the course of the study. Frequent blood sampling did have a significant impact on WBC numbers and caused an acute elevation in neutrophils and monocytes in the circulation. In order to differentiate the effects of dexamethasone treatment from repeated blood sampling, the captive experiment would have benefited from either a design in which both animals received the treatment and control in random order, or received the control then treatment then control to account for carry-over. An experiment with substantial

recovery period between trials would also have been advisable. This was prevented by time constraints and the availability of only two animals during this preliminary experiment. Future blood sampling protocols that involve repeated venipuncture should allow at least 24 hours recovery between samples to minimise the impact of stress and inflammation.

5.4.4. Summary

The dose of dexamethasone used here had an appropriate duration of action and magnitude of effect on cortisol levels to mimic a fast-induced rise in cortisol, and caused reversible and short-lived changes in HPA axis and immune function. Dexamethasone caused a dramatic reduction in cortisol that indicated a high perceived GC dose, which was sustained for up to 72 hours. This suggests that GR were activated by the dose of dexamethasone used and that it was reasonable to expect that any GR-mediated effects of dexamethasone on physiology and behaviour would be observed. Dexamethasone did not cause changes in TH, thus physiological and behavioural effects of the drug were not mediated through TH. This dose was therefore deemed suitable to investigate the effect of GCs on timing of departure from the breeding colony and fuel use during the postweaning fast of wild pups (chapter 6).

Chapter 6

**The effects of handling regime,
dexamethasone treatment and
energy reserves on fasting fuel
utilisation and departure from the
colony in grey seal pups**

6.1. Introduction

6.1.1. Summary

It is crucial for the survival of seal pups that they leave the breeding colony with adequate fuel to sustain them until they can forage effectively. Although this partly depends on the amount of fuel provided by the mother during suckling, it also requires careful management of these endogenous energy reserves after weaning. The amount of time that pups can spend fasting is dictated by the size and rate of utilisation of their fat and protein stores. Pups face a trade off between the need to grow and develop whilst fasting on land and the need to learn to forage successfully at sea before energy stores become critically depleted. Fuel allocation during fasting is likely to have a large impact on the length of time an animal can survive before it finds food. However, the mechanism that regulates both fasting fuel use and timing of departure from the colony is unknown.

This study used the direct interventions of supplementary feeding and dexamethasone treatment to investigate whether increased fuel availability and elevated glucocorticoid (GC) levels, as well as sex and the size of fuel reserves at weaning, influence mass and body composition changes during fasting, and the timing of departure from the colony in grey seal pups. The current study is also the first to examine the impact of handling regime on fuel use in grey seal pups. Predictions were made about the length of time that pups can survive at sea before they find food, based on fasting rates of fuel use and assumptions about the degree to which fat and protein reserves can be depleted before the animals starve to death.

6.1.2. Effect of fuel reserves on energy use and fast duration

Previous studies on the general pattern of mass loss and fuel use during fasting in grey seal pups have shown that more than 90% of energetic costs are met by fat catabolism (Nordoy and Blix, 1985; Worthy and Lavigne, 1987; Nordoy et al, 1990; Reilly, 1991). However, differences in size and body composition lead to substantial variability in the contribution of protein and lipid to overall energetic needs in rats, humans and polar bears (Cherel et al, 1992; Atkinson et al, 1996; Dulloo and Jacquet, 1999), as well as in other pinniped species (Carlini et al, 2001; Beauplet et al, 2003; Biuw, 2003; Noren et al, 2003; Noren and Mangel, 2004). This interindividual variability in the rate of fat and protein use can have a substantial impact on the ability to fast for extended periods (Cherel et al, 1992; Dulloo and Jacquet, 1999) and may influence how long seal pups are able to survive at sea before they must begin to forage (Biuw, 2003).

Postweaning fast duration is largely dictated by the size of initial energy reserves in southern elephant seals (Carlini et al, 2001). Larger, fatter pups tend to undergo a longer postweaning fast (Arnbom et al, 1993; Carlini et al, 2001; Biuw, 2003; Noren and Mangel, 2004). There is also a degree of consistency in mass at departure relative to mass at weaning in these animals (Arnbom et al, 1993; Carlini et al, 2001). In wild grey seal pups, the duration of the fast is positively correlated with percentage fat at weaning (Noren et al, 2003b), or negatively related to daily energy expenditure, (Reilly, 1991). Together this suggests that information about the size of fuel reserves is a component in the signal that not only dictates fuel allocation but also causes these pups to go to sea.

The impact of initial fuel reserves on the variation in energy expenditure and fuel allocation in grey seal pups warrants investigation, given the impact of condition on survival of these animals (Hall et al, 2001; Hall et al, 2002). Feeding experiments involving captive seals have explored a variety of questions related to the heat increment of feeding (Markussen et al, 1994), the gain in body mass, fat and protein after fasting (Worthy and Lavigne, 1983; Condit and Ortiz, 1987) and the impact of pollutants (Jenssen et al, 1995) on hormone and vitamin dynamics. The impact of endogenous energy reserves on fuel use has been explored in a number of otariid species and southern elephant seals (Arnould et al, 1996; Arnould et al, 2001; Donohue et al, 2002; Beauplet et al, 2003; Biuw, 2003). This is the first time supplementary food has been used to investigate directly the impact of energy availability on fuel allocation in wild seals.

6.1.3. Effect of sex on fasting energy use

Fuel allocation strategy in fasting otariids and southern elephant seal pups is dependent on sex. In Antarctic, Subantarctic and Northern fur seal pups, males are heavier than females and lay down more lean tissue, but have proportionally smaller fat reserves (Arnould et al, 1996; Arnould et al, 2001; Donohue et al, 2002; Beauplet et al 2003). Female otariid pups have higher mass-specific mass loss rates (Guinet et al, 1999), energy expenditure (Ono and Boness, 1996; Arnould et al, 2001) and a greater reliance on protein catabolism (Beauplet et al, 2003) than males during fasting.

Phocid seals are sexually dimorphic, therefore males and females are likely to vary substantially in their developmental and energetic requirements. They may adjust

their fuel allocation strategy during the postweaning fast to reflect these needs, in a similar way to otariid pups (Arnould et al, 1996; Guinet et al, 1999; Arnould et al, 2001; Donohue et al, 2002; Beauplet et al, 2003). However, previous work on both wild (Reilly, 1991) and captive (Nordoy and Blix, 1985; Nordoy et al, 1990) grey seal pups and other phocid species (Carlini et al, 2001; Noren et al, 2003a) showed no differences between males and females in the rate at which different tissue components are utilised during the postweaning fast. One study on southern elephant seal pups that included a wide range of weaning masses, the fuel utilisation strategy employed by lean pups depended on the sex of the animal (Biuw 2003). Since male and female grey seal pups show a large difference in survivorship (Hall et al, 2001), the possibility of sex differences in fuel allocation strategy in grey seal pups was explored further.

6.1.4. Effect of handling on energy use

Repeated restraint stress in rats causes a profound and sustained alteration in metabolism that persists for several days after the cause of the stress has been removed (Zhou et al, 1999). Fatty acid oxidation is increased and glucose uptake is reduced in adipocytes, resulting in an overall increase in the rate that fat tissue is utilised (Zhou et al, 1999). The mechanism that drives this change is unknown but may involve stress-induced elevation of GC levels.

The potential effect of restraint stress on fuel use patterns in seals has not been addressed. Energy expenditure is likely to increase during handling as a result of stress and higher activity levels if the animal struggles. Pups handled frequently may therefore experience greater tissue loss than those handled less often. It is also

possible that repeated handling stress causes long-term alterations to fuel utilisation in seal pups. This possibility necessitates an investigation into the effects of handling frequency on changes in mass loss and body composition during fasting.

6.1.5. Effect of GCs on energy use and fast duration

Effective fuel management and appropriate timing of departure require a mechanism whereby information about the state of fuel depots is relayed to the central nervous system (CNS) and periphery to effect appropriate changes in energy use and behaviour patterns. The co-ordination of an integrated behavioural and physiological response to changes in fuel availability is achieved in other mammals through the action of hormonal intermediaries, including GCs. These hormones are responsive to changes in fuel supply and metabolism and effect changes in energy acquisition and utilisation, thereby regulating long term energy balance.

GCs increase in fasting animals (Cherel et al, 1992; Cherel et al, 1988a,b and c; Robin et al, 1998; Friedl et al, 2000) and promote foraging behaviour in all mammals and birds studied so far (eg. Santana et al, 1995; Tataranni et al, 1996; Heath, 1997; Belthoff and Dufty, 1998; Robin et al, 1998; Tsipoura et al, 1999; Groscolas and Robin, 2001; Kern et al, 2001; Koch et al, 2002) through their actions on appetite centres in the brain (Debons et al, 1986; Chen and Romsos, 1996). They also elevate lipolysis and proteolysis, and their net effect on fuel use and body composition is dependent on the action of other hormones and metabolic cues (Divertie et al, 1991; Dallman et al, 1993; Challet et al, 1995; Santana et al, 1995; Strack et al, 1995; Weiler et al, 1997; Mantha and Deshaies, 2000; Ottoson et al, 2000; Djurhuus et al, 2002; Djurhuus et al, 2004).

The role of GCs in fuel use and feeding is poorly understood in seals. If cortisol, the major GC in pinnipeds, acts in a similar way in grey seals as it does in other mammals, it may be involved in the control of both fuel use during fasting and timing of departure from the colony. Cortisol has been implicated in both these roles in fasting northern elephant seal pups and adult female Subantarctic fur seals (Ortiz et al, 2001a and b; Guinet et al, 2004). In grey seal pups, cortisol levels are generally higher in the early stage of the postweaning fast (chapters 4 and 5) and are responsive to altered energy availability in grey seal pups (chapter 4), which suggests they may act as a signal of changing fuel supply. However, reported changes in cortisol throughout the postweaning fast are different between species (Nordoy et al, 1990; Nordoy et al, 1993; Ortiz et al, 2001a and b; Guinet et al, 2004, chapters 4 and 5). Clearly, the role of GCs in fuel reserve management and foraging behaviour in seals requires direct investigation. In this study, cortisol levels were manipulated using dexamethasone, a potent and long-acting artificial cortisol analogue, to establish directly whether a persistent elevation in GC levels can drive a change in fuel use or prompt departure from the colony. This is the first use of an artificial GC to investigate relatively long-term (several days) hormonal influences on fuel use and behaviour in wild seals.

6.1.6. Energy reserves, fuel allocation and survival at sea

Grey seal pups, like many other phocids, must learn to forage independently without any parental guidance after they have left the natal colony. Their endogenous fuel reserves must sustain them until they have both encountered prey and learnt to feed effectively. These animals have no prior experience of locating food resources,

which may be distant from the natal colony and unpredictable in time, space, quality and quantity. The amount of time that grey seal pups have to find food after departure from the colony and before the onset of terminal starvation is unknown. This depends on how rapidly body reserves are utilised and the amount of fat and protein depletion that can be tolerated.

The lower critical threshold of fat content at which phase III of fasting begins is 4-6% fat in humans (Friedl et al, 1994), 18-25% in rats (Belkhou et al, 1991; Cherel et al, 1992) and 5-20% fat in penguins (Robin et al, 1988; Robin et al, 1998; Groscolas and Robin, 2001). At this point the relative contribution of protein utilisation to energy expenditure is increased and residual fat depots are spared. Seals may be expected to be vulnerable to depletion of fat reserves because they rely on their subcutaneous blubber layer for insulation against cold water temperatures as well as fuel for metabolism. However the estimated number of days to starvation as a result of lipid depletion in southern elephant seal pups is insensitive to changes in the lower critical threshold of relative fat content for values between 10 and 25% (Biuw, 2003).

Animals can starve to death when they have substantial fat stores if body protein reaches critically low levels (Cherel et al, 1992) at which cardiac muscle function and the structural integrity of tissues is compromised. Reduction of body protein stores by 30-50% is incompatible with survival in humans (Garrow et al, 1965), and dogs fed a calorifically adequate but protein-free diet starve to death when they have lost two thirds of their initial protein reserves (Garrow, 1959). In captive fasting grey seals pups, these critical levels of protein depletion are not expected to be reached until ~60 days of fasting (Nordoy and Blix, 1985). Based on rates of change in fat and protein use during the postweaning fast, southern elephant seal pups, are

predicted to starve to death from protein depletion before fat reaches critical levels, irrespective of whether animals were large or small at departure (McConnell et al, 2002; Biuw, 2003). The effect of protein depletion on the amount of time wild grey seals pups have to find food after departure from the colony has not been explored.

6.1.7. Experimental aims

The aim of this study was to explore the mechanism that regulates fuel allocation during fasting. To this end, the impact of direct interventions of handling regime, supplementary feeding and dexamethasone, as well as natural differences in initial energy reserves and sex, on mass loss, fuel allocation and fast duration in wild, fasting grey seal pups were examined. The study also investigated the effect of fasting fuel allocation strategy on the length of time animals are expected to survive at sea, based on various assumptions about critical levels of fat and protein depletion.

6.2. Materials and Methods

6.2.1. Study animals and treatment groups

The 58 grey seal pups used in this study (Table 2.1) were born on the Isle of May in 2001 and 2002. Birth, weaning and departure dates were determined as described in chapter 2. Departure date was known for 24 of the 28 study animals in 2001 and all study pups from 2002.

Pups were penned as soon as possible after weaning and assigned to the treatment groups described in chapters 4 and 5, such that each group contained a range of animals of different sizes and an even sex ratio, where possible.

The effects of handling regime and supplementary feeding on fuel use and departure were investigated in 2001. Treatment groups were FED, HIGH, LOW and UNKNOWN, the first three of which had experienced handling prior to weaning. FED animals received supplementary food for five days after weaning, HIGH and LOW refer to handling frequencies of every three days and three times (*timepoints*) during the postweaning fast, respectively. UNKNOWN animals were handled with the same frequency as the LOW group but had not been handled prior to weaning.

The impact of dexamethasone was investigated in 2002. The treatment groups were CONTROL, SALINE and DEX, which referred to no injection, a saline or dexamethasone injection at ten days after weaning, respectively. The general handling and blood sampling procedures are described in chapter 2, and the handling frequency and time variables used in analysis in the two experiments are described in chapters 4 and 5.

6.2.2 Mass and body composition measurement

6.2.2.1. Mass

Mass and length measurements were taken and body condition calculated for each capture as described in chapter 2 to provide information about the rate of overall tissue loss.

The average daily rate of mass gain (DMG: $\text{kg}^{-1} \text{d}^{-1}$) was calculated for pups that were handled during suckling (Equation 1). Overall daily rate of mass loss during fasting ($\text{DML}_{(f)}$: $\text{kg}^{-1} \text{d}^{-1}$) was calculated for all pups (Equation 2). Pups were assumed to gain no mass on the day before weaning, and the day of weaning was assumed to be the first day of weight loss. Where mass at weaning (WM) was not known, it was estimated, based on DMG for pups handled during suckling (Equation 3), and on $\text{DML}_{(f)}$ for pups only handled after weaning (Equation 4). Where mass at departure (DM) was not known, it was estimated using Equation 5.

$$\text{DMG} = \frac{\text{mass}_{(LL)} - \text{mass}_{(EL)}}{\text{date}_{(LL)} - \text{date}_{(EL)}} \quad 1$$

$$\text{DML}_{(f)} = \frac{\text{mass}_{(FP)} - \text{mass}_{(LP)}}{\text{date}_{(FP)} - \text{date}_{(LP)}} \quad 2$$

$$\text{WM} = \text{mass at date}_{(LL)} + (\text{DMG} \times ((\text{date}_{(w)} - 1 \text{ day}) - \text{date}_{(LL)})) \quad 3$$

$$\text{WM} = \text{mass at date}_{(FP)} + (\text{DML}_{(f)} \times (\text{date}_{(FP)} - \text{date}_{(LP)})) \quad 4$$

$$\text{DM} = \text{mass at date}_{(LP)} - (\text{DML}_{(f)} \times (\text{date}_{(d)} - \text{date}_{(LP)})) \quad 5$$

Where subscripts $_{(EL)}$ = early lactation, $_{(LL)}$ = late lactation, $_{(FP)}$ = first postweaning capture, $_{(LP)}$ last postweaning capture, $_{(w)}$ = weaning, $_{(d)}$ = departure

In addition to $\text{DML}_{(f)}$, DML was calculated for the intervals between each capture after weaning to provide information about tissue loss on a finer timescale.

6.2.2.2. Body composition

Body composition was estimated using deuterium oxide (D₂O) dilution by the method described in Reilly and Fedak (1990). This procedure was performed in pups from branded females in both years early and late in lactation. In 2001, body composition estimation was performed within three days after weaning for UNKNOWN animals and for all pups at the final capture before release from the pen, on average 21 ± 4 (s.d.) days after weaning. In 2002, eleven study pups were recaptured for body composition estimation late in the fast, between 14 and 22 days postweaning (mean = 17 ± 3).

After a plasma sample was taken for background D₂O concentration ([D₂O]) measurement in body fluids, a preweighed dose of 3-5ml D₂O (99.9%; Sigma-Aldrich Chemicals) was injected, either subcutaneously (from a sealed vial) into the axillary region (suckling pups in both years and postweaned pups in 2001), or intravenously (from a sterile syringe) into the epidural vein (postweaned pups 2002). A second plasma sample was taken between three and six hours later to determine [D₂O] after equilibration with body water compartments. Pups were not restrained between samples.

Syringes for subcutaneous injections were weighed with the D₂O dose before injection and again after injection to determine total injectate mass. Syringes for intravenous injection were weighed dry and with the D₂O dose prior to injection. After intravenous injection the syringe was flushed with blood four times to ensure complete delivery of the preweighed dose. The protocol was changed from subcutaneous to intravenous injection in 2002 because pups developed localised swellings in the axillary region approximately ten days after subcutaneous injection.

This was thought to be an inflammatory reaction, either to the highly concentrated D₂O, which is a known IgE-dependent histamine-releasing factor (Gillespie, 1982; Maeyama et al, 1985), or to potential contamination in the preweighed vials. Rapid dilution of the D₂O by immediate mixing in the blood stream after intravenous injection was thought to avoid the localised allergic reactions seen in the previous year. This method also avoided potential contamination of the D₂O from the glass vials used previously and reduced the opportunities for spillage caused by the movement of the animal.

After centrifugation, described in chapter 2, four 50µl aliquots of each plasma sample were flame-sealed into capillary tubes, and stored at room temperature until analysis. [D₂O] in parts per million (ppm) in two sub-samples of each of the background and enriched blood samples and standards was measured in duplicate in a mass spectrometer at the University of Aberdeen. Where the variation between replicates was greater than 2% the samples were reanalysed.

Total body dilution space was calculated using Equation 6:

$$\text{Dilution space} = \frac{(\text{mass of injectate} \times [\text{D}_2\text{O}]_i)}{[\text{D}_2\text{O}]_E - [\text{D}_2\text{O}]_B} \times \frac{\text{MW}_{(\text{H}_2\text{O})}}{\text{MW}_{(\text{D}_2\text{O})}} \quad 6$$

where subscripts _i = injectate, _E = enriched sample, _B = background sample, and MW_{H₂O} = molecular weight of water and MW_{D₂O} = molecular weight of D₂O.

Absolute mass and percentage of each of the body components (fat, protein, water and ash) and total body gross energy content (TBGE) were determined from total body water content (TBW), using the equations derived by comparison of isotope dilution with whole-body chemical composition of grey seal carcasses (Reilly

and Fedak, 1990). The calculation of TBGE assumes an energy density of 39.5kJ g^{-1} for fat and 23.5 kJ g^{-1} for protein (Reilly and Fedak, 1990).

The average daily rates of gain in total body fat (TBF), protein (TBP), ash (TBA) and TBW for animals handled during suckling were calculated in the same way as DMG, substituting the appropriate values into Equation 1. Daily rates of change in TBF, TBP, TBA and TBW during fasting were calculated in the same way as $\text{DML}_{(t)}$ substituting the appropriate values into Equation 2, to give DFL, DPL, DAL and DWL, respectively.

TBGE and body composition at weaning and departure were calculated as described above for estimation of WM and DM, substituting the appropriate values into equations 3-5. Daily energy expenditure (DEE) was calculated from the loss of TBGE between weaning and the postweaning body composition measurement. Total energy (TEE), fat (TFU) and protein (TPU) utilised by departure, daily change in percentage fat content ($\Delta\text{ fat } \%$), percentage contribution of fat to DEE ($\% \text{DEE}_{\text{fat}}$) and percentage of initial TBP utilised by departure ($\% \text{TBP}_{\text{U}}$) were also calculated.

The composition of the herring used here in 2001 for supplementary feeding was not measured but was caught in the North Sea between June and September 2001, and was therefore likely have a relatively high fat content. The amount of energy, water, fat and protein ingested by each animal in the FED group during the whole feeding period was therefore estimated from the total amount of fish consumed multiplied by the proximate composition of whole Atlantic herring reported by Gallivan and Ronald (1981), which had a medium to high fat content (63% water; 18.5%fat; 15.5% protein; 9.29kJ g^{-1} energy density). The amounts ingested were corrected for the assimilation efficiency of 91% as determined from an experiment on harp seals fed a herring diet (Lawson et al, 1997) to give an estimate of the total

amount of energy (TE), water (TW), fat (TF) and protein (TP) available from the food for use by the animals. These values are given in Table 6.1.

Table 6.1: Meal size on each day of feeding, total mass of herring consumed and estimated total amounts of gross energy (GE), fat (F), and protein (P) consumed and assimilated for each animal in the FED group.

	Meal size (kg)					Total (kg)	Consumed			Assimilated		
	Day 1	Day 2	Day 3	Day 4	Day 5		GE (MJ)	F (kg)	P (kg)	GE (MJ)	F (kg)	P (kg)
Yeti	1.5	1	1.3	1.5	1.4	6.7	62.23	1.24	1.04	56.63	1.13	0.95
Bernie	1	0.9	1	0.9	1	4.8	44.58	0.86	0.74	40.57	0.79	0.68
Pascal	1	1.2	1.2	1.4	1.1	5.9	54.80	1.06	0.91	49.86	0.97	0.83
Leonardo	1.4	1.2	1.4	0.9	1.1	6.0	55.73	1.08	0.93	50.72	0.98	0.85
Newton	1.2	1.2	1	1.1	1.2	5.7	52.94	1.03	0.88	48.18	0.93	0.80
Roger	1	1.4	1	1.2	1.2	5.8	53.87	1.04	0.90	49.02	0.95	0.92
Mawson	1.6	1	1.4	1	1.2	6.2	57.59	1.12	0.96	52.41	1.02	0.87

The exact fate of these additional resources, in terms of whether they were used for growth or metabolism and when they were used, was not determined. However, because initial and final mass and body composition was known, the amount of fat and protein added to the system must have been metabolised at some point during the postweaning period, whether or not the actual metabolised component came from endogenous reserves or the supplementary food. TE, TF, TP and TW were therefore included in the calculations of DEE, DFL, DPL and DWL for the FED animals by dividing each component by fast duration and adding it to the appropriate value for tissue loss already calculated from the body composition information. A second DML ($DML_{(fish)}$) was also calculated for the FED group that accounted for the mass of the fish, to ensure that all mass, fat and protein loss values were consistent. Total energy, fat and protein utilised by departure and $\%DEE_{fat}$ for the FED animals also included TE, TF and TP in the calculations.

All rates of change could only be calculated for those animals for which body composition was estimated at weaning and late in the fast. Departure body composition values could only be estimated for those animals for which date of departure was also known. Details of the number of animals in each group for which body composition information was available at weaning, late in the fast and departure is given in Table 6.2.

Table 6.2. Number of males (M) and females (F) from each group for which body composition information could be obtained at weaning, late fast and departure. Bold outline shows COM and LO/UN groups, where there were insufficient animals for analysis in the original treatment groups.

Year	Group	overall			weaning			late fast			departure		
		Total	M	F									
2001	FED	7	4	3	7	4	3	7	4	3	6	3	3
	HIGH	7	4	3	6	4	2	6	4	2	6	4	2
	LOW	7	4	3	5	3	2	4	2	2	4	2	2
	UNKNOWN	7	2	5	5	0	5	4	0	4	2	0	2
	No. animals	28	14	14	23	11	12	21	10	11	18	9	9
2002	CONTROL	9	6	3	7	6	1	2	2	0	2	2	0
	SALINE	10	5	5	8	4	4	4	1	3	4	1	3
	DEX	10	5	5	8	4	4	5	2	3	5	2	3
	No. animals	29	16	13	23	14	9	11	5	6	11	5	6
Both years total		57	30	27	47	26	21	32	15	17	29	14	15

6.2.3. Statistical analysis

The effects of group, sex, year and body energy stores on mass, body composition parameters and fast duration were investigated. All proportion or percentage data were arcsine transformed, and continuous data that did not have a normal distribution were log-transformed before analysis. F tests, Bartlett's tests (parametric) or Levene's tests (non-parametric) were used to determine whether variance between categories was equal.

Body composition and mass variables were closely related to each other and often derived from the same data. In these circumstances, where differences between categories (group, sex etc.) were under investigation, multivariate analysis of variance (MANOVA) was performed to determine whether the category had an overall effect on the first discriminant function of the dependent variables (dv). Pillai's trace was used as the test statistic. Given a significant effect of the categorical variable in the MANOVA, which indicated that dvs could be treated separately, the univariate tests were then examined to determine which dvs were influenced by the categorical variable.

Where dependent variables were continuous, and where multiple tests on the same data were necessary due to small sample size, a Bonferonni correction was applied to reduce the possibility of type I error.

6.2.3.1. Treatment effects

Within each year, data from males and females were pooled within each group to test for group differences in WM, $DML_{(t)}$, DM and fast duration in all study animals. WM, $DML_{(t)}$ and DM were considered together in a MANOVA since both WM and DM were in part derived from mass change data. Fast duration was considered separately. In 2001, DM was also considered separately because DM was known for fewer animals than WM.

The effects of *timepoint* and group on the change in DML between the early (early to mid *timepoint*) and late (mid to late *timepoints*) portions of the fast were investigated in all four groups in the 2001 study, using linear mixed effects models (LMEs). Mass was included as a covariate to account for the effects of progressive

reduction in body mass on DML. *Timepoint* was replaced with *day* to examine differences between FED and HIGH groups in more detail and to investigate changes in DML in the 2002 study.

To determine whether those animals for which body composition information was available (BC pups) were representative of all the study animals within each year, MANOVAs and ANOVAs were used to compare the information that was known (WM, $DML_{(f)}$, DM and fast duration) between BC pups and non-BC pups. A Bonferroni correction was applied to account for the fact that the same data had been used previously to examine group differences in the same variables.

Since all body composition data was derived from mass and TBW, group differences within each year in WM and $TBW_{(w)}$, $DML_{(f)}$ (or $DML_{(fish)}$) and DWL and DM and $TBW_{(d)}$ were investigated initially to avoid multiple comparisons using the same information. Where a significant effect of group in the MANOVA indicated that the responses of mass and TBW were sufficiently different to be treated separately, group effects on individual body composition components were then investigated using MANOVA. Where a significant effect of group in the MANOVA indicated that the responses of different body composition variables were sufficiently different to be treated separately, the univariate analyses were examined to determine which of those variables were influenced by group.

Body composition at departure was measured in only two females from the UNKNOWN group (see Table 6.2). For the purpose of analysis, the departure body compositions for LOW and UNKNOWN groups were therefore combined and referred to as the LO/UN group, since these groups experienced identical handling regimes after weaning. Similarly, in 2002, there were insufficient animals in the SALINE and CONTROL groups alone at late fast and departure to compare body

composition derived information between groups. Instead, SALINE and CONTROL groups were combined for statistical tests on body composition estimates, and will be referred to as the combined control group (COM). Where group differences were apparent at departure when data were available for fewer animals, further tests were performed using only those pups to establish whether those differences had been present at weaning or during the fast.

The small sample sizes prevented simultaneous testing for differences between groups and sex. To establish whether group differences could be driven by a mismatch in the sex ratio between groups (see Table 6.2), the effect of sex on mass and body composition information and fast duration was investigated by pooling data from all groups within each year. Sex effects were considered significant at $p < 0.025$, since the same data had already been used to investigate group effects.

6.2.3.2. Energy reserves

There were differences between years in the timing of body composition measurements and the duration that the animals were penned. This could have caused error in body composition estimates if mass loss and utilisation of individual tissue components did not occur in a linear way over time, which is often the case in fasting seals (Nordoy et al, 1990; Rea and Costa, 1992; Nordoy et al, 1993; Biuw, 2003; this chapter). The effects of year on mass and body composition information and fast duration were therefore investigated to establish whether data from both studies could be pooled to increase sample size.

Where appropriate, mass and body composition information from animals in both years was pooled to investigate the effects of initial energy reserves at weaning

and sex on changes in mass and body composition parameters using linear models.

This excluded FED and DEX animals. The effect of sex and WM on $DML_{(t)}$ and DM was investigated in both BC and non-BC pups from the remaining groups. The effects of sex, WM, $TBF_{(w)}$, $\%TBF_{(w)}$, $TBP_{(w)}$ and $TBGE_{(w)}$ on $DML_{(t)}$, DFL, DPL, DEE $\%DEE_{fat}$, DM, $\%TBF_{(d)}$, $\%TBP_U$ and fast duration were investigated in BC pups from those groups.

6.3 Results

Details of all LMEs are given in Appendix 4.

6.3.1. Handling and feeding effects

There was a significant effect of group on the combination of WM and $DML_{(f)}$ (MANOVA: $F_{(6,48)} = 2.543$, $p = 0.032$) in all animals in 2001 but there was no effect of group on DM or fast duration. The results of the univariate tests on WM and $DML_{(f)}$ in 2001 are given in Table 6.3. $DML_{(f)}$ was significantly lower in the FED group than in the other three groups (when the mass of the fish was not included in the calculations). This created an apparent inconsistency, in that the FED pups did not fast for longer or have a higher DM, despite a lower $DML_{(f)}$ than the other groups. The data from the unfed groups were therefore pooled (UNFED) to increase sample size, and t tests were performed to try identify whether the feeding effect on $DML_{(f)}$ impacted on DM or fast duration. The results are also shown in Table 6.3 and again show no significant difference between the FED and UNFED groups in WM, DM or fast duration but a highly significant difference between the groups in $DML_{(f)}$.

The effects of handling regime and feeding on DML during the early and late portions of the fast are shown in Figure 6.1. HIGH, LOW and UNKNOWN groups showed a significant reduction in DML from the early to the later portion of the fast. This reduction was also significant when body mass was included as a covariate.

DML and the pattern of change in DML during both the early and late portions of the fast, were not significantly different between the unfed groups. In contrast, DML was significantly lower in the FED group than in the other three groups in the

early portion of the fast, but not in the late portion of the fast, and increased significantly in FED animals from the early to the late portion of the fast.

Figure 6.1: DML during early and late portions of the fast in FED (red), HIGH (blue), LOW (green) and UNKNOWN (black) pups. Values are mean \pm standard deviation. \star denotes a significant difference ($p < 0.05$) between FED group and the other three groups. *a* and *b* denote a significant decrease and increase, respectively, from earlier to later portions of the fast within groups and are colour-coded by group

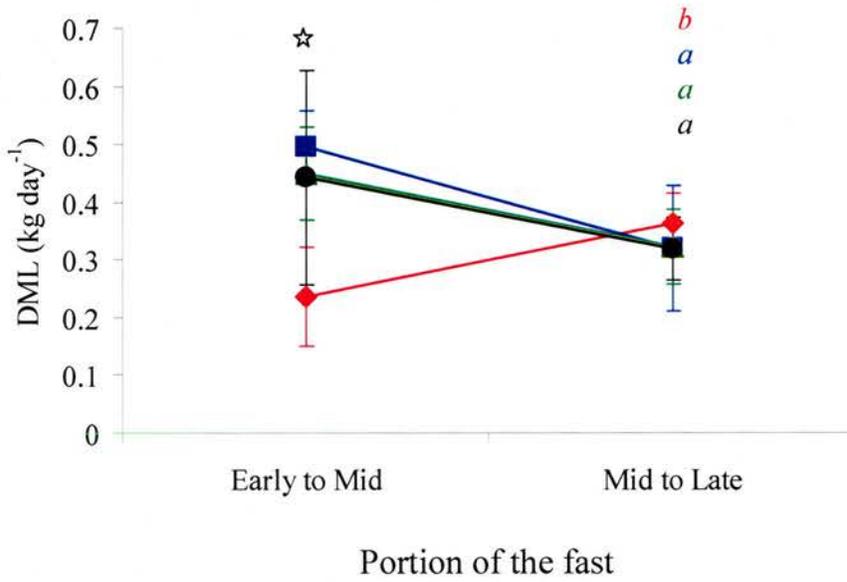


Table 6.3: Results of a. ANOVAs and b. t tests comparing WM and DML₍₀₎, DM and fast duration between all four groups (a.) and between FED and UNFED groups (b.) for all animals in 2001. Table shows mean values ± standard deviation (s.d.), number of individuals (n) and the range of values. Bold font highlights the group that was significantly different from the others (p<0.05).

	FED			HIGH			LOW			UNKNOWN			F	p	
	mean	s.d.	n	mean	s.d.	n	mean	s.d.	n	mean	s.d.	n			range
WM (kg)	46.7	9.7	7	45.0	4.5	7	46.1	4.3	7	44.0	8.3	7	28.6-53.7	0.08	0.973
DML ₍₀₎ (kg day ⁻¹)	0.28	0.04	7	0.40	0.07	7	0.41	0.07	7	0.38	0.08	7	0.22-0.46	5.24	0.006
DM (kg)	33.7	6.1	6	32.2	5.4	7	33.4	2.8	7	32.2	5.1	4	26.4-37	0.14	0.936
Duration (days)	28.5	7.4	6	27.1	4.1	7	23.4	3.3	7	20.8	7.5	4	10-26	2.11	0.131

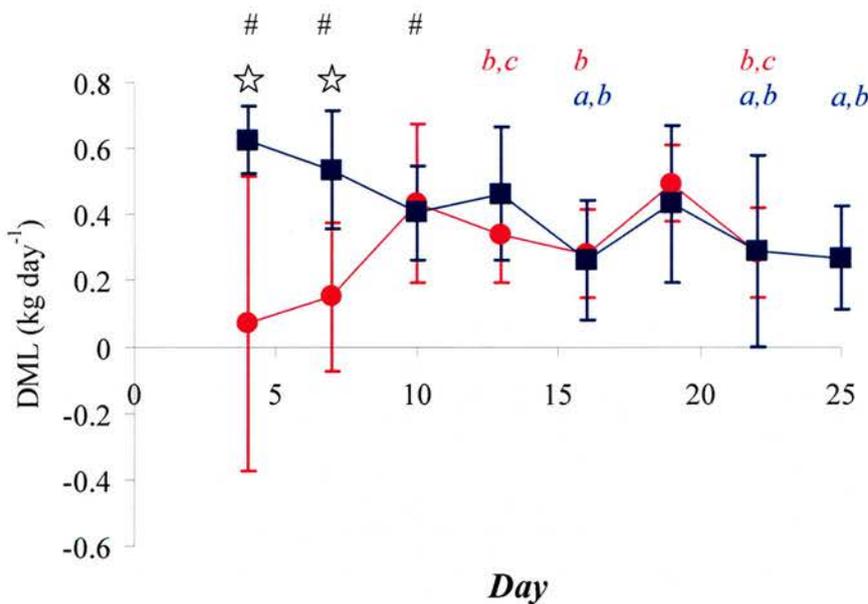
	FED			UNFED			T	p
	mean	s.d.	n	mean	s.d.	n		
WM (kg)	46.7	9.7	7	45.0	5.7	21	0.57	0.574
DML ₍₀₎ (kg day ⁻¹)	0.28	0.04	7	0.40	0.07	21	3.98	< 0.0001
DM (kg)	33.7	6.1	6	32.7	4.22	18	0.44	0.666
Duration (days)	28.5	7.4	6	24.3	5.1	18	1.56	0.132

b.

The changes in DML over three-day intervals during the fast were significantly different between groups and are shown in Figure 6.2. The pattern of change in DML was the same when mass was included as a covariate. DML was significantly lower in the FED group than in the HIGH group during the first seven days after weaning. The effect persisted until ten days after weaning when mass was included as a covariate.

In the FED group, DML increased from initially low values, during the first seven to ten days after weaning, to levels comparable with those in the HIGH group by *day* 13, and did not change substantially thereafter. In contrast, DML decreased progressively over the first 13 days of the fast in the HIGH group, such that they were significantly lower by *day* 16 than during the first seven days after weaning. DML remained low then increased from *day* 16 to 19.

Figure 6.2: DML at three-day intervals in FED (red) and HIGH (blue) pups. Values are mean \pm standard deviation. \star and # denote a significant difference between groups ($p < 0.05$), in DML alone and in DML when mass was included as a covariate, respectively. *a*, *b* and *c* denote a significant difference ($p < 0.05$) from *day* 4, 7 and 10, respectively, within group and are colour coded accordingly.



There was no significant difference between BC and non-BC pups in the combination of WM and $DML_{(f)}$ (MANOVA: $F_{(2,25)} = 0.603$, $p = 0.555$) or in DM in 2001. Fast duration was longer in BC pups than in non-BC pups. The results of the univariate tests on WM, $DML_{(f)}$, DM and fast duration between BC and non-BC pup within each year are given in Table 6.4.

Table 6.4: Results of ANOVAs comparing WM, $DML_{(f)}$, DM, and fast duration between BC and non-BC pups within each year. Tables show mean values \pm standard deviation (s.d.), number of individuals (n) and the range of values. Bold font highlights significant difference after Bonferonni correction ($p < 0.025$).

	BC pups				Non BC pups				F	p
	mean	s.d.	n	range	mean	s.d.	n	range		
2001										
WM (kg)	46.1	6.5	18	39.3-66.0	44.2	7.4	10	28.6-53.7	0.51	0.481
$DML_{(f)}$ (kg day ⁻¹)	0.36	0.08	18	0.23-0.52	0.38	0.08	10	0.22-0.50	0.39	0.539
DM (kg)	33.3	4.6	18	25.9-44.3	32.1	5.2	6	26.4-41.0	0.28	0.603
Duration (days)	26.9	5.0	18	17-38	20.5	6.2	6	10-29	6.69	0.017
2002										
WM (kg)	45.4	7.5	11	36.4-61.0	43.5	6.4	18	35.4-56.0	0.52	0.475
$DML_{(f)}$ (kg day ⁻¹)	0.47	0.13	11	0.33-0.76	0.48	0.14	18	0.33-0.96	0.13	0.719
DM (kg)	31.9	4.3	11	27.1-40.5	33.5	5.3	18	25.4-42.3	0.71	0.408
Duration (days)	23	5.6	11	15-34	16.6	3.6	18	12-23	14.17	0.001

Group comparisons of body composition parameters within 2001 for BC pups are shown in Table 6.5. The fasting values in this case account for the additional mass, fat protein and energy from the supplementary food. There were no differences between groups in the combination of WM and $TBW_{(w)}$ (MANOVA: $F_{(6,38)} = 0.471$, $p = 0.471$) in 2001. However, there was a difference between groups in the combinations of $DML_{(f)}$ and DWL (MANOVA: $F_{(6,34)} = 2.608$, $p = 0.035$) and DM and $TBW_{(d)}$ (MANOVA: $F_{(4,30)} = 4.603$, $p = 0.005$), allowing group effects on body composition information during fasting and at departure to be examined in more detail. The results of the univariate analyses are given in Table 6.5.

$DML_{(fish)}$ was significantly higher in FED animals compared with the $DML_{(f)}$ of the other three groups. There was a significant difference in the group effects on

DFL, DPL, DEE and %DEE_{fat} (MANOVA: $F_{(12,48)} = 3.137$, $p = 0.002$). DFL ($p = 0.071$) and DEE ($p = 0.062$) showed a tendency to be higher in the FED group compared with the others. Neither DPL nor %DEE_{fat} were different between groups. Δ fat % was analysed separately because it was not derived from the same data as the other fasting parameters in the FED group. It was negative in the FED group and positive in the other groups, and this difference was approaching significance ($p = 0.066$).

There was a significant difference in the group effects on TBF_(d), TBP_(d), TBGE_(d), %TBF_(d) and %TBP_U (MANOVA: $F_{(8,26)} = 2.585$, $p = 0.032$). DM, TBF_(d) and TBGE_(d) were higher in the LO/UN group than in the other two groups. %TBF_(d) was lower in the FED group than in the LO/UN and HIGH groups. The effects of group on TEE, TFU and TPU were significantly different (MANOVA: $F_{(6,28)} = 5.005$, $p = 0.001$). TEE and TFU showed a tendency to be higher in the FED animals, whereas TPU was not different between groups.

Weaning and fasting body composition information was reanalysed using only those animals for which body composition information at departure was available to investigate how group differences at departure may have arisen. There were no differences between groups in the combination of WM, TBP_(w) and %TBF_(w) (MANOVA: $F_{(6,28)} = 1.117$, $p = 0.390$), where TBGE_(w) and TBF_(w) were too highly correlated with WM to be included in the analysis.

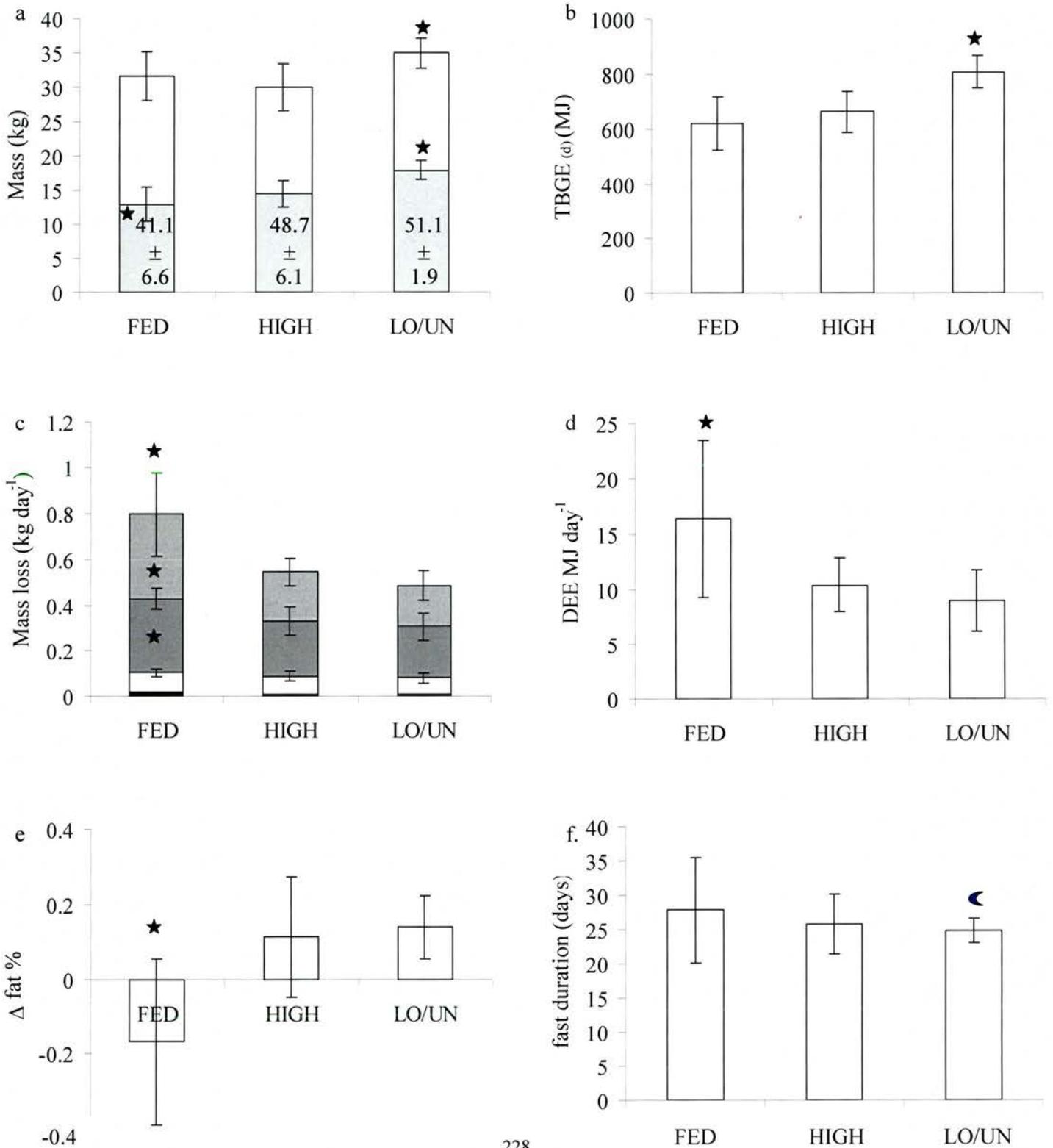
The difference in the effects of group on the combination of DML_(f) and DWL was approaching significance for this subset of animals (MANOVA: $F_{(4,30)} = 2.635$, $p = 0.054$). In the univariate analyses these FED pups had significantly higher DML_(fish) than DML_(f) in the other groups (ANOVA: $F_{(2,17)} = 7.68$, $p = 0.005$) and higher DWL (ANOVA: $F_{(2,17)} = 5.40$, $p = 0.017$) than the other two groups. Group had significantly

different effects on DFL, DPL, DEE, %DEE fat (MANOVA: $F_{(8,26)} = 3.621$, $p = 0.006$). In the univariate analyses the FED pups had significantly higher DFL (ANOVA: $F_{(2,17)} = 4.38$, $p = 0.032$) and DEE (ANOVA: $F_{(2,17)} = 4.27$, $p = 0.034$) than in the other two groups, but %DEE_{fat} was not significantly higher in these animals (ANOVA: $F_{(2,17)} = 2.18$, $p = 0.147$). Δ fat % was significantly different between the FED animals and the other two groups (ANOVA: $F_{(2,17)} = 6.29$, $p = 0.010$). FED animals showed a decrease in Δ fat %, whereas the other groups showed an increase, as a result of the higher rates of fat loss in FED animals. DPL (ANOVA: $F_{(2,17)} = 0.47$, $p = 0.636$) and fast duration were not different between groups (Kruskal-Wallis: $H_{(2)} = 1.10$, $p = 0.577$), but there was significantly less variance in fast duration in the LO/UN animals than in the other two groups (Bartlett's test: $T = 8.158$, $p = 0.017$). These differences between FED, HIGH and LO/UN groups are shown in Figure 6.3.

Table 6.5. Mass and body composition parameters in each group in 2001. Mean value, standard deviation (s.d.) and sample size (n), are given for each group. F and p values are shown for univariate ANOVA analysis on body composition data where MANOVA revealed a significant group effect on mass and TBW. Bold font indicates the group that was significantly different from the others. With the exception of Δ fat %, fasting values include mass and energy derived from supplementary food for the FED group.

	FED		HIGH		LOW		UNKNOWN		F _(3,20)	P
	mean	s.d.	n	mean	s.d.	n	mean	s.d.		
Weaning										
WM(kg)	46.74	9.70	43.93	3.83	47.33	3.87	43.21	8.35	7.31	0.002
TBF _(w) (kg)	21.84	5.56	19.98	1.54	22.03	2.98	20.47	5.87	2.80	0.071
TBP _(w) (kg)	5.58	0.92	5.39	0.69	5.67	0.55	5.08	0.45	1.46	0.260
%TBF _(w)	46.42	2.65	45.62	2.69	46.46	3.55	46.44	6.02	2.95	0.062
TBGE _(w) (MJ)	1006.26	242.32	928.40	70.43	1016.24	118.51	939.71	244.52	1.16	0.354
Fasting										
DML _(f) (kg day ⁻¹)	0.77	0.19	0.55	0.11	0.54	0.12	0.40	0.01	2.89	0.066
DFL (kg day ⁻¹)	0.34	0.18	0.21	0.06	0.19	0.08	0.14	0.04		
DPL (kg day ⁻¹)	0.08	0.02	0.08	0.02	0.08	0.02	0.06	0.01		
DEE (MJ day ⁻¹)	15.20	7.19	10.42	2.46	9.68	3.23	6.97	1.42		
%DEE _(fat)	85.11	7.80	81.68	6.15	78.96	6.58	77.63	9.54		
Δ fat (%)	-0.11	0.25	0.11	0.16	0.16	0.09	0.15	0.11		
									F _(2,17)	p
Departure										
DM (kg)	31.67	3.56	30.06	3.37	34.97	2.18			3.92	0.043
TBF _(d) (kg)	12.97	2.56	14.56	1.91	17.95	1.40			9.58	0.002
TBP _(d) (kg)	4.3	0.81	3.44	0.75	3.76	0.28			2.61	0.107
%TBP _(d)	24.5	9.89	36.49	8.47	31.24	9.28			2.75	0.096
%TBF _(d)	41.09	6.58	48.70	6.09	51.14	3.44	6		6.61	0.009
TBGE _(d) (MJ)	621.44	97.74	664.06	75.21	807.03	58.36			9.14	0.003
TEE (MJ)	415.36	265.06	264.35	52.62	219.27	69.65			3.35	0.063
TFU (kg)	10.04	6.70	5.44	1.41	4.44	1.71			3.23	0.068
TPU (kg)	2.24	0.67	1.95	0.47	1.73	0.43			1.39	0.279

Figure 6.3: Differences between groups in pups for which body composition was available at weaning and departure from 2001 in a. DM (total bar height), TBF_(d) (grey portion of bar) and %TBF_(d) (value on bar), b. TBGE_(d) c. DML_(f) or DML_(fish) (total bar height), DAL (black), DPL (white), DWL (dark grey) and DFL (light grey), d. DEE and e. Δ fat % and f. fast duration. \star denotes the group that was significantly different from the other two ($p < 0.05$). \blacktriangleleft denotes the group with significantly different variance from the other two. Error bars show standard deviation.



There was a significant effect ($p < 0.025$ after Bonferonni correction) of sex on the combination of WM and $TBW_{(w)}$ in all BC pups (MANOVA: $F_{(2,20)} = 6.286$, $p = 0.008$) and in the smaller sample of animals for which departure body composition was also available (MANOVA: $F_{(2,15)} = 9.365$, $p = 0.002$). This allowed mass and body composition data at weaning to be treated separately.

Differences between males and females in body composition variables at weaning could also be treated separately, both in BC pups (MANOVA: $F_{(3,19)} = 7.255$, $p = 0.002$) and in the smaller sample (MANOVA: $F_{(3,14)} = 7.300$, $p = 0.003$). $TBP_{(w)}$ was significantly higher in males (mean = 5.74 ± 0.51 kg) than in females (mean = 5.17 ± 0.74 kg) in all BC pups (ANOVA: $F_{(1,22)} = 4.57$, $p = 0.044$). In the smaller sample, this difference was approaching significance (ANOVA: $F_{(1,17)} = 3.99$, $p = 0.063$), and $\%TBF_{(w)}$ was significantly lower in males ($44.73 \pm 1.70\%$) than females ($48.09 \pm 2.71\%$) (ANOVA: $F_{(1,17)} = 8.30$, $p = 0.011$).

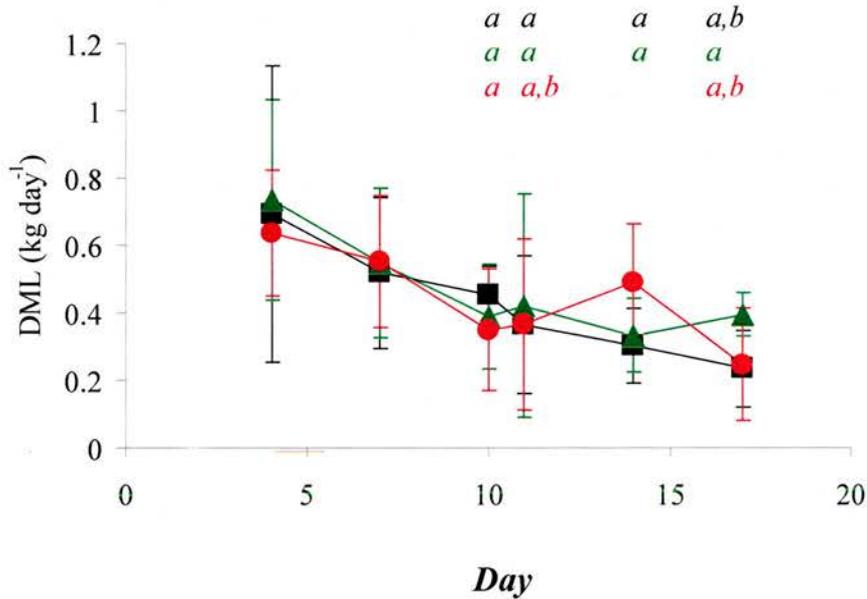
There were no sex differences in $DML_{(f)}$ and DWL in all BC pups (MANOVA: $F_{(2,20)} = 1.810$, $p = 0.190$) or in the smaller sample size (MANOVA: $F_{(2,15)} = 1.560$, $p = 0.242$), or in DM and $TBW_{(d)}$ (MANOVA: $F_{(2,15)} = 0.590$, $p = 0.567$).

6.3.2. Dexamethasone effects

There was no effect of group on the combination of WM, $DML_{(f)}$ and DM (MANOVA: $F_{(6,50)} = 0.399$, $p = 0.876$) or on fast duration (ANOVA: $F_{(2,28)} = 0.06$, $p = 0.938$) in 2002.

The changes in DML over three-day intervals during the fast were significantly different between groups and showed the same pattern when mass was included as a covariate. The pattern of change is shown in Figure 6.4.

Figure 6.4: DML at three-day intervals in CONTROL (black), SALINE (green) and DEX (red) pups. Values are mean \pm standard deviation. *a* and *b* denote significant differences from *day* 4 and 7, respectively, and are colour coded by group



All groups showed a progressive decline in DML over the first ten days after weaning to a lower level that did not change substantially thereafter. DML was not significantly different between groups during any of the three-day time intervals. However, in the DEX group there was an increase in DML after dexamethasone treatment to levels comparable with those at the start of the fasting period. The change in DML was apparent between one and three days after dexamethasone treatment (*day* 11-14). DML declined to previous levels by *day* 17, and this reduction was approaching significant.

In 2002, there was a significant difference between BC and non-BC pups in the combination of WM, DML_(t) and DM (MANOVA: $F_{(3,25)} = 3.474$, $p = 0.031$),

indicating that the three variables could be treated separately. However, there was no effect of group on each variable in the univariate analysis. Fast duration was longer in BC pups than in non-BC pups (Table 6.3).

Table 6.6. shows group comparisons of body composition parameters within 2002 for BC pups. There were no differences between groups in WM and $TBW_{(w)}$ in all BC pups (MANOVA: $F_{(4,40)} = 0.523$, $p = 0.719$) or in the eleven remaining at departure (MANOVA: $F_{(2,8)} = 0.088$, $p = 0.917$). There were no group differences in $DML_{(f)}$ and DWL (MANOVA: $F_{(2,8)} = 0.572$, $p = 0.586$) or in DM and $TBW_{(d)}$ (MANOVA: $F_{(2,8)} = 0.531$, $p = 0.607$). Fast duration was not significantly different between groups (T test: $T_{(9)} = 0.46$, $p = 0.653$).

Table 6.6. Mass and body composition parameters in each group in 2002. Mean value, standard deviation (s.d.) and sample size (n), are given for each group.

		CONTROL			SALINE			DEX		
		mean	s.d.	n	mean	s.d.	n	mean	s.d.	n
Weaning	WM (kg)	44.58	7.40		45.45	4.34		45.66	8.79	
	$TBF_{(w)}$ (kg)	21.38	6.18		20.02	2.34		21.10	5.03	
	$TBP_{(w)}$ (kg)	5.16	1.14	7	5.71	0.59	8	5.51	0.84	8
	% $TBF_{(w)}$	47.48	8.75		44.03	2.68		975.35	2.34	
	$TBGE_{(w)}$ (MJ)	978.03	241.74		936.91	103.54		45.93	219.31	
		COM								
		mean		s.d.	n					
Fasting	$DML_{(f)}$ (kg day ⁻¹)	0.61		0.25			0.69		0.27	
	DFL (kg day ⁻¹)	0.19		0.13			0.29		0.19	
	DPL (kg day ⁻¹)	0.10		0.03	6		0.09		0.02	5
	DEE (MJ day ⁻¹)	10.1		5.71			13.7		7.97	
	%DEE _(fat)	68.50		25.2			80.28		8.6	
	Δ fat (%)	1.28		0.78			1.88		0.89	
Departure	DM (kg)	30.22		4.48			30.25		4.35	
	$TBF_{(d)}$ (kg)*	15.32		2.73			14.48		2.63	
	$TBP_{(d)}$ (kg)	3.26		0.53	6		3.51		0.58	5
	% $TBP_{(U)}$	41.17		9.28			36.76		8.36	
	% $TBF_{(d)}$	50.72		3.44			47.87		4.76	
	$TBGE_{(d)}$ (MJ)*	687.65		117.5			662.92		111.2	

There were no significant differences ($p < 0.025$ after Bonferonni correction) between males and females in WM and $TBW_{(w)}$ in all BC pups (MANOVA: $F_{(2,20)} = 1.524$, $p = 0.242$) or in the eleven remaining at departure (MANOVA: $F_{(2,8)} = 3.051$,

$p = 0.104$). There was a significant difference between males and females in $DML_{(f)}$ and DWL (MANOVA: $F_{(2,8)} = 7.188$, $p = 0.016$) that allowed changes in mass and body composition variables to be examined separately, but there were no significant sex differences in body composition variables (MANOVA: $F_{(4,6)} = 1.896$, $p = 0.230$). There was no significant sex difference in DM and $TBW_{(d)}$ (MANOVA: $F_{(2,8)} = 2.456$, $p = 0.147$).

6.3.3. Impact of fuel reserves and sex on fuel utilisation strategy

There were no significant differences ($p < 0.0125$ after Bonferonni correction) between years in WM and $TBW_{(w)}$ (MANOVA: $F_{(2,43)} = 0.037$, $p = 0.964$), $DML_{(f)}$ and DWL (MANOVA: $F_{(2,29)} = 1.391$, $p = 0.265$) or DM and $TBW_{(d)}$ (MANOVA: $F_{(2,26)} = 0.037$, $p = 0.210$).

Since FED and DEX groups were not representative of all animals in terms of the pattern of change in mass loss and body composition, these animals were excluded from the analysis of effects of fuel reserves on fuel allocation. In all remaining pups from both years, $DML_{(f)}$ was significantly higher in males than in females (mean: males = 0.472 ± 0.124 kg day⁻¹; females = 0.383 ± 0.08 kg day⁻¹; $T_{(38)} = 3.12$, $p = 0.003$). However, as shown in Figure 6.5, males in this sample also tended to be heavier at weaning (mean: males = 46.14 ± 5.30 kg; females = 42.88 ± 6.04 kg; $T = 1.82$, $p = 0.077$) and $DML_{(f)}$ increased significantly as a function of WM in these animals, although the relationship was weak. DM was strongly dependent on WM (Figure 6.6) but was not affected by sex.

Figure 6.5: Relationship between $DML_{(f)}$ and WM in pups from both years that were not in the FED or DEX groups. LM: $DML_{(f)} = 0.0055 (WM) + 0.19$, $T_{(1,38)} = 2.171$, $p = 0.036$, $R^2 = 0.0752$. Males (blue) and females (red) are shown separately to highlight the tendency for males to be heavier and have a higher $DML_{(f)}$ than females.

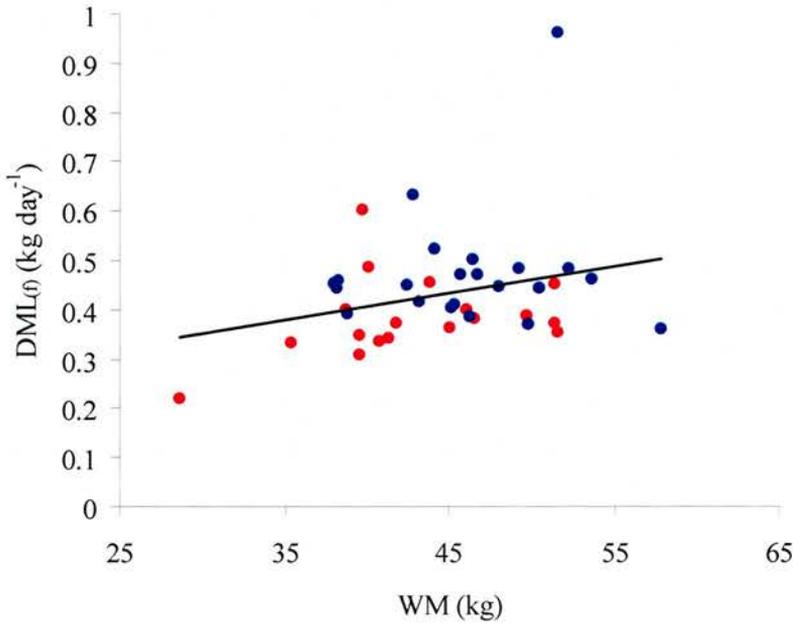
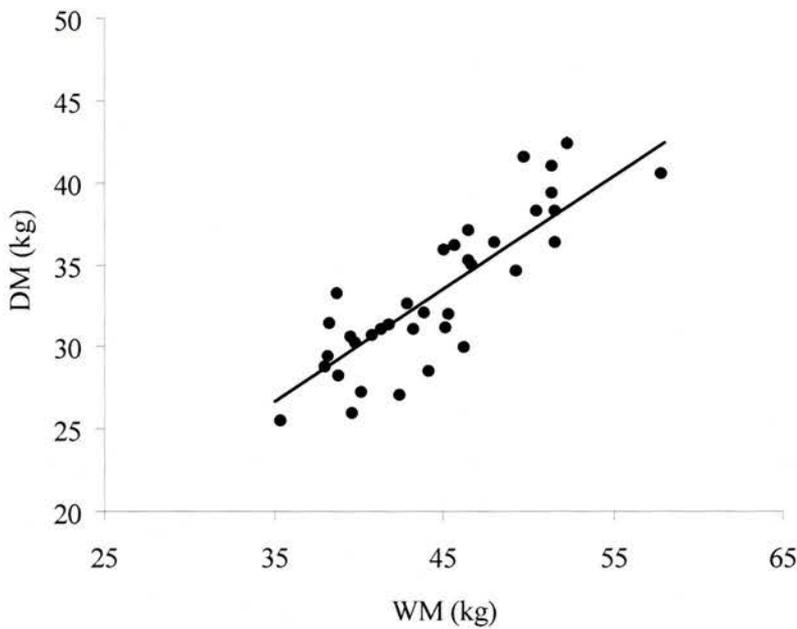


Figure 6.6: Relationship between DM and WM in pups from both years that were not in the FED or DEX groups. LM: $DM = 0.686 \times WM + 2.642$, $T_{(1,38)} = 9.512$, $p = 3.09 \times 10^{-11}$, $R^2 = 0.7131$.



In all BC pups, %TBF_(w), TBGE_(w), TBP_(w) and TBF_(w) increased significantly (p considered significant at < 0.0125 following Bonferroni correction) as a function of WM, although the relationship between %TBF_(w) and WM was weak:

*	%TBF _(w)	=	0.0008 WM	+ 35.51	F _(1,44) = 9.51	p = 0.0035	r ² = 0.159
*	TBF _(w)	=	0.5821 WM	- 5.42	F _(1,44) = 207.40	p = 9.69 x 10 ⁻¹⁰	r ² = 0.821
*	TBP _(w)	=	0.0868 WM	+ 1.52	F _(1,44) = 59.85	p = 9.69 x 10 ⁻¹⁰	r ² = 0.5667
*	TBGE _(w)	=	25.40 WM	- 179.99	F _(1,44) = 355.40	p = 2.20 x 10 ⁻¹⁶	r ² = 0.8873

The effect of sex and initial energy reserves on rates of tissue utilisation, DEE, departure body composition and fast duration in BC pups (excluding FED and DEX animals) are given in Tables 6.7 and 6.8. Several individual body mass and composition parameters had a significant effect on fuel allocation independently of the others, even after Bonferonni correction, but there were no additive effects or interactions between them.

After Bonferonni correction, sex did not have a significant effect on mass or body composition or on fast duration. DML_(f) increased as a function of WM, TBP_(w) or TBGE_(w) but not as a function of %TBF_(w). The effect of TBF_(w) on DML_(f) was approaching significance. DFL and DEE increased as a function of WM, TBF_(w) and TBGE_(w), and DPL was higher in animals with higher TBP_(w). %DEE_{fat} increased as a function of %TBF_(w) but not as a function of WM, TBP_(w), TBF_(w) or TBGE_(w).

Table 6.7: Equations of the lines that describe the linear change in $DML_{(t)}$, DFL, DPL, DEE and $\%DEE_{fat}$ with and sex and mass and body composition at weaning. The slope of the line and the intercept (I) are given with standard error (s.e.), F, p and r^2 values. Bold font indicates that the slope of the regression is significantly different from zero after Bonferonni correction (p considered significant at <0.008). * indicates parameters that describe more than 50% of the variability in the dependent variable. Red font highlights the parameter that best describes changes in the dependent variable.

	parameter	slope (kg or %)	s.e.	I	s.e.	F _(1,16)	p	r ²
DML _(t)	Sex	0.170	0.0697	0.467	0.049	5.94	0.0269	0.2250
	WM*	0.028	0.0057	-0.694	0.258	23.52	0.0002	0.5699
	TBF _(w)	0.034	0.0109	-0.132	0.230	9.05	0.0083	0.3212
	TBP_(w)	0.213	0.0527	-0.613	0.290	16.32	0.0009	0.4741
	TBGE_(w)	0.001	0.0002	-0.265	0.241	11.64	0.0036	0.3849
%TBF _(w)	0.003	0.0127	0.414	0.582	0.06	0.8158	0.0587	
DFL	Sex	0.037	0.0417	0.177	0.030	0.79	0.3882	0.0127
	WM*	0.015	0.0031	-0.441	0.140	20.94	0.0003	0.5398
	TBF_(w)*	0.022	0.0045	-0.267	0.095	24.16	0.0002	0.5767
	TBP _(w)	0.076	0.0343	-0.220	0.189	4.91	0.0415	0.1871
	TBGE_(w)*	0.001	0.0001	-0.326	0.101	26.88	9.0x10⁻⁵	0.6035
%TBF _(w)	0.012	0.0059	-0.362	0.271	2.42	0.0561	0.1602	
DPL	Sex	0.032	0.0113	0.067	0.008	8.23	0.0111	0.2983
	WM	0.003	0.0013	-0.049	0.060	4.91	0.0416	0.1869
	TBF _(w)	0.002	0.0023	0.043	0.048	0.75	0.4006	0.0152
	TBP_(w)	0.032	0.0099	-0.092	0.054	10.53	0.0051	0.3592
	TBGE _(w)	5.9x10 ⁻⁵	5.4x10 ⁻⁵	0.026	0.052	1.24	0.2821	0.0139
%TBF _(w)	-0.003	0.0020	0.208	0.094	1.76	0.2031	0.0429	
DEE	Sex	2.261	1.728	8.704	1.222	1.71	0.209	0.0402
	WM*	0.632	0.1203	-18.79	5.474	27.64	7.8x10⁻⁵	0.6104
	TBF_(w)*	0.9341	0.1956	-9.593	4.110	22.80	0.0002	0.5619
	TBP _(w)	3.810	1.369	-11.03	7.532	7.745	0.0133	0.2841
	TBGE_(w)*	0.023	0.004	-12.39	4.314	26.97	8.8x10⁻⁵	0.6044
%TBF _(w)	0.793	0.4455	-23.99	19.02	3.167	0.0941	0.1131	
%DEE _{fat}	Sex	0.700	0.6600	80.98	0.332	1.13	0.3047	0.0072
	WM	0.007	0.0080	40.79	15.752	0.89	0.3599	0.0064
	TBF _(w)	0.073	0.0154	24.36	6.662	4.70	0.0456	0.1787
	TBP _(w)	0.056	0.5939	87.26	16.966	0.09	0.7629	0.0563
	TBGE_(w)	3.649	9.1485	22.57	8.367	3.99	0.0630	0.1495
%TBF_(w)	0.119	0.0001	23.20	19.266	12.13	0.0031	0.3957	

Table 6.8: Equations of the lines that describe the change in fast duration, DM, %TBF_(d), and %TBP_U with mass and body composition at weaning, sex and DML, DPL, DFL, DEE and %DEE_{fat}. The co-efficient (value) of the slope of the line and the intercept (I) are given with standard error (s.e.), F, p and r² values. Bold font indicates that the slope of the regression is significantly different from zero following Bonferonni correction (p considered significant at <0.005). * indicates parameters that describe more than 50% of the variability in the dependent variable. Red font highlights the parameter that best describes changes in the dependent variable.

	parameter	value	s.e.	I	s.e.	F _(1,16)	p	r ²
Fast duration	Sex	1.7780	1.956	25.78	9.46	0.83	0.3770	0.0104
	WM	-0.2772	0.2080	37.44	9.46	1.78	0.2012	0.0437
	TBF _(w)	-0.1710	0.3334	28.45	7.00	0.26	0.6150	0.0453
	TBP _(w)	-2.6970	1.7120	39.65	9.42	2.48	0.1348	0.0801
	TBGE _(w)	-0.0052	0.0079	29.85	7.70	0.42	0.5254	0.0352
	%TBF _(w)	0.2699	0.3044	12.52	13.99	0.79	0.3885	0.0128
	DML	-7.6520	5.8410	29.11	3.36	1.72	0.2087	0.0404
	DPL	-69.7810	31.6530	30.72	2.78	4.86	0.0425	0.1850
	DFL	1.0680	11.7350	24.68	2.51	0.01	0.9286	0.0620
	DEE	-0.0749	0.2752	25.63	2.89	0.07	0.7889	0.0576
	%DEE _{fat}	0.2059	0.0901	12.19	5.62	5.22	0.0363	0.1989
DM	Sex	0.7927	1.9420	31.36	1.373	0.17	0.6885	0.0516
	WM*	0.6556	0.1365	2.077	6.211	23.06	0.0002	0.5648
	TBF _(w)	0.9447	0.2262	12.11	4.754	17.44	0.0007	0.4916
	TBP _(w)	3.4970	1.5630	12.61	8.600	5.01	0.0398	0.1908
	TBGE_(w)*	0.0232	0.0053	9.44	5.096	19.49	0.0004	0.5210
	%TBF _(w)	0.4044	0.2861	31.21	13.147	2.00	0.1766	0.0555
	DML	6.9490	5.7220	27.92	3.292	1.48	0.2422	0.0272
	DPL	17.0020	34.9090	30.33	3.074	0.24	0.6328	0.0470
	DFL	16.1480	10.6840	28.59	2.281	2.28	0.1502	0.0702
	DEE	0.3800	0.2510	28.02	2.63	2.29	0.1496	0.0706
	%DEE _{fat}	0.0319	0.1007	29.79	6.28	0.10	0.7554	0.0559
	duration	-0.5434	0.2018	45.22	5.09	7.25	0.0160	0.2689
%TBF _(d)	Sex	0.0510	0.0366	51.31	0.02	1.39	0.2552	0.0226
	WM	<0.0001	4.7072	46.43	0.97	0.15	0.7071	0.0529
	TBF _(w)	0.0002	0.0010	40.84	0.44	2.06	0.1701	0.0589
	TBP _(w)	0.0497	0.0304	62.23	0.92	1.64	0.2188	0.0362
	TBGE _(w)	<0.0001	<0.0001	42.29	0.55	1.47	0.2423	0.0271
	%TBF_(w)	0.0043	0.0007	21.97	1.46	6.14	0.0248	0.2321
	DML	0.1930	0.3609	47.76	0.12	0.53	0.4752	0.0281
	DPL	17.3680	11.3079	46.60	0.09	1.57	0.2280	0.0325
	DFL	0.0283	1.354	50.51	0.06	0.02	0.8870	0.0611
	DEE	<0.0001	<0.0001	44.97	1.64	0.01	0.9326	0.0621
	%DEE _{fat}	1.3128	0.1063	52.42	0.41	0.12	0.7290	0.0543
	duration	1.4663	6.1691	49.23	0.39	0.02	0.8794	0.0609
%TBP _U	Sex	0.3484	0.1663	32.76	0.08	2.10	0.1670	0.0606
	WM	<0.0001	0.0022	34.91	4.58	0.01	0.9750	0.0624
	TBF _(w)	<0.0001	0.0052	41.46	2.29	0.15	0.7020	0.0525
	TBP _(w)	0.0780	0.1534	21.81	4.57	0.51	0.4860	0.0298
	TBGE _(w)	<0.0001	3.0055	40.36	2.80	0.09	0.7706	0.0567
	%TBF _(w)	0.0039	0.0043	63.83	8.82	0.91	0.3547	0.0054
	DML	0.3307	1.3933	23.43	0.46	4.19	0.0575	0.1580
	DPL	79.2705	31.0918	20.36	0.27	11.94	0.0033	0.3915
	DFL	0.0735	6.3012	35.05	0.29	0.01	0.9162	0.0617
	DEE	<0.0001	<0.0001	34.65	3.55	0.35	0.5634	0.0399
	%DEE _{fat}	0.0005	1.5600	48.47	1.84	0.95	0.3438	0.0029
	duration	0.0012	0.0028	27.57	1.80	0.42	0.5262	0.0353

Fast duration was negatively related to DPL and positively correlated with %DEE_{fat}, but these relationships were not significant after Bonferonni correction. DM was positively correlated with WM, TBF_(w) and TBGE_(w). There was a significant positive relationship between DPL and %TBP_U.

%TBF increased significantly by ~ 4% from $45.85 \pm 3.31\%$ at weaning to $50.19 \pm 4.08\%$ at departure (paired t-test: $T_{(18)} = 4.74$, $p < 0.0001$). Although %TBF_(d) was positively correlated with %TBF_(w), the relationship was not significant after Bonferonni correction.

6.3.4. Estimating days to starvation

Approximate days to starvation after the end of the fast were calculated for all animals for which body composition was estimated at weaning and departure. As in similar studies on southern elephant seal pups (McConnell et al, 2002; Biuw, 2003), it was assumed that pups did not begin to feed and continued to lose body tissue components at the same rate at sea as they had during the postweaning fast.

Animals must retain some residual fat as a percentage of body mass to maintain tissue integrity (Robin et al, 1988; Belkhou et al, 1991; Cherel et al, 1992; Friedl et al, 1994; Robin et al, 1998; Dulloo and Jacquet, 1999; Groscolas et al, 2001). Since the pups in this study increased in %TBF whilst fasting as a result of protein loss, the effect of fat depletion on starvation was not examined. Animals were assumed to starve to death when they reached either 70%, 60% or 50% of their protein mass at weaning, irrespective of remaining fat reserves, based on information from starvation in dogs and humans (Garrow, 1959; Garrow et al, 1965).

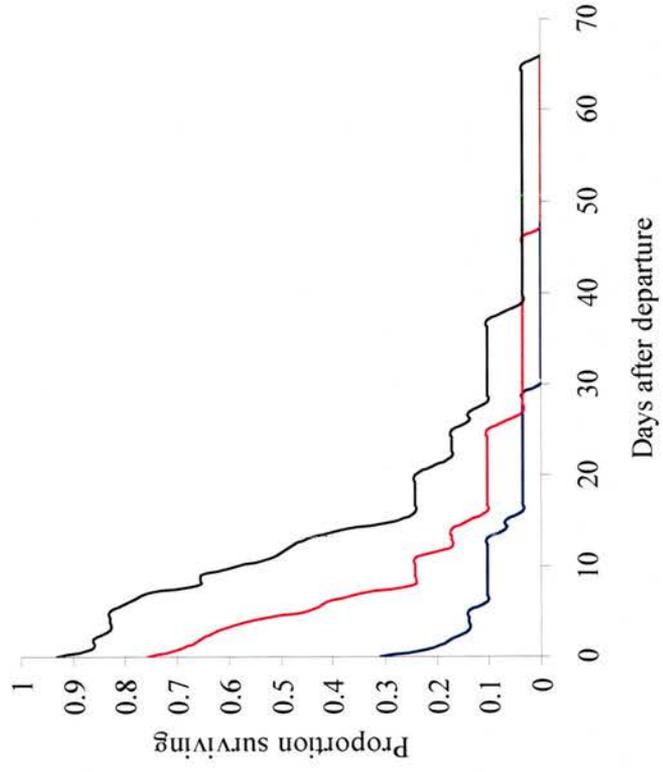
For each animal for which body composition was measured at weaning and late fast, the number of days it would be able to survive was calculated from the difference between its $TBP_{(w)}$ and its TBP at the given threshold value, divided by DPL. These values were then compared with known fast duration to determine how long each animal could survive at sea after departure before it found food.

The number of days to starvation at each critical protein depletion threshold is shown in Table 6.9. The proportion of animals expected to remain alive on each day after departure from the colony at each level of tolerated protein depletion is shown in Figure 6.7.

Table 6.9: Number of days to starvation at each critical threshold of protein depletion in total and after departure from the colony base on each individual's DPL and TBP_(w)

Critical threshold	Total					At sea						
	Minimum	Lower quartile	Median	Mean \pm s.d.	Upper quartile	Maximum	Minimum	Lower quartile	Median	Mean \pm s.d.	Upper quartile	Maximum
70%	12	18	21	23.7 \pm 9.0	26	55	-15	-6	-2	-1.2 \pm 9	0	30
60%	16	24	28	31.6 \pm 12.1	35	73	-8	0	5	6.7 \pm 11	8	47
50%	20	30	36	39.5 \pm 15.1	44	92	-3	5	12	11.3 \pm 14	16	66

Figure 6.7: Proportion of study pups expected to survive each day after departure from the colony, based on their fasting rate of protein use and on critical threshold values for depletion of protein mass to 70% (blue), 60% (red) and 50% (black) of weaning values.



6.4. Discussion

6.4.1. Effects of handling on mass and energy use

Repeated handling during both suckling and fasting, at frequencies commonly used in studies on wild seal pups, had no impact on body mass or composition changes, fuel allocation or metabolic rate. Since there were no differences between the UNKNOWN pups and LOW and HIGH groups, and the range of weaning masses and rates of mass loss were comparable to those found in grey seal pups elsewhere (Coulson, 1959; Reilly, 1991; Mellish et al, 1999; Hall et al, 2002), the pups in this study were assumed to be representative of grey seal pups in general.

Mass, body composition and the size of energy reserves at weaning were not different between pups that were handled twice during suckling and those that had never been handled prior to weaning. Stress experienced during aggressive encounters with adults occurs more frequently and likely has a greater impact on suckling behaviour, and therefore weaning mass and body composition, than stress caused by two brief handling episodes during suckling.

Energy expenditure, mass loss and the relative contribution of fat and protein to fuel use were not influenced by handling frequency during the postweaning fast. The rates of mass loss (Coulson, 1959; Nordoy and Blix, 1985; Worthy and Lavigne, 1987; Nordoy et al, 1990; Reilly, 1991; Mellish et al, 1999; Hall et al, 2002) and energy expenditure (Reilly, 1991) were comparable to those found in grey seal pups elsewhere. The handling regimes used here were of much lower intensity than those that cause lasting changes in metabolism in other animals. A sustained reduction in body mass occurs in rats exposed to three hours of restraint stress per day, even after

cessation of the stress and return of food intake to previous levels (Zhou et al, 1999). This persistent reduction in body mass is caused by long-lived alterations in metabolism after stress, primarily due to elevated rates of fatty acid utilisation and impaired glucose uptake by adipocytes (Zhou et al, 1999). By comparison, pups in the current study handled during suckling were sampled on two occasions ten days apart prior to weaning for a total of ~20 minutes each time. Postweaning handling bouts lasted a maximum of 15 minutes and were separated in time by three to ten days. Together these results indicate that the levels of stress experienced by seal pups during comparatively short handling episodes, even as regularly as every three days, were not high enough to have any discernible impact on fuel allocation, metabolic rate and therefore body mass and composition.

Animals that had only been handled three times during the postweaning period were heavier, had larger fat reserves and, consequently, greater total energy stores at departure than those that were handled more frequently. These differences were apparent at the end of the fast, despite the similarity between the same animals in initial mass and body composition, energy expenditure, mass loss and allocation of fat and protein to fuel use. The discrepancy therefore likely results from the difference in variability in fast duration rather than handling frequency. In those animals for which body composition was estimated at departure, variation in fast duration was up to three days greater in the HIGH group and six days greater in the FED group, than in the animals that were handled at low frequency. As a result some individuals in the FED and HIGH groups had the opportunity to utilise more of their energy reserves than the other pups.

6.4.2. Effects of feeding on mass and energy use

Supplementary feeding for five days after weaning altered fuel use dramatically during the postweaning period. Although the overall mass loss rate was higher in FED pups when the mass of the fish was included in the calculations, it was reduced in FED animals during the early portion of the postweaning period. FED pups showed lower rates of mass loss, compared with animals from the HIGH group, during the first seven to ten days after weaning. Some animals maintained or gained weight. This reduction in the rate of mass loss was probably partially due to the presence of digesta in the gut. It is also likely that the rate of mass loss decreased in FED animals during the early portion of the postweaning period because the additional energy supply reduced the immediate need to utilise endogenous fuel reserves.

The animals may have assimilated the energy gained during feeding, which could account for the reduction in mass loss rate. Feeding could have augmented their blubber reserves by promoting fat storage, perhaps through the elevation in cortisol levels shown in chapter 4 and an increase in free fatty acid concentration (FFA), the combination of which increase fat deposition in rats and humans (Samra et al, 1998; Mantha and Deshaies, 2000). Alternatively, the reduction in the rate of mass loss in FED animals may have resulted from a temporary increase in protein mass in response to feeding. Harp seal and northern elephant seal pups fed after a period of fasting selectively gain protein rather than fat (Worthy and Lavigne, 1983; Condit and Ortiz, 1987).

Daily energy expenditure increased dramatically in response to feeding, to levels 45-118% higher than those seen in the unfed groups. This increase is likely to

be due, in part, to the energetic cost of digestion, termed specific dynamic action (SDA) or the heat increment of feeding (HIF). HIF is caused by both the physical aspects of digestion, such as gut motility, and the biochemical processing of food, including secretion of hydrochloric acid, enzymes and hormones and excretion of nitrogenous waste (Secor, 2003). Some of the elevation in energy expenditure in the current study is likely to be a result of HIF. It is not a fixed amount, but varies with meal size and composition (Forbes et al, 1944; McCue et al, 2005). When expressed as a percentage of the energy content of particular macronutrients, HIF is approximately 6% for carbohydrates, 13% for fat and 30% for protein (Forbes et al, 1944). In pinnipeds, HIF has been reported to range from 4.7 to 16.82% of the gross energetic content of a meal and to raise metabolic rate to 1.31 to 2.13 times resting levels (Gallivan and Ronald, 1981; Markussen et al, 1994; Rosen and Trites, 1997 and references therein; Sparling 2003). The magnitude of HIF is dependent on meal size. In seals fed similar quantities (1-2kg) of herring to the meals in the current study, HIF ranged from 4.7 to 15.74% of gross energetic content of the meal, and 1.1-1.9 times resting metabolic rate. The duration of the elevation in metabolic rate was also dependent on meal size and persisted for up to 12 hours after feeding (Gallivan and Ronald, 1981; Markussen et al, 1994; Rosen and Trites, 1997 and references therein; Sparling 2003).

These reported values for HIF in pinnipeds appear to be insufficient in either magnitude or duration to account for the elevation in energy expenditure in the FED animals above that in the other groups. This may be partially due to the comparison of the energy expenditure of FED animals with fasting pups. HIF in pinnipeds has been measured relative to the postabsorptive metabolic rate in captive animals that were accustomed to regular feeding (Gallivan and Ronald, 1981; Markussen et al, 1994;

Rosen and Trites, 1997; Sparling, 2003). The metabolic rate of northern elephant seal pups after 2 weeks of feeding is 19% higher than that of fasting pups (Rea and Costa, 1992) and grey seal pups also reduce metabolic rate during the postweaning fast. The size of HIF is therefore smaller relative to the metabolic rate of postabsorptive animals than to the metabolic rate of fasting pups. The impact of HIF on the FED pups may therefore appear larger because their energy expenditure was compared to that of pups that had fasted for an extended period. However, published HIF values for pinnipeds are still insufficient to account for the higher energy expenditure of FED animals when HIF is expressed as a percentage of gross energetic content of the food. If HIF was 4.7–15.74% of the gross energetic content of the meals in this study, the additional energy required for digestion would have been only 0.44–2.19 MJ on each of the days that the animals were fed. This extra energy requirement clearly does not account for the higher DEE in FED pups than in the other groups. The disparity is even greater if the increased energy expenditure only occurred during and immediately following the feeding period, which is likely if the increased energy expenditure was due to HIF, instead of throughout the fast as the DEE values imply.

The elevated energy expenditure in FED animals may have been a result of upregulation and reorganisation of the gastrointestinal tract and other organs involved in nutrient processing in order to cope with the sudden presence of food in the gut and the change in diet. HIF tends to be relatively modest in animals that eat relatively small meals and feed regularly because the gut is never empty and thus some digestive processes are constantly underway (Secor et al, 1994; Secor and Diamond, 1995). The energetic cost of feeding is higher for animals that have previously been fasting because they must rapidly reverse the gut atrophy that occurs during starvation (Karasov and Diamond, 1983; Secor et al, 1994). The gut is an expensive organ to

maintain (Secor et al, 1994) and starvation leads to a reduction in intestinal length or mass, villus height and number, and downregulation of many transporter proteins (Karasov and Diamond, 1983). Gut atrophy can occur within 2-3 days of the onset of fasting in rodents (Karasov and Diamond, 1983) and begins after defecation in rattlesnakes (Secor et al, 1994). Although the FED pups in the current study were given supplementary food soon after weaning, they may have experienced a reduction in the size and function of the gut and other organs between the cessation of suckling and the onset of the feeding period.

Downregulation of gut function must be reversed in response to refeeding and in amphibians and reptiles the magnitude of HIF correlates with the degree of gut hypertrophy that occurs after food intake (Wang et al, 2001). Snakes that eat large meals following extended fasting periods provide an extreme example of the extensive tissue regeneration and consequent elevated metabolic rate that can occur in response to refeeding (Secor et al, 1994; Wang et al, 2001). Rattlesnakes (*Crotalus cerastes*) and Burmese python (*Python molorus*) experience up to a 17-fold increase in metabolic rate within two days of eating a meal of roughly 25% of their body mass (Secor et al, 1994; Secor and Diamond, 1995; Overgaard et al, 1999). This is due to increased secretion of hydrochloric acid and enzymes, a 5-22-fold increase in transporter activities, a twofold increase in intestinal mass and 26-53% increases in stomach, kidney and liver masses (Secor et al, 1994; Secor and Diamond, 1995; Secor, 2003). These changes persist for the duration of digesta in the gut (Secor et al, 1994). A large proportion (26->70%) of HIF in these animals is due to *de novo* protein synthesis (Secor, 2003; McCue et al, 2005). The FED pups in the current study are unlikely to have experienced such dramatic changes in organ function and metabolic rate as these snakes. Seal pups have less scope for elevating metabolic rate

than reptiles because they are endothermic and thus have a higher basal metabolic rate. In addition, their fasting period was shorter than the intervals between meals in rattlesnakes and pythons, their meals were much smaller relative to their body masses and the food was less costly to digest because it had a higher fat content and much lower protein content. However, it is likely that they required some degree of tissue restructuring to process their meals. This may have included synthesis of different enzymes from those used during suckling, to digest the novel diet not normally encountered so soon after weaning. The magnitude of their response to feeding is likely to lie between that of animals fed on a regular basis and that of snakes that feed at extremely infrequent intervals.

Unfortunately, it is unclear whether the large feeding-induced increase in energy expenditure in the FED group was a dramatic and immediate response to feeding that occurred in the early postweaning period, or whether energy expenditure remained elevated throughout the fast. It is possible that the guts of FED pups remained active after the feeding period and contributed to an ongoing higher DEE than in the other groups.

It is possible that the DEE of the fed animals was overestimated as a result of errors in the assumptions for the calculation of energy intake and assimilation. The herring used here were assumed to be relatively fat and energy dense because they were caught in the North Sea between June and September, thus values of 9.29kJ g⁻¹ and 18.5% fat (Gallivan and Ronald, 1981) were thought to be reasonable. However, Atlantic herring may be as lean as 10% fat with an energy density of only 7.4kJ g⁻¹. Recalculating DEE for the FED group gives a value of 14.82MJ day⁻¹ (data not shown), which is still roughly 4-8MJ day⁻¹ greater than the mean DEE of the unfed groups. The digestibility of the protein component of herring may be as low as 65%

(Trumble et al, 2003), which could have led to an overestimate of the contribution of protein to the energy budget of the FED animals. In addition the assimilation efficiency of the animals may have been overestimated because these animals had not experienced a fish diet before and their gastrointestinal tracts may not have been adequately prepared to digest this food. The faeces of the FED animals were grey, extremely liquid and quite fatty (pers. obs.), which may suggest that the capacity of recently weaned pups to absorb the fat and protein in the food was lower than in adult seals. However, if, as an extreme example, the contribution of the fish to energy expenditure was completely ignored, the FED animals still had a mean calculated DEE of $13.31 \text{ MJ day}^{-1}$ (data not shown), which remains between 3 and 6 MJ day^{-1} higher than the mean DEE of the unfed animals.

Further difficulties in establishing the exact size of the increase in energy expenditure, and thus the shortfall in HIF to account for the increased DEE in FED animals, were the extreme variability of DEE in the FED group, the lack of consistency in DEE between unfed groups, the particularly low DEE of unfed animals compared with the average DEE of 12 MJ day^{-1} in fasting grey seal pups reported by Reilly (1991), and the fact that DEE of all groups in this study lay within the range of fasting values reported by Reilly (1991).

The higher overall rate of mass loss and the increase in energy expenditure in FED animals was not due to an increase in the rate of protein utilisation. FED pups did not spare the additional protein from the supplementary food. Although they had used slightly less protein by departure as a relative amount of their initial endogenous reserves than the other groups, the total amount of protein used and rate of protein utilisation remained comparable with the other groups. Instead, the provision of additional food caused an increase in the rate of fat utilisation and a resulting decrease

in relative fat content during fasting. There was a tendency for FED animals to show a greater proportional contribution of fat to energy expenditure, but this difference was not large enough to be statistically significant. The impact of feeding on fuel utilisation was large enough to result in a lower relative fat content at departure in FED animals. A comparable effect of endogenous fuel reserves on fuel utilisation strategy has been demonstrated in wild fasting Subantarctic fur seal and elephant seal pups, which show greater proportional contribution of fat breakdown to energy expenditure in animals with larger absolute and relative fat stores (Carlini et al, 2001; Beauplet et al, 2003; Noren et al, 2003a; Biuw, 2003; Noren and Mangel, 2004). This is the first direct evidence that fasting grey seal pups vary fuel allocation depending on energy availability.

The infrequent sampling regime in the current study prevented determination of when feeding-induced changes in body composition, energy expenditure and fuel allocation occurred. The changes in body composition and fuel allocation reported here represent an average over the whole fast. The use of plesythmography or ultrasound would be desirable to investigate shorter-term changes in body composition.

The absence of a difference between FED and unfed animals in either mass at departure or fast duration is inconsistent with the different rates of mass loss in the FED animals. The absence of a difference between groups in departure mass is likely to be an artefact of the criteria for release from the pen in 2001. Animals were not released until they had reached 70% of weaning mass or 30kg, whichever happened sooner (chapter 2). Since the FED group took longer to reach these values, they were held for slightly longer than unfed animals. This translates into approximately four days difference in fast duration between FED and unfed animals (Table 6.3), which is

not statistically significant, probably as a result of the confounding effect of high variability in fast duration between animals and the small sample size in the FED group. It remains unclear whether FED pups would have left the colony at a higher body mass or after a longer period of fasting given the choice.

6.4.3. Effect of dexamethasone on mass, energy use and departure

Dexamethasone caused a small, short-lived increase in mass loss that may have resulted from elevated energy expenditure or a change in the rate of utilisation of either fat or protein. This brief change did not alter average rates of tissue use over the whole fast or final mass or body composition. Despite these effects of dexamethasone on fuel use, it did not affect behaviour and prompt departure from the colony.

The short-lived increase in mass loss occurred between one and four days after dexamethasone treatment. This suggests that sustained high GC levels (chapter 5) are able to alter short-term energy expenditure in seals. GCs increase energy expenditure and drive an increase in mass loss in rats and humans (Strack et al, 1995; Tataranni et al, 1996). This is the first demonstration of a direct effect of a GC on tissue loss in a fast-adapted animal.

GCs can cause mass loss through their impact on both lipolysis and proteolysis (Darmaun et al, 1988; Divertie et al, 1991; Santana et al, 1995; Weiler et al, 1997; Mantha and Deshaies, 2000; Ottoson et al, 2000; Djurhuus et al, 2002; Djurhuus et al, 2004). It has been suggested that cortisol promotes a high rate of lipolysis to maintain a largely fat-based metabolism in fasting northern elephant seal pups (Ortiz et al, 2001 a and b). In this study it was not possible to distinguish whether the dexamethasone-induced increase in mass loss was driven by an overall elevation in energy

expenditure as a result of both higher fat and protein breakdown, or a shift towards preferential use of either tissue type. The effect of dexamethasone was not large or long-acting enough to impact on mass or body composition at departure. Since body composition was measured only twice after weaning, the rates of change in body components represented the average over the entire fast duration. This made it impossible to detect short-term changes in body composition and energy expenditure that may have occurred in response to dexamethasone treatment over the same time scale as the increased mass loss rate. The small sample sizes in this study may also have prevented detection of small but significant changes in body composition and energy expenditure. Again, techniques that can measure body composition more frequently and less invasively, including plesytmography or ultrasound, would have been useful and informative in this study.

Since GCs appear to increase the rate of tissue utilisation in the short-term in grey seal pups, it is likely that natural changes in cortisol during fasting are instrumental in controlling fuel use in grey seal pups. The reduction in the rate of mass loss during the fast, mirrors changes seen in cortisol levels (chapter 4), although mass loss rate and cortisol concentrations were not directly comparable because the two parameters were measured over different time-scales. Cortisol concentrations taken at any given instant provide a snapshot of an extremely dynamic system, whereas mass loss rates are a result of slower processes over a period of three days. Clearly, the impact of GCs on energy expenditure and fuel allocation requires further attention.

There was no direct effect of dexamethasone on departure at a dose large enough to induce complete suppression of endogenous cortisol secretion for 24 to 72 hours (chapter 5). This indicates that a sustained increase in cortisol does not prompt

departure from the colony in grey seals. This conclusion is in agreement with the absence of an increase in cortisol throughout fasting or towards the end of fasting in captive grey and harp seal pups (Nordoy et al, 1990; Nordoy et al, 1993). It does not support the suggested role of increasing cortisol levels as a signal that prompts departure from the colony in Subantarctic fur seal females (Guinet et al, 2004) and northern elephant seal pups (Ortiz et al, 2001 a and b).

Elevated cortisol levels are associated with the transition into phase III in other animals (Cherel et al, 1988 a, b and c; Cherel et al, 1992; Challet et al, 1995; Robin et al, 1998; Friedl et al, 2000), but there is no evidence that healthy grey seal pups enter phase III during the normal course of the postweaning fast (Nordoy et al, 1990). The fact that pups did not leave the colony in response to high GC levels provides further evidence that departure is unrelated to the onset of phase III of fasting. These findings do not exclude the possibility that cortisol provides a cue to forage in pinnipeds when fat reserves are very low. A persistent elevation in cortisol is still likely to increase hunger and cause departure if it is perceived as a signal of low energy reserves. Dexamethasone treatment did not prompt departure in grey seal pups, which may suggest that GCs do not initiate foraging behaviour at all in these animals.

However, it is more likely that dexamethasone was administered at an inappropriate time. Many of the study pups from all three groups left the colony almost immediately after treatment, making it difficult to distinguish those pups that departed from the colony in response to endogenous cues from those that may have been prompted to leave by dexamethasone. Since some dexamethasone treated animals remained on the colony for several days after the injection, this explanation alone is not sufficient to explain the lack of a response to dexamethasone. Grey seal pups may not respond to artificially high GC levels as though it were a signal of the

onset of phase III if other hormonal and metabolic cues are not also present. For example, a dramatic change in fatty acid oxidation and BUN, prolactin and glucagon concentrations occur at the same time as elevated GC levels in animals on entry into phase III (Le Maho et al, 1981; Cherel et al, 1988 a, b and c; Robin et al, 1998; Groscolas and Robin, 2001; Bernard et al, 2002). Artificially high GC levels may not induce departure in grey seal pups if other conflicting signals take precedence. The impact of GCs on behaviour can be modified by information from a variety of sources to ensure that the appropriate response occurs. The stimulatory effect of GCs on food intake can be dampened by the antagonistic effect of insulin (Strack et al, 1995), which indicates recent feeding and prevents over-eating if cortisol levels are high for reasons other than low energy reserves. In breeding male king penguins, the abandonment of the egg that occurs in response to elevated corticosterone, glucagon and BUN levels at the end of the incubation period can be postponed for several days if the bird has not been relieved by its mate (Groscolas et al, 2000). Clearly, in grey seal pups elevated GCs alone are not sufficient to prompt departure. Although cortisol may be involved in fuel utilisation and as an emergency signal that triggers departure when fuel reserves are critically low, it is necessary to look elsewhere for endocrine cues that ordinarily prompt departure in healthy pups.

It is possible that in this study dexamethasone did not gain access to the central nervous system to impact on those neural circuits that control appetite and feeding behaviour. The same mass specific dose of dexamethasone as that used in this study, administered to rats subcutaneously, reduced GR availability to corticosteroids in the pituitary and spleen but had no effect on GR occupancy in the hypothalamus, hippocampus or cortex (Cole et al 2000). This demonstrates the inability of this relatively low dose of dexamethasone to overcome the exclusion mechanism at the

blood brain barrier. The use of a larger dose of dexamethasone, to ensure that the exclusion mechanism at the blood brain barrier was saturated, may have provided a better indication of the effects of GCs both on fuel use and behaviour in fasting seal pups.

6.4.4. Effect of energy reserves on fasting fuel utilisation

This is the first report of an impact of energy reserves at weaning on energy expenditure, the rate of utilisation of tissue components and the contribution of fat to energy expenditure in fasting grey seal pups.

Heavier grey seal pups were in better condition than smaller pups, since absolute fat and protein mass and total body energy content were strongly related to weaning mass. Relative fat content at weaning was comparable with (Biuw, 2003), or slightly higher than (Carlini et al, 2001), that seen in other phocids, and was positively correlated with weaning mass. This relationship was found previously in grey (Reilly, 1991, Iverson, 1993) and northern elephant seal pups (Noren et al, 2003a), but not southern elephant seal pups (Carlini et al, 2001, Biuw 2003). Grey seal pups appear to have a relatively consistent lean mass, such that variation in body mass between animals is largely due to differences in fat mass. Weaning mass is therefore a relatively good indicator of initial energy reserves, and specifically fat content, in these animals.

Pups that were heavier at weaning, and therefore possessed larger absolute fat and energy stores, showed higher rates of mass loss, specifically loss of fat mass, and had higher DEEs than did initially lighter pups. Daily protein loss increased with initial protein mass. In addition, the percentage contribution of fat to energy

expenditure increased with relative fat content at weaning, irrespective of the mass of the animal or its absolute fat or protein reserves at weaning. These results compare well with findings from fasting elephant seal and Subantarctic fur seal pups and polar bears, in which energy expenditure, the contribution of fat to energy use and the loss of fat tissue increase as a function of the size of initial fat reserves (Atkinson et al, 1996; Carlini et al, 2001; Beauplet et al, 2003; Biuw, 2003; Noren et al, 2003a; Noren and Mangel, 2004).

DEE increased as a function of weaning mass and correlated with absolute body fat mass and total body gross energy rather than protein. This may seem unusual given that general allometric relationships between resting energy expenditure and body mass largely result from the metabolic costs associated with increasing lean body mass (Kleiber, 1975). A relationship between metabolic rate and lean body mass may not have been apparent here because DEE was measured, which was not standardised with regard to activity levels and represented an average value over a number of days or weeks. Higher energy expenditure in this study in larger individuals is unlikely to be caused by their greater fat mass because adipose tissue does not contribute substantially to energy expenditure (Aarseth et al, 1999). Larger, and therefore fatter, animals may be able to afford greater rates of tissue breakdown, although the reason for greater energy use in fatter animals is unclear. It is unlikely that energetic costs incurred as a result of activity and movement in larger and fatter pups were substantially greater than those in smaller, leaner animals because all fasting pups spend a large proportion of their time resting and sleeping. Instead, the relationship between body mass, fat mass or gross energy and energy expenditure may be accounted for by changes in the size of fat stores rather than lean body mass

simply because there was substantial variation in fat mass but relative consistency in protein mass between individuals.

In previous studies, fat contributed 94% to energy expenditure in wild and captive grey seal pups (Nordoy and Blix, 1985; Worthy and Lavigne, 1987; Nordoy et al, 1991; Reilly, 1991). The values of proportional contribution of fat breakdown to energy expenditure seen here are substantially lower and more variable, and are comparable with values reported in fasting subadult harbour seals (Markussen et al, 1992), harp seal pups (Worthy and Lavigne, 1987) and elephant seal pups (Biuw, 2003; Noren et al, 2003a). The animals in the current study remained on the colony for longer than they may have otherwise chosen to because they were penned. They lost 7-36% of their mass at weaning, whereas free ranging pups lose 12-24% of their weaning mass (Reilly, 1991). These animals may have begun to increase protein utilisation when they were unable to leave in response to natural cues, resulting in an increase in the average relative contribution of protein to energy expenditure over the whole fast. However, if this were the case, an increase in the rate of mass loss would be expected in those animals towards the end of the fast, and this did not occur. In captive fasting grey seal pups, no signs of increased protein use were seen until after 38 days of fasting (Nordoy et al, 1992). The difference in fuel allocation between pups in this study and the captive animals may result from differences in activity levels, since the captive pups were held in confined cages and the pups here were able to range freely in a large enclosure and interact with other animals.

There was no difference in mass at weaning or departure, or mass loss rate between BC pups and non-BC pups, but BC pups fasted for longer. This was not due to a longer period spent in the pen, but because they voluntarily remained on the colony after release. It is therefore possible that the fuel utilisation strategies

employed by animals in the current study are representative of pups that chose to undergo a longer postweaning fast than average.

Despite heavy reliance on fat breakdown to meet fasting energy requirements, the fuel allocation strategy of individual grey seal pups is flexible and depends on fuel availability. Although they have substantial fat reserves in absolute and relative terms, healthy pups must still regulate fat use carefully because it must be used as fuel for metabolism and for insulation at sea. These future requirements must be accounted for in the fuel allocation strategy employed during the fast. There is clearly a greater premium on fat reserves in leaner pups and this shifts the balance of fuel use towards protein utilisation and a greater degree of lipid-sparing in these animals. Fatter, heavier southern elephant seal pups can rely almost entirely on fat without compromising their reserves at departure, whereas leaner pups have to be more frugal if they are to have sufficient fat reserves at departure (Biuw, 2003). The current results suggest that this is also true for grey seal pups.

The fuel allocation strategy is unlikely to be based on fuel availability at one point in time and is probably adjusted in response to changes in fuel demand and availability throughout the fast. The effect of initial energy reserves on energy use demonstrated here is therefore relatively simplistic and a state-dependent model similar to that developed by Noren and Mangel (2004) may be a more accurate reflection of the fuel allocation decisions made as the fast progresses.

Despite large inter-individual variation in rates of tissue utilisation and a substantial reduction in body mass during the postweaning fast, there is a remarkable degree of consistency in relative fat content between weaning and departure. This is similar to findings in other phocid seal pups (Nordoy and Blix, 1985; Rea and Costa, 1992; Carlini et al, 2001; Biuw, 2003; Noren et al, 2003a) and suggests that animals

attempt to maintain their relative level of adiposity within relatively narrow limits, despite large reductions in mass. The hormone leptin may be involved in this process if it has a similar role in body fat regulation in seals as does in terrestrial mammals (Collins et al, 1996; Friedman and Halaas, 1998). Leptin appears to be unrelated to adiposity in northern elephant seals (Gurun et al, 2001; Ortiz et al, 2001b; Ortiz et al, 2003a), but the results from those studies may be based on an inappropriate method of hormone measurement (Hammond et al, in press; chapter 3). The possibility that leptin regulates fat storage and utilisation in seals has not been explored thoroughly and requires further investigation.

Mass loss rate declined progressively during the first 13 days after weaning to a low level that did not change substantially for the remainder of the fast. A similar decline in the rate of mass loss has been reported in captive grey seals over the first two weeks of the postweaning fast, driven by a 45% reduction in metabolic rate that occurs during the first ten days of the fast (Nordoy et al, 1990). A fall in mass loss rate as a result of a fall in metabolic rate in response to fasting is well documented in pinnipeds (Rea and Costa, 1992; Arnould et al, 2001), and other mammals (Castellini and Rea, 1992) and birds (Le Maho et al, 1981; Cherel et al, 1988 a, b and c). The reduction in mass loss rate in this study was also likely due to a fall in metabolic rate, although short term changes in energy expenditure were not monitored in this study. A decline in metabolic rate may be due to inactivity and the lack of food processing in the gut (Reilly, 1991) as well as depression of cellular metabolism (Rea and Costa, 1992). The lack of an increase in the rate of mass loss later in the fast again indicates that the animals in this study did not enter phase III, despite remaining on the colony longer than they may have done if they had not been penned. This provides further

evidence that the signal to leave the colony differs from the cue to forage in penguins (Robin et al, 1998; Groscolas et al, 2000; Groscolas and Robin, 2001).

In this study, weaning mass, absolute and relative fat stores at weaning and the rate of fat loss were unrelated to fast duration, similar to previous results in free ranging grey seal pups (Reilly, 1991). However, other work on a larger sample of grey seals has shown that fatter pups fast for longer (Noren et al, 2003b). The absence of a relationship with body fat at weaning with fast duration in this study is perhaps a result of the artificial extension to the fast imposed by penning.

Fast duration tended to be shorter in pups that utilised protein at a higher rate and had a lower contribution of fat to energy expenditure. The contribution of fat to energy expenditure increased with relative fat content at weaning. In elephant seal pups, fast duration is positively correlated with lipid stores at weaning (Noren et al, 2003a), and fatter pups have a greater contribution of lipid to energy expenditure (Carlini et al, 2001; Biuw, 2003; Noren and Mangel, 2004). While the relative and absolute size of fat reserves dictate fasting fuel utilisation strategy, whereby fatter pups rely more heavily on fat breakdown and can fast for longer than leaner animals, it is the availability of protein rather than fat reserves, that appears to limit fast duration.

6.4.5. Effect of sex on fasting fuel utilisation

Sex differences in fuel use were not apparent during the postweaning fast of grey seal pups in this study and are not seen generally in fasting phocid pups (Nordoy and Blix, 1985; Nordoy et al, 1990; Reilly, 1991; Carlini et al, 2001; Noren et al, 2003a). One recent study found that the contribution of fat to energy expenditure is

greater in fatter southern elephant seal pups of both sexes, but that lean males utilise more fat than lean females (Biuw, 2003). The lack of a sex difference in fasting fuel use in the current study and elsewhere in phocids may reflect smaller sample sizes and the absence of extremes of fat and thin animals, most notably lean individuals. It is also possible that sex differences become apparent at different times between species depending on life history. For example, southern elephant seal pups can fast for up to three months, whereas free-ranging grey seal pups tend to fast for less than 30 days (Reilly, 1991; this chapter). Sex differences may not become apparent during the shorter fasting period in grey seals. Alternatively, the less extreme size dimorphism in grey seals compared with southern elephant seals may explain the absence of a sex difference in the smaller species. Differences in future developmental requirements of males and females may not impact on fasting fuel allocation so early in the life of grey seal pups.

6.4.6. Post departure survival

Protein depletion, rather than fat utilisation, had a substantial impact on the probability of survival after departure from the colony in grey seal pups. This is similar to findings in southern elephant seal pups (McConnell et al, 2002; Biuw, 2003). Larger, fatter animals were better equipped to survive because they rely heavily on fat catabolism to meet energetic requirements and minimise protein utilisation. Lighter, leaner pups were under greater energy constraints because they contributed a larger fraction of protein stores to meet metabolic costs. Higher rates of protein use whilst fasting in these animals may have compromised their ability to survive at sea because it reduced the time available to find food before the onset of

terminal starvation. As in southern elephant seal pups (Biuw, 2003), those animals that met 20-30% of energetic costs from protein utilisation may have approached critical levels of protein before departure from the colony.

The limiting effect of protein reserves on survival during starvation is well documented in other animals. Irrespective of the size of fat reserves, animals can starve to death before fat reserves are depleted due to exhaustion of protein reserves and the resultant loss of tissue integrity (Garrow, 1959; Garrow et al, 1965). Obese rats, which do not experience the biochemical changes that normally occur when fat stores are depleted at the onset of phase III, can starve to death from protein loss whilst they still possess extensive fat reserves (Cherel et al, 1992). If the pups in the current study had continued to fast until they depleted protein reserves to any of the three critical thresholds, their relative fat contents would still have been greater than 50% in most cases.

Non-obese terrestrial animals starve to death if protein reserves are depleted by 30-50% (Garrow, 1959; Garrow et al, 1965; Cherel et al, 1992). If grey seal pups were only able to tolerate a 30% reduction in initial protein reserves, 20 of the 29 animals in the current study would have starved to death before they left the colony. Similarly, if southern elephant seal pups starve to death when 30% of their initial lean mass has been utilised then 75% are expected to die within 26 days, which means that a large portion of them also reach critical protein levels before they leave the colony (Biuw, 2003). None of the pups in this study died whilst fasting on land and several of those that had approached or reached what would normally be considered critical protein levels whilst ashore survived for several months at sea, as indicated by satellite telemetry data (chapter 7). Clearly, grey seal pups, as well as southern elephant seal pups, can tolerate a greater fractional loss of their body protein reserves

than previously thought. Obese rats can withstand a 57% reduction in protein reserves compared to only 29% in their lean litter mates (Cherel et al, 1992), but the mechanism that allows fatter animals to withstand greater protein losses is unknown. Their extensive fat reserves may allow seal pups to differ in their ability to tolerate protein depletion from animals that do not routinely undergo prolonged periods of fasting as an integral part of their life history.

Grey seal pups in captivity can sustain a postweaning fast of up to 52 days without exhausting protein stores (Nordoy et al, 1990) and it has been predicted that they can sustain a fast of ~ 60 days before protein reserves are depleted by 30-50% (Nordoy and Blix, 1985). None of the pups in the current study could have fasted for this length of time if they could tolerate only 30% reduction in protein reserves. Indeed, a fast of 60 days is at the extremes of what the pups in this study were predicted to tolerate even if 40-50% of protein reserves were utilised. The relative contribution of protein to energy expenditure was higher in this study, which may explain why the animals were predicted to starve to death earlier than in captive animals (Nordoy and Blix, 1985).

The current results suggest that, if animals can tolerate 40% protein depletion then 75% of pups must find food on the day that they leave the colony in order to survive, and 50% can survive for five days before they have to feed. If pups can tolerate the loss of half their initial protein stores then 75% of animals can survive the first week at sea before they find food, and 50% can survive for 11 days after departure. This is consistent with the fact that lean tissue is not very energy dense but very metabolically costly, and as a result, animals usually carry little protein in excess of their immediate needs. The length of time that naïve grey seal pups spend at sea before they begin to forage is unknown and depends on the proximity and

predictability of food sources and the speed at which they learn to feed effectively.

Since the Isle of May is relatively close to known grey seal feeding grounds (McConnell et al, 1999) it is reasonable to assume that grey seal pups could encounter prey within their first week at sea. Captive studies show that grey seal pups rapidly learn hunting techniques within five days of first encountering live fish (Kastelein et al, 1995).

The assumption that animals continue to utilise fuel reserves at the same rate at sea as on land may be incorrect. Metabolic requirements are likely to change dramatically between the inactive fasting state on land and the onset of diving behaviour in cold water in grey seals. For example, metabolic rate is higher at sea than whilst fasting on land in suckling ringed seals (Lydersen and Hammill, 1993) and female Antarctic fur seals (Arnould et al, 1996). If energy expenditure of grey seal pups also increases when they go to sea, they may have less time to find food than predicted here. In addition, higher metabolic costs at sea will be exacerbated if fat reserves are depleted to the point at which the blubber layer is too thin to provide adequate insulation and a further increase in energy expenditure is needed for heat production. The onset of starvation will be accelerated further in pups that reach this point.

Captive grey seals have a lower metabolic rate whilst diving than when resting at the surface (Sparling and Fedak, 2004). Free-ranging pups at sea may also undergo metabolic depression on a dive-by-dive basis. Over a longer timescale, at-sea metabolic rate decreases as the proportion of time at sea spent diving increases in female Antarctic fur seals (Arnould et al, 1996). Similarly, grey seal pups may reduce energy expenditure by spending as great a proportion of time underwater as they are physiologically able, given constraints on oxygen storage capacity and the ability to

manage those oxygen stores. Pups could survive for longer at sea if diving reduces energy expenditure sufficiently to defer the costs of increased activity and thermoregulation until they can feed effectively. However, it is unclear whether this is a feasible energy saving strategy. Diving itself may not be the main cause of the lower metabolic rate seen in foraging Antarctic fur seals that spend a greater proportion of time diving (Arnould et al, 1996). It may instead result from a decrease in time spent swimming at the surface between food patches, and an increase in time spent resting at the surface, which causes an overall reduction in energy expenditure (Arnould et al, 1996). There may be no net benefit to increased time spent diving if elevated metabolic rates at the surface or during haul-out periods compensate for metabolic depression during diving. The possibility that grey seals can adjust metabolic rate in response to changes in activity levels and food intake is currently under investigation in the captive facility at SMRU. The observation that animals can gain more weight at low food intake and high activity levels than when food intake is higher and activity levels are reduced may suggest seals make some metabolic adjustments of this nature (C. Sparling, *pers comm*).

Pups may be able to survive for much longer than predicted if they manage to consume even small amounts of food. Northern elephant seal and harp seal pups fed after the postweaning fast can maintain weight and gain protein mass even when energy intake is well below metabolic requirements by preferentially utilising fat reserves and laying down protein obtained from food (Worthy and Lavigne, 1983; Condit and Ortiz, 1987). Wild grey seal pups may be able to delay or prevent starvation in a similar way once they have gone to sea by relying on blubber reserves for energetic needs and feeding opportunistically on any available prey, even low quality items, to bolster protein reserves.

6.4.7. Summary

Stress caused by repeated handling at frequencies commonly used in studies on wild seals does not cause long-term changes to fuel use in fasting grey seal pups. A sustained (up to 72 hours) elevation in GC concentration drives an increase in overall tissue utilisation. This suggests that natural changes in cortisol levels could contribute to the regulation of fuel use during fasting. This effect is relatively short lived and may only persist for the amount of time that the GC levels are high. The short-term impact of high GC levels on fuel allocation in fasting seal pups is unknown.

Elevated GCs alone are not sufficient to cause departure from the colony. This supports the findings in chapter 4. It suggests that while increased cortisol levels could be a subsidiary signal that acts in conjunction with other hormonal and metabolic changes to alter behaviour under some circumstances, perhaps for example starvation, it is not a part of the normal cue that prompts pups to go to sea.

Energy availability, both from endogenous reserves and from supplementary feeding, has a considerable impact on long-term fuel disposal strategy in grey seal pups. Fatter animals or those provided with additional food, rely more heavily on fat to meet energetic requirements than leaner pups. This is true of both males and females and there is no apparent difference in fuel utilisation between the sexes.

Fast duration is limited by the rate of protein depletion and by the contribution of fat to energy expenditure. The fuel allocation strategy employed during fasting has a large impact on survival after departure from the colony. Leaner animals, which utilise their protein reserves more rapidly than their fatter conspecifics, have a much shorter time margin in which to find food. Although grey seal pups appear to have a greater tolerance to protein depletion than other mammals and do not enter phase III

of fasting whilst ashore, they may reach critical protein levels soon after departure from the colony. These animals are therefore under considerable pressure to begin to feed very soon after they have gone to sea for the first time, despite possessing extensive fat reserves.

Chapter 7

Development of diving and foraging capabilities in grey seal pups in their first year at sea

7.1. Introduction

7.1.1. Summary

Body fat reserves contribute to increased probability of survival during the first year in a variety of pinniped species, including southern elephant seals (McMahon et al, 2000; Biuw, 2003), northern fur seals (Baker and Fowler, 1992) and Weddell seals (Burns, 1999). First year survival in grey seals is higher for animals that are heavier and possess greater fat reserves at weaning, and is greater in females than in males (Hall et al, 2001; Hall et al, 2002). The mechanism that underlies these trends may be linked to early development of diving and foraging skills. Body size and composition not only determine the amount of energy available to sustain pups as they learn to forage, but also impact on the ability to dive through their effects on muscle power, oxygen storage capacity, metabolic rate and buoyancy. This chapter describes preliminary investigations of the effect of sex and body condition on movements, behavioural characteristics and development of physiological capabilities of twenty grey seal pups, tracked using satellite telemetry during their first year.

7.1.2. First-year survivorship

Female grey seals expend a considerable amount of their stored energy in rearing pups because they fast throughout lactation and, in doing so, they incur substantial costs. They lose roughly 40% of their post-partum mass and may suffer reduced pupping success in the subsequent year (Fedak and Anderson, 1982;

Anderson and Fedak, 1987; Pomeroy et al, 1999). This large expenditure may be a good investment if it results in enhanced first-year survival probability for the pups because they are bigger and in better condition at weaning as a result (Hall et al, 2001; Hall et al, 2002).

The proximate mechanism that drives differences in survivorship is unknown. Survival to the end of the first year is dependent on events after weaning as well as maternal provisioning, and is likely to be influenced by the early development of diving and foraging skills. Individual characteristics that may contribute to the development of these skills include sex, body size and composition, differences in the allocation of fuel reserves to energy expenditure, the duration of fasting on land and experience at sea.

7.1.3. At-sea behaviour of grey seals

Adult grey seals spend 90% of their time at sea submerged (Thompson et al, 1991; Fedak and Thompson, 1993a), performing dives usually between three and seven minutes in duration (Fedak and Thompson, 1993b). They undertake both long distance trips (>100km) between known haul out sites, and repeated, short duration trips from the same haul-out site to discrete foraging grounds, often within 40km offshore (Thompson et al, 1991; McConnell et al, 1992; Hammond et al, 1993; McConnell, et al, 1999). Most of these return foraging trips last for three days or less and account for 12-14% of the activity budget (Thompson et al, 1991; Hammond et al, 1992; McConnell et al, 1993; McConnell et al, 1999). However, the amount of time allocated to these activities varies considerably between years, sites and

individuals (Hammond et al, 1992; McConnell et al, 1992; McConnell et al, 1993; McConnell et al, 1999).

Since adult behaviour patterns, on the whole, are likely to represent successful foraging strategies, pups that develop similar behavioural patterns during their first year of life should be more likely to survive. However, pups may be unable to utilise the same foraging areas or strategies as a result of physiological or behavioural limitations due to their small size and relative inexperience. The pressure to begin to forage and the areas and depths available to pups may vary with size and condition, and this is likely to be reflected in their movements and behaviour.

7.1.4. Physiological constraints on diving capability

The ability to remain at depth for long enough to find and eat sufficient prey may be contingent upon adequate oxygen storage capacity and effective management of those reserves, both within individual dives and over more extended periods of time. The acquisition of adequate foraging skills by grey seal pups may therefore be limited by their physiological capabilities if feeding requires animals to remain submerged for a long time, for instance if prey is scarce, difficult to capture or is found in deep water.

The ability to rely on aerobic metabolism whilst submerged maximises the time that can be spent underwater over periods longer than individual dives. Dives in which a switch to anaerobic metabolism is necessary to meet energetic needs require a long post-dive surface recovery period or subsequent repeated short, aerobic dives to eliminate the resultant lactate accumulation (Fedak and Thompson, 1993a). The

theoretical aerobic dive limit (tADL) is the maximum dive duration beyond which net whole-body lactate production is expected to begin as a result of anaerobic metabolism, calculated from estimates of body oxygen reserves and metabolic rate (Kooyman et al, 1980). Time is maximised by performing a greater number of shorter dives that lie within the tADL, rather than fewer, prolonged dives that exceed it (Fedak and Thompson, 1993; Boyd, 1997). Most dives performed by adult grey seals are within their tADL, thus anaerobic dives are infrequent in adult grey seals. Dives exceeding the tADL are rarely followed by extended surface periods or short aerobic dives, which suggests that these animals employ oxygen-conserving strategies whilst at depth (Fedak and Thompson, 1993a and b). Evidence to support this comes from captive grey seals diving voluntarily in a quasi-natural setting, which exhibit lower metabolic rates during diving than when resting at the surface (Sparling and Fedak, 2004).

Development of diving capabilities, in terms of oxygen storage capacity and cardiovascular control, has been suggested as one of the major reasons for the prolonged land-based fast that occurs after weaning before the seal pups leave the colony for the first time (Thorson and Le Boeuf, 1994; Noren et al, 2003b). Oxygen storage capacity increases as animals grow and develop, as a result of increased muscle tissue, blood volume, haematocrit and haemoglobin and myoglobin concentrations (Bryden and Lim, 1972; Kodama et al, 1977; Thorson and Le Boeuf, 1994; Noren et al, 2000; Noren et al, 2003b). Oxygen utilisation in diving seals is managed by selective vasoconstriction (Davis et al, 1983; Ostholm and Elsner, 1999), which diverts blood flow to the brain and heart, regulates heart rate (Reed et al, 1994) and promotes the release and utilisation of oxygen bound to myoglobin in underperfused tissues, including the working muscles. This cardiovascular control

develops as pups mature (Thorson and Le Boeuf, 1994). Those pups that undertake a longer fast, which are often those that are larger at weaning, are thus likely to benefit from greater breath-hold capabilities when they first go to sea (Hindell et al, 1999; Noren et al, 2003b).

Juvenile animals have elevated mass-specific metabolic rates compared to adults. Grey seal pups in their first year of life have metabolic rates that average 1.48 to 2.66 times those predicted for an adult conspecific of the same mass (Boily and Lavigne, 1997; Sparling, 2003). Oxygen storage capacity is proportional to body mass, but oxygen consumption is proportional to $\log^{0.75}$ mass. Given the effect of age, smaller animals should therefore be unable to remain submerged for as long as larger individuals.

Body composition as well as mass can influence both oxygen storage capacity and utilisation. Body size and composition could influence metabolic requirements through effects on buoyancy and thermoregulation. For example, descent rate is significantly affected by changes in buoyancy in adult grey seals and juvenile northern elephant seals (Webb et al, 1998; Beck et al, 2000). Relatively higher buoyancy in fatter animals may place additional constraints on already limited oxygen reserves if they experience greater costs on descent or maintaining depth than leaner animals, and cannot compensate during the ascent phase. Alternatively, it is possible that greater insulative capacity of a thicker blubber layer in fatter pups reduces the oxygen demand for thermoregulation, thus allowing animals to remain underwater for longer.

7.1.6. Evaluating dive performance in wild seals

The maximum physiological diving capabilities of free-ranging seals cannot be measured directly, but can be inferred from behavioural information collected remotely using satellite telemetry (Bennett et al, 2001). Both physiological and ecological constraints influence diving behaviour, as measured by the amount of time spent submerged during individual dives and over longer periods of time (hours to days), and the amount of time spent at the surface between dives. Maximum physiological capabilities are likely to be apparent in the extremes of these elements of their diving behaviour because animals will occasionally choose to push themselves to their limits. The diving capabilities of adult southern elephant seals have been inferred from the 95th percentile of dive durations (Bennett et al, 2001). This approach attempts to separate behavioural choice from physiological limitations and is used here to examine the early development of the maximum ability of grey seal pups to remain submerged.

Dive duration is to some extent dependent on dive depth because a minimum length of time is required to swim a given distance (eg. DeLong and Stewart, 1991; Hindell et al, 1992). This study attempts to control for the potentially confounding effects of depth on maximum dive durations. However, dive durations beyond the minimum time required reflect the choice of the animal to remain submerged. Seals may therefore choose to stay underwater for as long as they are physiologically able, irrespective of dive depth, such that maximum dive durations are likely to be relatively independent of depth.

The amount of time animals spend at the surface between dives is again influenced by both ecological and physiological factors. The post-dive surface interval required depends, in part, on the duration of the preceding dive, since more time is required after longer dives to replenish oxygen stores and eliminate lactate (Fedak and Thompson, 1993). The minimum time at the surface needed after a dive of a given duration will be influenced by the size of oxygen reserves, the degree to which those reserves are depleted during diving, the extent to which they are replenished and the rate at which oxygen is loaded into the blood and tissues whilst at the surface. A similar approach to that described above is used here to explore the ontogeny of minimum (5th percentile) surface intervals after dives of given durations.

Foraging opportunities tend to be maximised if animals spend as much time as possible underwater in profitable areas over periods longer than individual dives (Boyd, 1997). The total proportion of time animals can spend submerged relies on a combination of both the ability to remain submerged and the minimum time that is needed at the surface. The maximal time that can be spent at spent depth for long periods may not involve repeated long dives if that strategy requires anaerobiosis. The maximum time allocated to diving over periods of several hours is thus another measure of diving performance used here to investigate development of diving capabilities.

7.1.7. Behavioural and survival consequences of constraints on diving capability

Physiological limitations, caused by body size and condition, constrain the behavioural options and foraging strategies of juvenile elephant seals and Weddell

seals (Thorson and Le Boeuf, 1994; Burns and Castellini, 1996; Burns et al, 1997; Burns, 1999; Hindell et al, 1999; Irvine et al, 2000). Northern elephant seal pups exceed their tADL on 30-47% of dives during their first three months after weaning (Thorson and Le Boeuf, 1994) and underyearling southern elephant seals exceed their tADL on an average of 22% of dives (Irvine et al, 2000). Weddell seal pups and juveniles feed at similar depths to adults but must operate much closer to their physiological diving capabilities and often have to exceed their tADL to do so (Burns, 1999).

Yearling Weddell seals and southern elephant seal pups show differences in diving and foraging strategies related to body size (Burns et al, 1997; Hindell et al, 1999; Irvine et al, 2000). Heavier southern elephant seal pups perform deeper and longer dives than lighter conspecifics (Hindell et al, 1999; Irvine et al, 2000). Small underyearlings are less able to follow vertically migrating prey to deeper depths than larger animals (Irvine et al, 2000). At any given dive depth, smaller Weddell seals are able to spend less time at the bottom of the dive, where foraging is assumed to take place, than larger animals. Larger animals perform longer shallower dives than smaller individuals, which perform deeper, more frequent dives (Burns et al, 1997). This strategy in smaller Weddell seal pups seems to compound the problem of limited oxygen reserves and the reason that they adopt it is unclear.

Although small body size may limit the ability to remain submerged, it may not compromise survival if the pups employ different foraging strategies that are sufficient for their requirements (Burns et al, 1997; Irvine et al, 2000). Small southern elephant seal underyearlings that forage successfully gain more blubber relative to their body mass than larger animals and are thus not disadvantaged by their small size (Irvine et al, 2000; Biuw, 2003).

The ability to remain submerged may be most critical in the initial few days and weeks at sea. Grey seal pups must rapidly learn to locate prey and forage effectively before fuel reserves are depleted to dangerous levels. Critical levels of protein depletion may be reached very shortly after departure from the colony despite the presence of substantial fat reserves (Biuw, 2003; chapter 6). Naïve pups are therefore under considerable pressure to begin to forage (chapter 6) and this pressure is greater in leaner animals, which utilise protein reserves more rapidly than fatter conspecifics (Dulloo and Jacquet, 1999; Biuw, 2003; chapter 6). The maximum diving capabilities of grey seals in the first days and months at sea, in relation to body size, condition, sex and the length of time spent fasting on land, are explored here.

Survivorship cannot be estimated accurately using satellite telemetry because it is difficult to distinguish tag failure from death of the animal. These devices may cease transmitting as a result of battery failure, antenna breakage or because they have become detached, all of which are independent of the fate of the animal. Despite the possibility of these confounding factors, however, this study investigated the relationship between body size, condition and sex and the duration of the tracking period as a rough index of potential survivability of the pups.

7.1.8. Experimental aims

The aims of this chapter are to describe the movement patterns of grey seal pups in their first year of life. Differences in mass, body composition and fast duration between pups that were tracked and those that were not are examined initially to ensure that the pups in this study are representative of all the pups in the thesis.

Changes in physiological diving capabilities, including maximum dive duration, minimum surface interval and proportion of time spent diving, were investigated using the extremes of these values (95th percentile and 5th percentile) as indices of maximum diving abilities. These changes are related to sex, mass and body composition to determine whether they may provide a mechanism for the differential survivorship seen in grey seal pups. Diving capabilities of pups are also compared with those of adults and two yearlings that had spent their first year in captivity to examine differences in dive performance between animals of different age classes and experience. The results presented are not a comprehensive analysis of all the available data and avenues for intended further research are discussed.

7.2. Materials and Methods

7.2.1. Attachment of Satellite Relayed Data Loggers

Satellite Relayed Data Loggers (SRDLs) were deployed on a subset of 21 of the 58 study animals in this thesis (Table 2.1). SRDLs weigh roughly 400g, which equates to 0.06-1.5% of the range of body masses recorded in the animals in this study. Mass and, where possible, body composition both at weaning and departure were estimated for each animal as described in chapter 6.

Pups were anaesthetised with a 0.025mg kg^{-1} intravenous dose of Zoletil₁₀₀ (Virbac, France). The fur behind the head at the base of the skull was dried and cleaned with ethanol and acetone before the SRDL was glued on using a two-part, rapid setting epoxy resin (RS components) as described elsewhere (Fedak et al., 1983; McConnell et al, 1999). The SRDLs were positioned on the back of the neck just behind the head to allow the aerial to emerge when the pups surfaced but to minimise any impact of the SRDL on mobility of the head and neck and to avoid the antenna entering the pups' field of vision. The epoxy resin was allowed to harden and the pups allowed to recover fully from the effects of the anaesthetic before they were released.

7.2.2. Data collection

The way in which dive information is collected using SRDLs is described in detail elsewhere (McConnell et al, 1992; Fedak et al, 2001) and is described briefly here. The SRDL consists of a dive computer and an ultra high frequency (UHF) transmitter. The computer compiles information from sensors detecting pressure,

surface periods when dry, and swimming speed to create a compressed record encoding dive depth, duration, surface interval and swim speed for individual dives.

In addition, dive behaviour is summarised for six-hourly periods and placed into one of three categories: “dive” represents periods of time when the submergence sensor indicates that the SRDL is wet and the pressure gauge records a depth of over 6m for longer than six seconds. Behaviour is classified as “cruise” when depth is above 6m, and as “haul-out” when the sensor is dry for longer than 240 seconds.

The compressed information about individual dives and the summary data are stored and selected for transmission by a pseudo-random process to ensure that all times of day are equally represented irrespective of diurnal changes in animal behaviour and satellite availability.

When the antenna of the SRDL is at the water surface the transmitter attempts to relay the data, at 40-second intervals, to Service Argos UHF receivers on board three polar orbiting National Oceanic and Atmospheric Administration (NOAA) satellites. The Argos system identifies the transmitter and estimates its location based on the Doppler shift of the signal from the SRDL as the satellite passes through its arc of visibility. The data are sent to a ground station and processing centre where they are assigned an index of location accuracy, termed Location Quality (LQ), from -2 to 3, before they are sent to the Sea Mammal Research Unit (SMRU), where they are stored in an Oracle database.

7.2.3. Data processing and visualisation

7.2.3.1. Movements

To account for potential error in location fixes, as a result of irregular uplinks, the data were filtered to remove those locations that would require animals to travel in excess of their assumed maximum sustainable speed capabilities of 2 m s^{-1} (McConnell et al, 1992; McConnell et al, 1999). An algorithm was applied to the raw locations that provided an estimate of position at six-hourly intervals (J. Matthipoulos: SMRU, unpublished). These smoothed locations were used to produce maps of the movements of grey seal pups in ArcView 3.2. One animal (Zap) was excluded from analysis since uplinks from the tag were extremely sporadic and unreliable.

Dive information was visualised and explored using the MAMVIS system, which allows seal movements to be animated in three dimensions against a background of parameters such as coastline and bathymetry (Fedak et al, 1996). Haul out sites used by pups were identified and their latitude and longitude were used to calculate the distance away from the nearest haul out at each smoothed location. Distance from the nearest haul out throughout the tracking period was plotted using R.1.9.1 (R Development Core Team, 2003; Ihaka and Gentleman, 1996). The trip duration was defined as the number of days between haul-out periods.

7.2.3.2. Dive performance

Dive duration, post dive surface interval and percentage time spent in “dive” throughout the tracking period were plotted. The duration of the tracking period for

each animal was divided into four-day time bins. This bin-size was a long enough period for each bin to contain some dives that stretched an animal to its physiological limit, whilst allowing adequate resolution of rapid temporal changes in dive parameters. The 95th percentile of both dive duration ($d95$) and of percentage time spent in “dive” ($\%dive$) within each time bin were used as indices of the maximum capabilities of an animal to remain submerged for individual dives and for six-hour summary periods, respectively. The 5th percentile of post-dive surface interval ($SI5$) was used as index of the minimum time an animal required at the surface after a dive.

For each individual, $d95$ was determined within each time bin for dives of all depths. In addition, to account for the potentially confounding effects of increasing dive depth on dive duration as animals moved into deeper water, $d95$ was also determined for dives within each of four depth bands ($max\ depth$) that included the range of depths performed by all animals (10-25m, 25-50m, 50-75m and 75-100m).

Since longer surface intervals are likely to be required after longer dives, all dives for each animal were placed into one-minute duration bands ($duration$), over the range of longer dives performed by the animals (2-5minutes). For each individual, $SI5$ was then determined within each time bin for each duration band.

For the six-hour summary information, periods containing haul-out behaviour were excluded to ensure that the behaviour represented purely at-sea behaviour. For each individual, $\%dive$ was then determined within each time bin.

$d95$, $\%dive$ and $SI5$ at the onset of diving ($_{first}$) and the maximum ($d95_{max}$ or $\%dive_{max}$) or minimum ($SI5_{min}$) that occurred within three months of the onset of diving were identified in each case. The mid-point date of the time bin in which those values occurred and their timing with regard to weaning and departure were determined. The changes in these values were not linear over time and the rate and

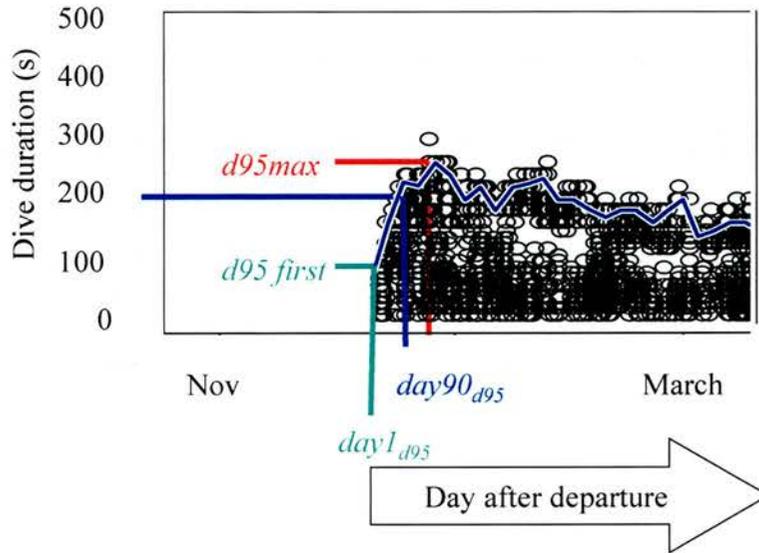
pattern of change varied between animals. As a result, the number of days after departure for each animal to achieve either 90% of its $d95_{max}$ ($day90_{d95}$) and $\%dive_{max}$ ($day90_{\%dive}$) or 10% of $SI5_{min}$ ($day10$) were used as indices of the amount of time taken to approach maximum capabilities. The number of days from departure to the mid-point of the time bin in which dives of each depth (for $d95$) or duration (for $SI5$) first occurred was also recorded ($day1_{d95}$) and ($day1_{SI5}$). The way in which these values were derived is shown for dive duration as an example in Figure 7.1.

These values were also obtained for two female yearlings, Kylie and Nora, that had spent their first year in captivity, during which they had extensive experience of diving in a quasi-natural setting. These animals were fitted with SRDLs immediately prior to release from the facility at the SMRU in November 2002. This provided a comparison between animals of a similar size with no prior diving experience (pups) with animals that had extensive diving experience but were similarly inexperienced in terms of foraging in the wild (yearlings).

7.2.4 Data analysis

Statistical procedures were performed in Minitab13.32 or R 1.9.1. The best linear models (LMs) or linear mixed models (LMEs) to describe the variation in the dependent variables were derived by stepwise regression as described in chapter 2. Details of the explanatory variables used are given below. All values are reported \pm standard deviation, unless otherwise stated.

Figure 7.1: Illustration of technique used to derive first and maximum values for d_{95} , and the day after departure that dives were first performed ($day1$) and dives within 90% of the max value were first performed ($day90$). A complementary method was used to derive the same values for $SI5$ and $\%dive$



7.2.4.1. Explanatory variables

Body mass, body fat as a percentage of body mass (%TBF) and total body protein (TBP), were used in the analyses investigating the effect of size and condition on dive parameters. %TBF represents body condition and TBP is likely to be related to oxygen storage capacity and metabolic costs.

Date of departure was defined as the day on which the animal showed a rapid transition from continuous haul-out behaviour at the breeding site to cruising and shallow diving and movement away from the colony. The date of departure was verified where possible with resighting information from the daily searches of the island described in chapter 2 and there was a close match between the two methods. Fast duration was defined as the difference between the date of departure derived from SRDL information and the weaning date (chapter 2).

Differences in fast duration, weaning mass (WM) and departure mass (DM), between animals with and without SRDLs were examined using T tests to determine whether pups with SRDLs were representative of all the study pups. Differences in %TBF and TBP were examined using MANOVA, since these variables were initially derived from the same mass and total body water information (chapter 6).

Sex differences in fast duration, mass and body composition information at weaning and departure of the pups fitted with SRDLs were also examined to determine whether effects of any of these variables in later analyses could be attributed to sex differences or, conversely, whether sex effects may primarily be caused by these other variables.

7.2.4.2. Dive performance

Explanatory variables included in each analysis are shown in Table 7.1. The time taken for each animal to achieve its maximum diving capabilities was examined in relation to sex, year, fast duration and condition. The effects of sex, year, day since weaning, day since departure, WM and DM on $d95_{\text{first}}$, $\% \text{dive}_{\text{first}}$, day1_{d95} and day1_{SIS} were investigated using LMs.

The maximum dive durations performed by an individual in each *max depth* category are not independent. The same is also true of minimum surface intervals in *duration* bands. To account for this lack of independence, LMEs were used to investigate the effects of the same variables on $d95_{\text{first}}$ (when depth category was included) and SIS_{first} .

In each case, the effects of total body protein and percentage body fat at weaning (TBPw and %TBFw) and at departure (TBPd and %TBFd) were also examined, using the subset of animals for which body composition had been estimated at both points (chapter 6). Where there was no effect of departure body composition, the larger subset of animals for which only weaning body composition estimates were available was used to investigate the effect of TBPw and %TBFw. Those models that incorporated all animals are denoted by *a*, whereas those that included only pups for which body composition information was available at weaning or departure are denoted by *w* and *d* respectively.

LMs and LMEs were also used to investigate variation in $D95_{\text{max}}$, $\% \text{dive}_{\text{max}}$ and SIS_{min} . In addition to the explanatory variables used previously, the effect of the first value in each case was included to account for the possibility that maximum capabilities are partially dependent on initial capabilities. The effects of first and max

values, year, sex, fast duration, WM, DM, and body composition information, where available, on $day90_{d95}$, $day90_{\%dive}$ and $day10$ were also investigated.

Table 7.1: List of explanatory variables used in stepwise LMs and LMEs to investigate variation in first and max (or min) values, and $day1$ and $day90$ (or $day10$). * indicates where those variables were appropriate

Subset of seals	first	max (or min)	$day1$	$day90$ (or $day10$)
a,w,d	WM	WM	WM	WM
	DM	DM	DM	DM
	Fast duration	Fast duration	Fast duration	Fast duration
	Year	Year	Year	Year
	Sex	Sex	Sex	Sex
		Day after departure	Day after departure	Day after departure
	First value	First value	First value	First value
	* <i>Max depth</i>	* <i>Max depth</i>	* <i>Max depth</i>	* <i>Max depth</i>
	* <i>Duration</i>	* <i>Duration</i>	* <i>Duration</i>	* <i>Duration</i>
w,d	%TBFw	%TBFw	%TBFw	%TBFw
	TBPw	TBPw	TBPw	TBPw
d	%TBFd	%TBFd	%TBFd	%TBFd
	TBPd	TBPd	TBPd	TBPd

7.2.4.3. Track duration

LMs were used to explore possible relationships between sex, year, WM, DM and body composition, where known, and the duration over which individuals were tracked.

7.3. Results

7.3.1. Differences between animals with and without SRDLs

There were no differences in fast duration, WM or DM between animals with and without SRDLs (Table 7.2). %TBF and TBP were not significantly different between pups with and without SRDLs either at weaning (MANOVA: $F_{(2,43)} = 2.066$, $p = 0.139$) or at departure (MANOVA: $F_{(2,26)} = 0.523$, $p = 0.599$)

Table 7.2: Comparison of fast duration, WM and DM from pups with SRDLs and those without. Mean values \pm standard deviation are given for each category and T, degrees of freedom (df.) and p values for each T test. # denotes tests that assumed unequal variance

	SRDL	No SRDL	T	d.f.	p
#Fast duration	22.85 \pm 7.97	21.27 \pm 5.35	0.78	29	0.439
#WM (kg)	46.99 \pm 8.46	43.69 \pm 5.37	1.58	27	0.126
DM (kg)	34.08 \pm 5.44	32.25 \pm 4.19	1.38	51	0.175

7.3.2. Sex differences in pups with SRDLs

The results of the sex comparison of fast duration and body condition measures in pups with SRDLs are shown in Table 7.3. Female pups fasted for longer than males and, in pups for which there were estimates of body composition at departure, males had a significantly higher DM than females.

Table 7.3: Results of T tests comparing fast duration and the body condition measures used in this chapter between male and female pups. Mean values \pm standard deviation, number of pups (*n*), T statistic, degrees of freedom (*d.f.*) and *p* value are given in each case. The tests refer to either all animals fitted with SRDLs (*a*) where *p* is considered significant for WM and DM at 0.025, or the subset of those pups for which body composition was available at weaning (*w*), where *p* is considered significant for mass and body composition variables at 0.0125, and at departure (*d*), where *p* is considered significant for mass and body composition variables at 0.008.

	variable	male	n	female	n	T	d.f.	p
	Fast duration (days)	19.18 \pm 5.16		26.33 \pm 8.62		2.25		0.038
<i>a</i>	WM (kg)	46.30 \pm 7.20	11	47.80 \pm 10.20	9	0.40	18	0.697
	DM (kg)	33.32 \pm 4.37		31.98 \pm 5.31		0.62		0.543
	Fast duration (days)	19.10 \pm 5.86		27.57 \pm 8.89		2.38		0.031
<i>w</i>	WM (kg)	47.11 \pm 7.04	10	48.60 \pm 11.1	7	0.35	15	0.733
	DM (kg)	33.71 \pm 4.40		30.51 \pm 4.62		1.45		0.169
	%TBFw	42.83 \pm 2.33		46.17 \pm 2.95		0.05		0.963
	TBPw (kg)	5.68 \pm 0.62		5.82 \pm 1.04		0.37		0.718
	Fast duration (days)	23.33 \pm 2.73		30.00 \pm 6.72		2.25		0.048
<i>d</i>	WM (kg)	48.92 \pm 6.06	6	48.2 \pm 12.1	6	0.14	10	0.894
	DM (kg)	33.87 \pm 3.94		29.03 \pm 2.68		2.48		0.032
	%TBFw	42.87 \pm 2.06		46.63 \pm 2.93		0.23		0.820
	TBPw (kg)	5.91 \pm 0.50		5.69 \pm 1.07		0.45		0.660
	%TBFd	49.49 \pm 5.44		44.57 \pm 6.26		1.02		0.332
	TBPd (kg)	3.78 \pm 0.91		3.62 \pm 0.56		0.34		0.740

7.3.3. Movements of grey seal pups at sea

The movements of the grey seal pups in the study are shown in Figure 7.2. They show large variability in movements between individuals and wide dispersal from the colony, up to 700km from the Isle of May. Distance from the nearest haul-out over the duration of each track is shown in Figure 7.3. Summary information about trip duration and the total tracking period duration for each animal is given in Table 7.4.

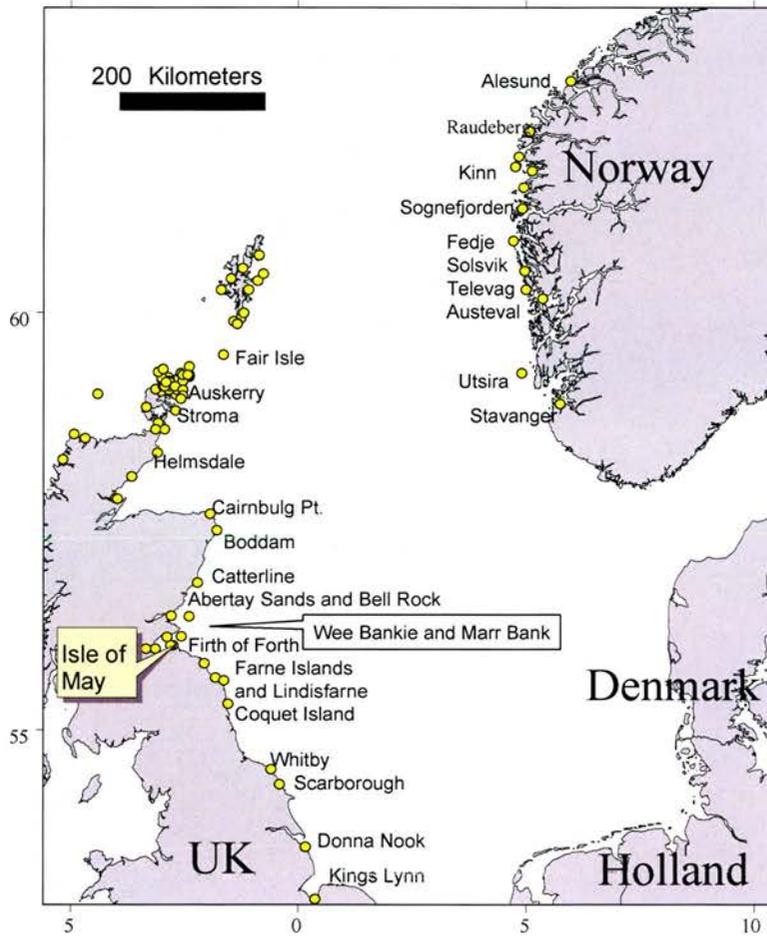
Movements of most pups were initially coastal and usually in the locality of the Isle of May, in and around the Firth of Forth and St Andrews Bay. Some pups ventured further afield, north along the coast south of Aberdeen and south to the Farne Islands. This early phase was punctuated by frequent periods of haul out, often

at sites used by other age classes of grey seals, and was extremely variable in length, from four up to roughly 42 days. In contrast to this coastal pattern, four animals swam rapidly out to sea on departure from the colony and showed little coastal movement in the early part of the tracking period.

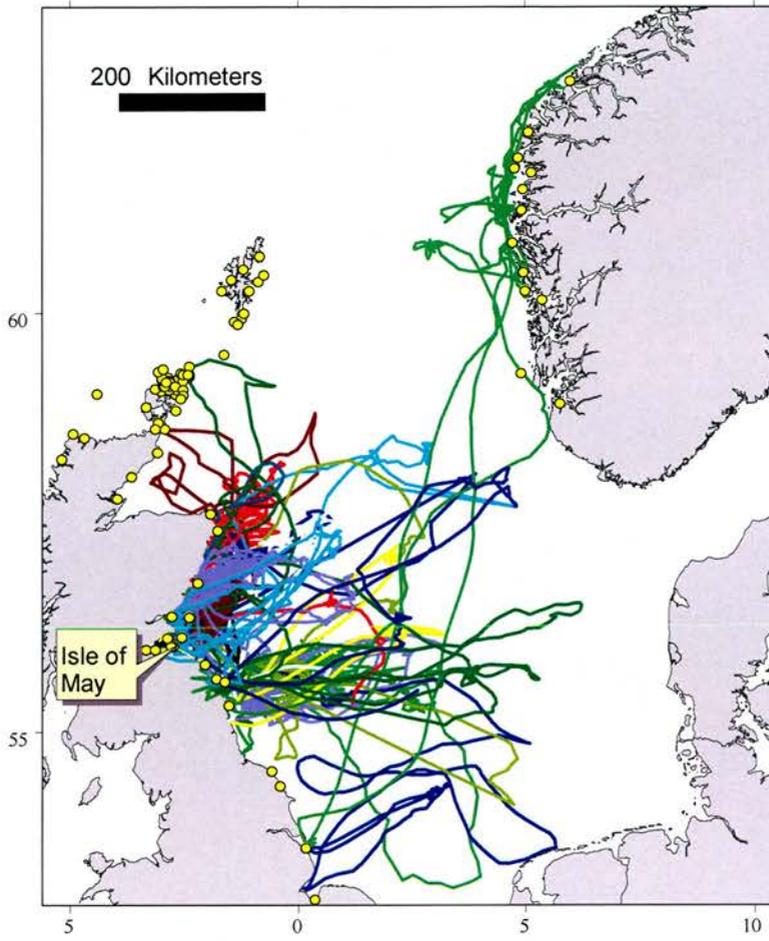
The end of the period of coastal movement was often marked by sudden, directed movement further offshore, in most cases for longer than ten days. Most pups undertook one or more prolonged (>7 days), long-distance trips to offshore areas, often initially exhibiting directed, rapid travel, followed by lengthy periods of meandering movement and some focussed diving in discrete areas. The first of these long trips (>7 days) offshore lasted on average 28.4 days and there was a gradual decrease in the duration of successive long trips. After April, pups began to perform shorter trips of between two and six days (short trips) from the same haul out, or group of haul outs, to nearby foraging areas.

Figure 7.2: Maps of the North Sea showing a. the major grey seal haul-out sites (indicated in yellow) on the east coast of the UK and west coast of Norway. Names refer to those sites or groups of sites in areas used by pups in this study, b. tracks of all study pups during their first year after departure from the Isle of May (tracks colour coded by seal) and c. individuals tracks for each animal.

a.
Haulouts

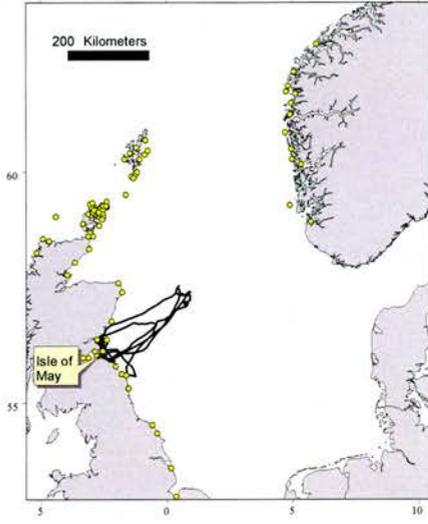


b.
All seals

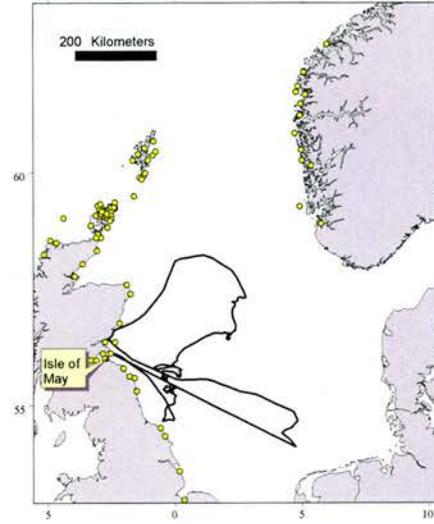


C.

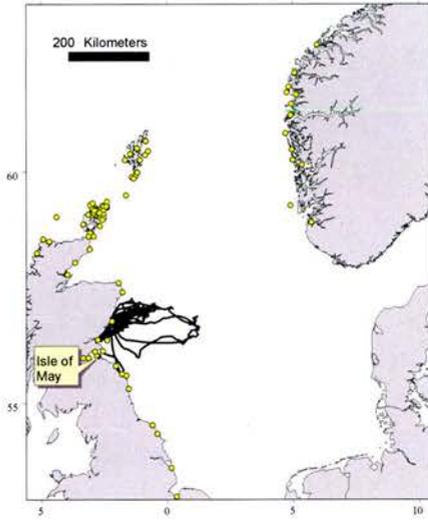
Yeti



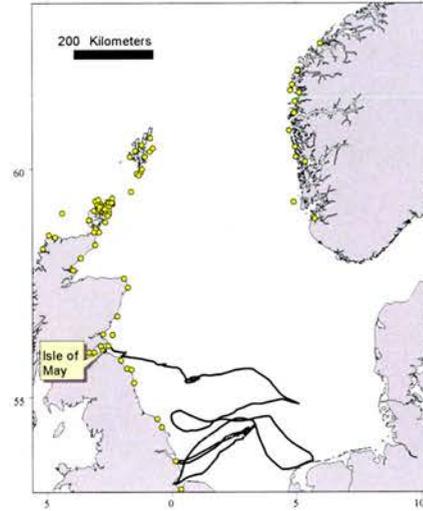
Yogi

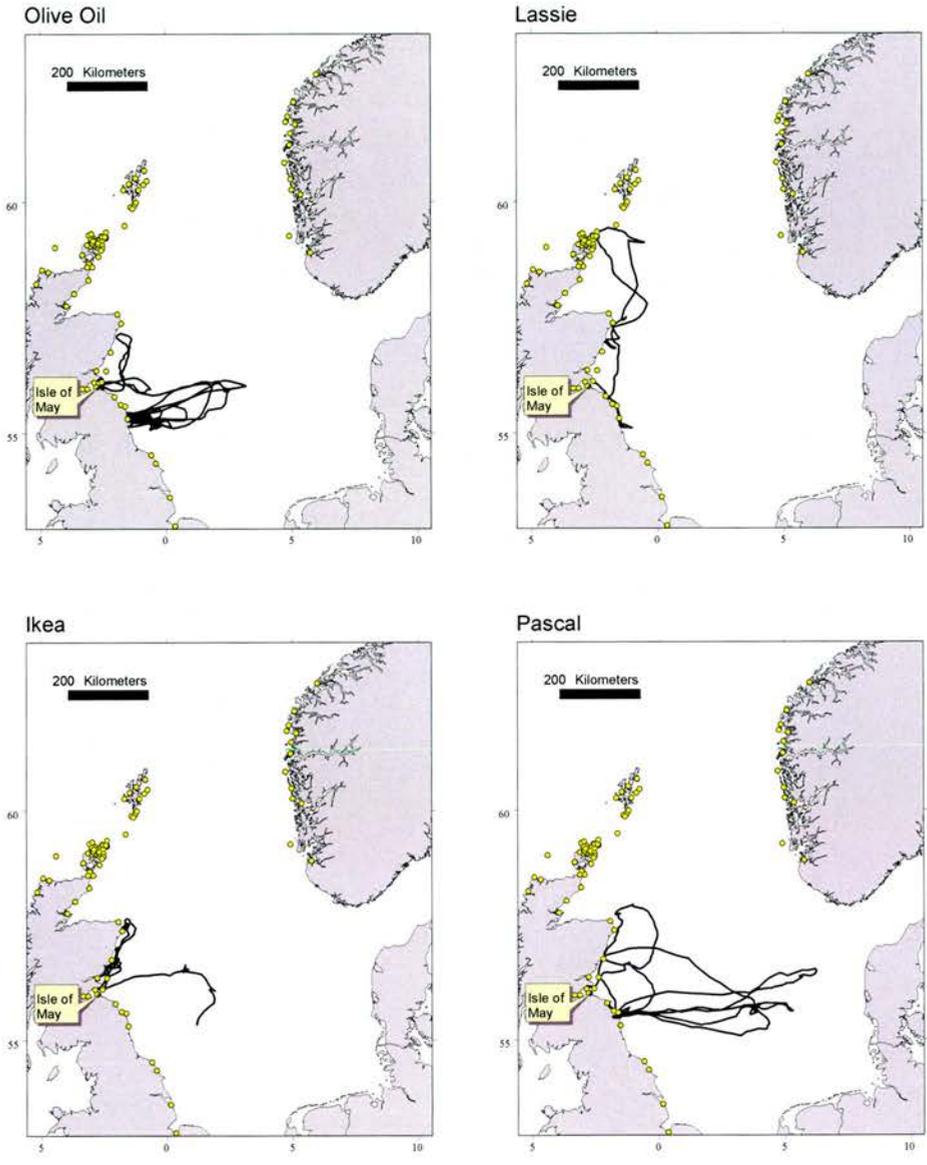


Queenie

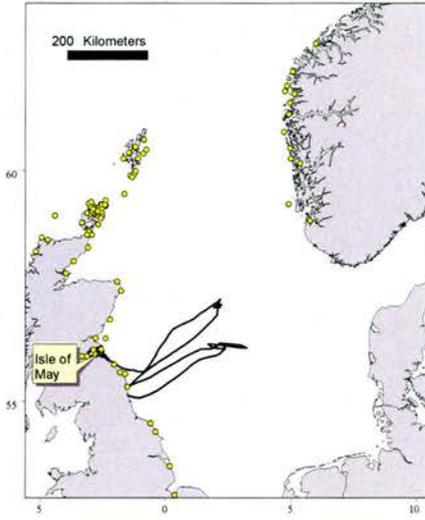


Scooby

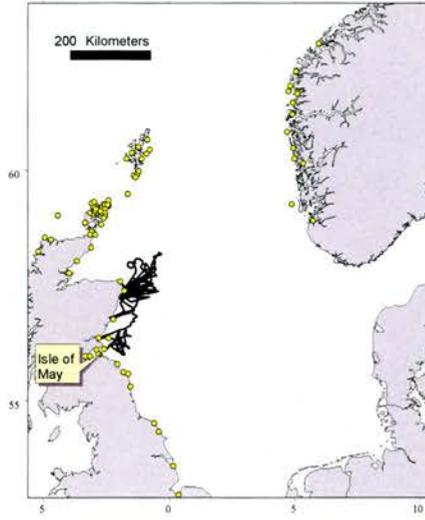




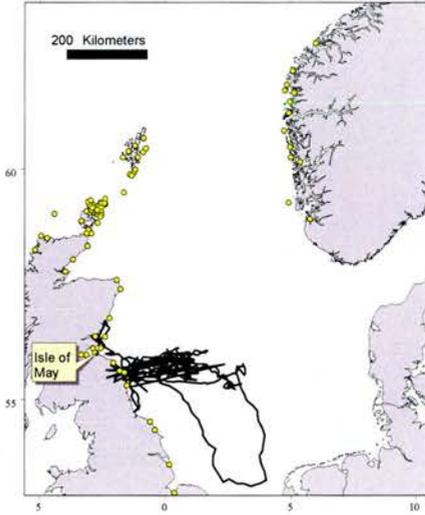
Zebedee



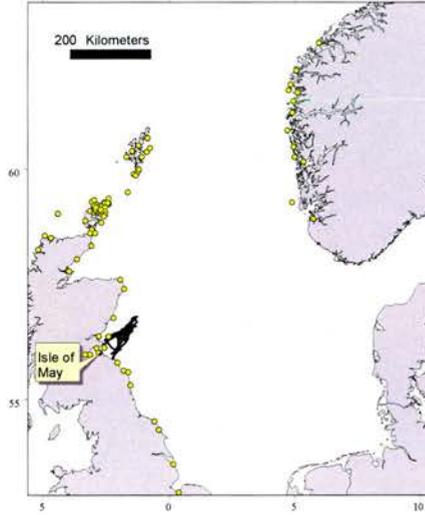
Captain Caveman

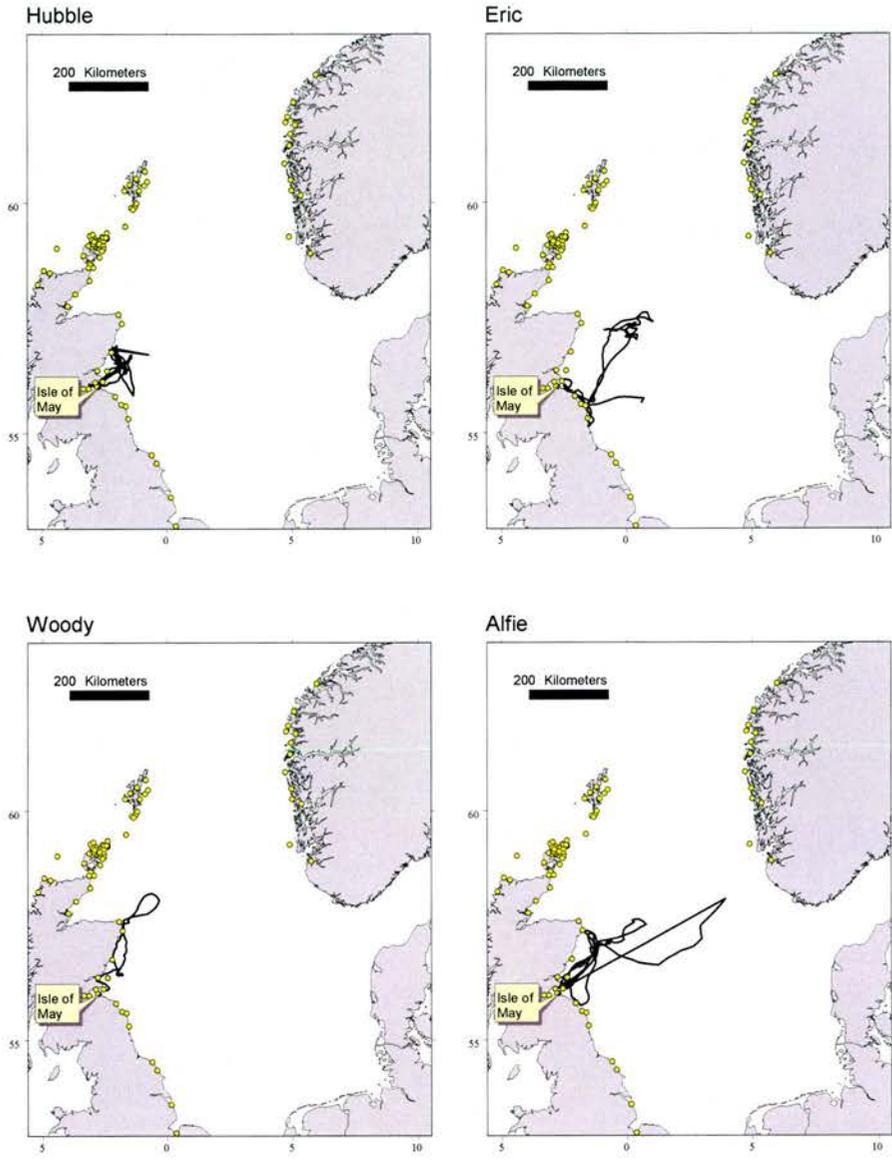


Kermit

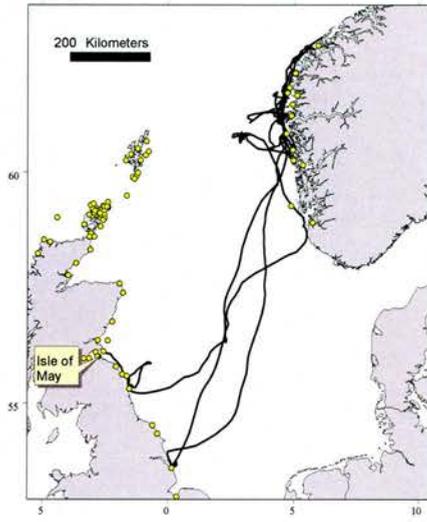


Leonardo

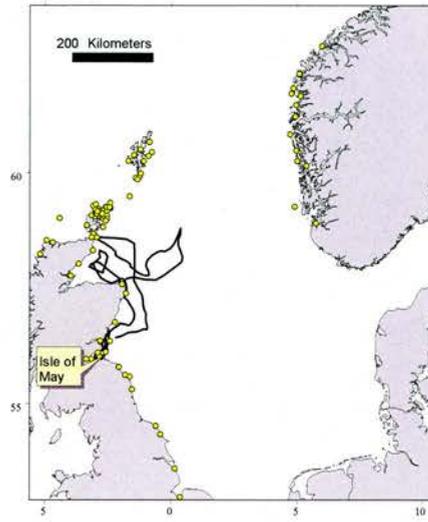




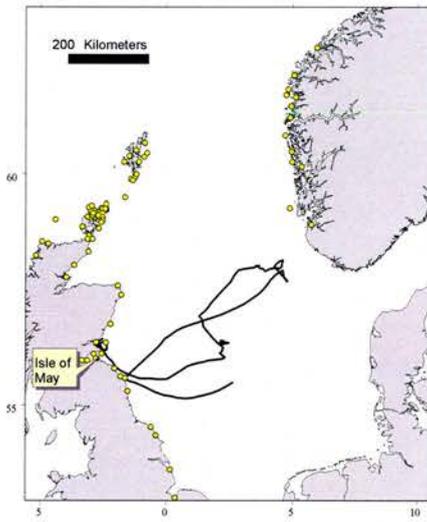
Mawson



Dumbo



Volt



Ulysses

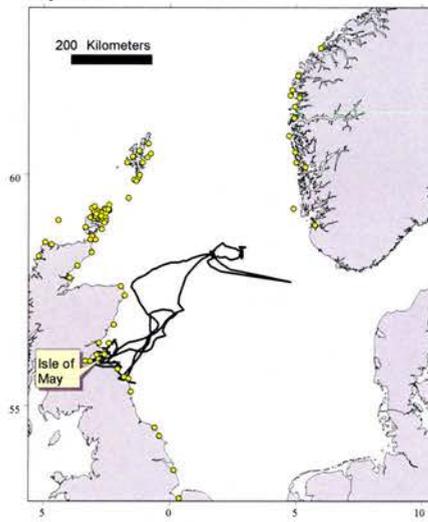
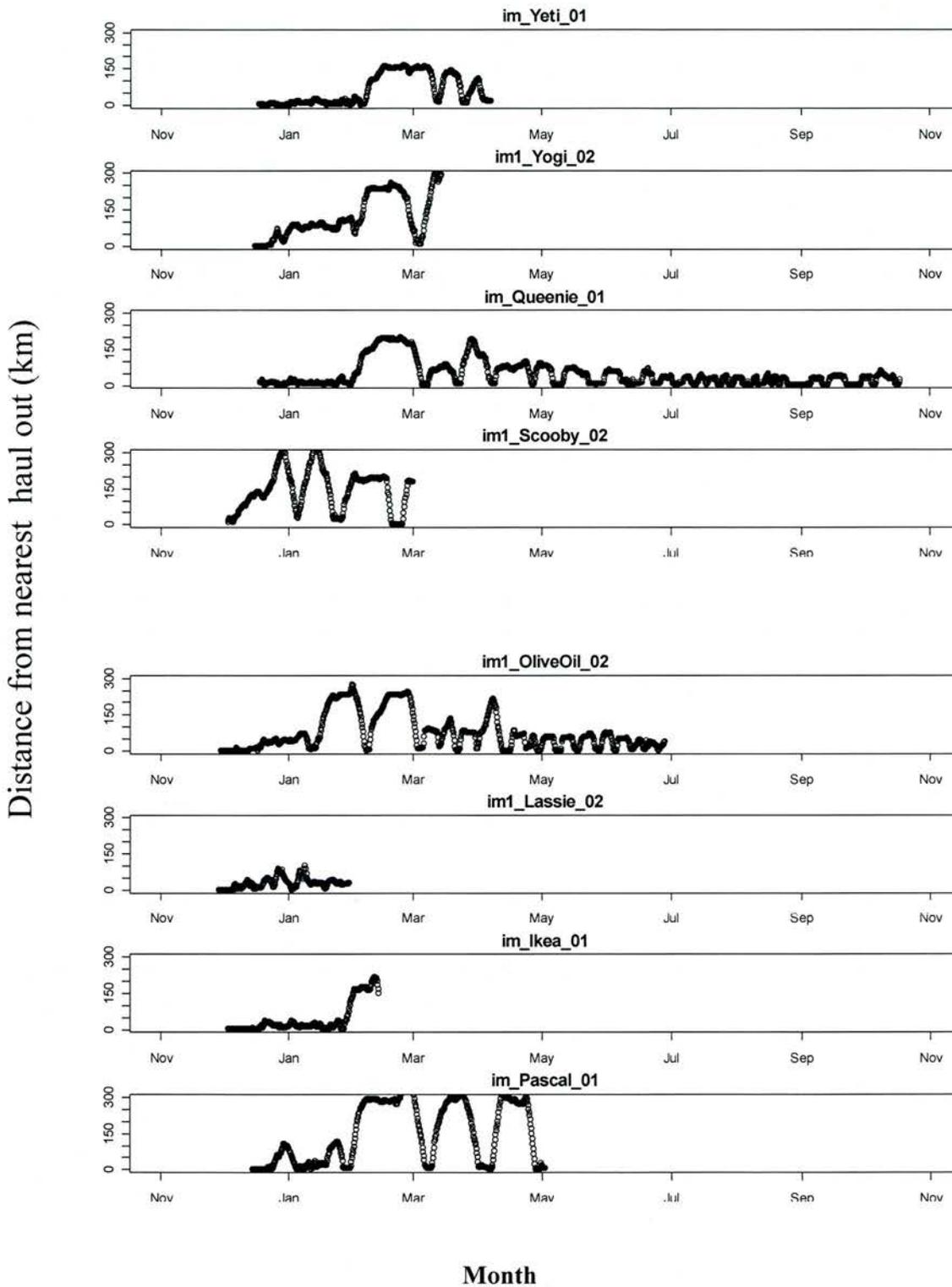
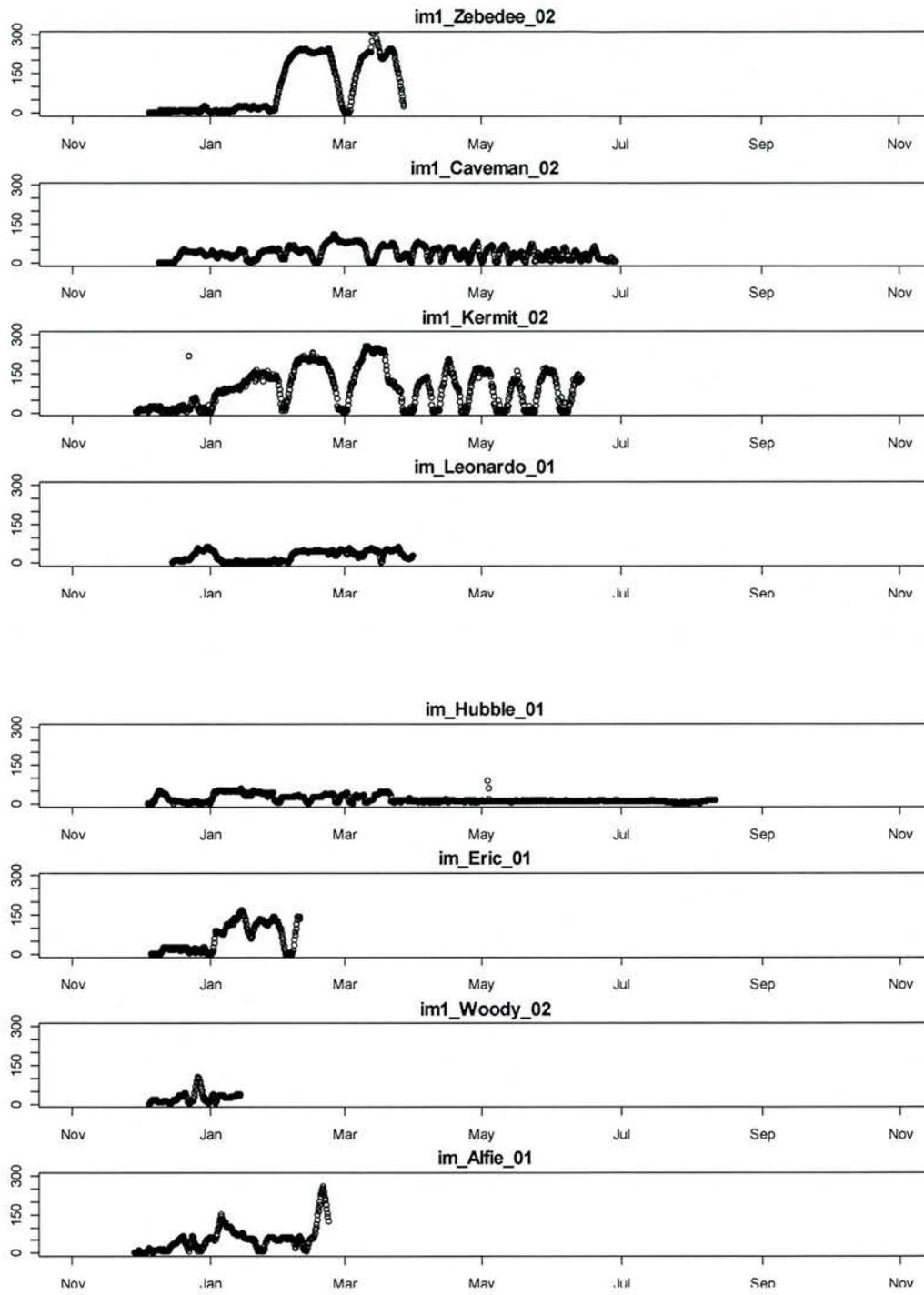


Figure 7.3: Distance from nearest haul out for six-hourly smoothed locations throughout the tracking period for each animal



Distance from nearest haul out (km)



Month

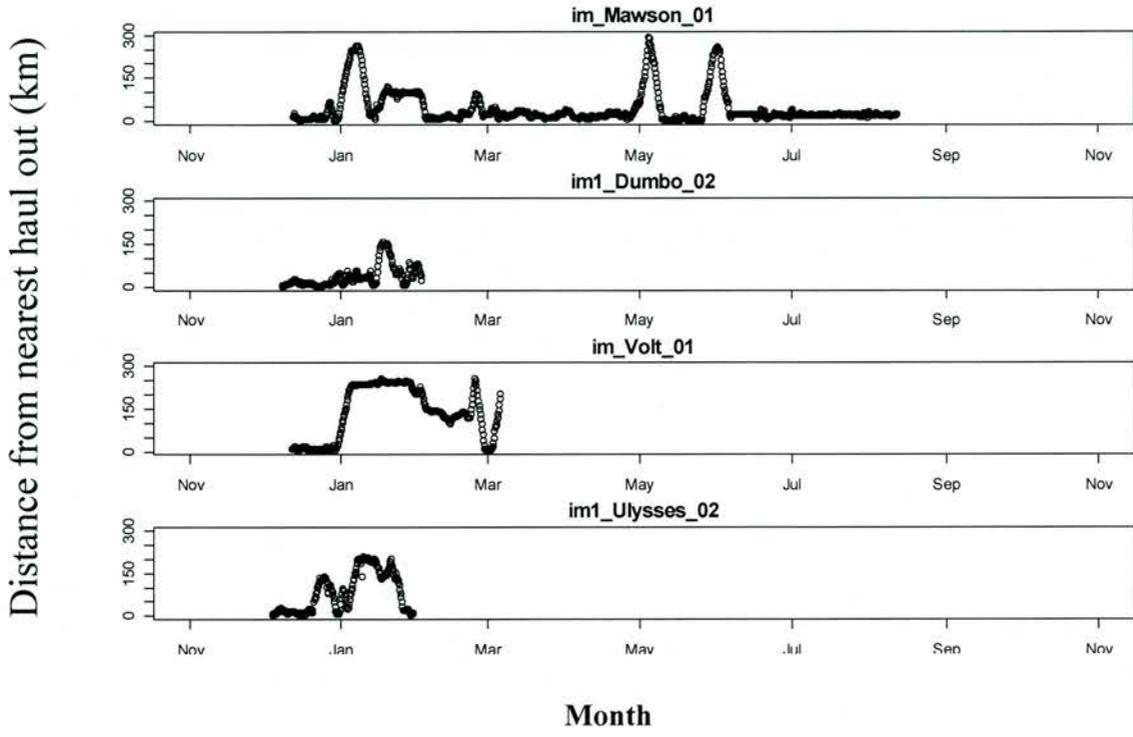


Table 7.4: Details of duration (days) of the initial period of coastal movement (coastal), first extended trip to sea (first trip), average duration of long (>7 days) trips and total tracking period (track duration). For each animal (ranked by weaning mass within each sex) the number of long trips undertaken (no.) and whether they began to exhibit short duration, repeated movement out to sea (short trips) is shown, along with mass (DM and WM) and percentage body fat (%TBF) and total body protein (TBP) at weaning (w) and departure (d), fast duration and year. Average values and standard deviation (s.d.) for all animals are given. Within each sex, animals are presented in descending order of weaning mass.

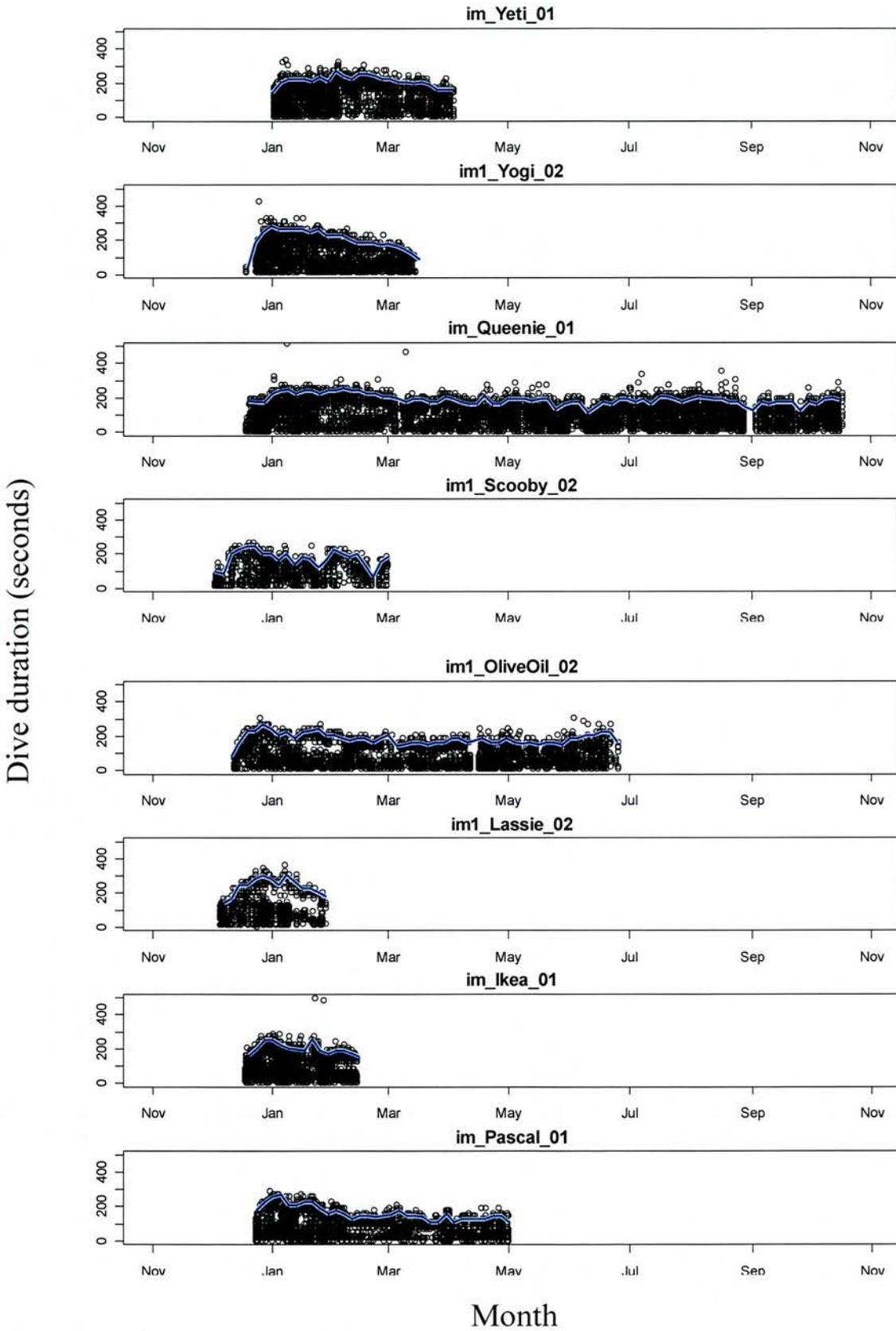
sex	seal	coastal first trip	average long trips	no. short trips	track duration	DM	WM	%TBFw	%TBFd	TBPw	TBPd	Fast duration	year
	Yeti	37	33	3	n	33.8	66.1	50.18	32.8	7.23	5.38	39	2001
	Yogi	0	70	2	n	27.5	61	49.19	44.5	6.85	3.45	30	2002
	Queenie	39	31	7	y	41	51.4					28	2001
	Scooby	0	78	2	n	39.4	51.4	43.34		6.62		13	2002
	Olive Oil	0	23	7	y	29.3	41.8	45.42	48.5	5.15	3.35	25	2002
	Lassie	11	14	3	n	27.6	41.2	44.69	42.5	5.15	3.62	20	2002
	Ikea	40	?	1	n	26.2	39.6	47.83	51.7	4.6	2.76	32	2001
	Pascal	0	11	5	n	29.8	39.3	45.2	47.4	5.16	3.15	34	2001
	Zebedee	42	28	2	n	33.2	38.8					16	2002
	Caveman	4	29	4	y	36.6	57.9	49.24	52.4	6.49	3.4	19	2002
	Kermit	31	58	9	y	41.7	56	49.61		6.22		13	2002
	Leonardo	5	14	2	n	36.8	52.1	46.93	49.1	6.18	4.15	26	2001
	Hubble	0	7	2	y	36	50.5	45.53	46.1	6.19	4.37	21	2001
	Eric	20	29	2	n	36.1	48.1	46.24	52.1	5.79	3.77	24	2001
	Woody	18	7	2	n	32	45.3	46.5		5.44		12	2002
	Alfie	8	14	3	n	27.9	44.2	43.1	52.9	5.73	2.84	25	2001
	Mawson	12	12	4	y	29.8	40.7	44.96	40.8	5.06	4.06	25	2001
	Dumbo	20	11	3	n	31.4	38.3	46.21		4.62		12	2002
	Volt	8	60	2	n	29.4	38.2					20	2001
	Ulysses	14	11	3	n	28.8	38	42.19		5.03		14	2002
	Average	15.45	28.42	3		32.72	47.00	46.26	46.73	5.74	3.69	22.4	
	s.d.	14.86	22.16	2		4.73	8.48	2.35	5.92	0.79	0.73	7.80	

7.3.4. Dive performance

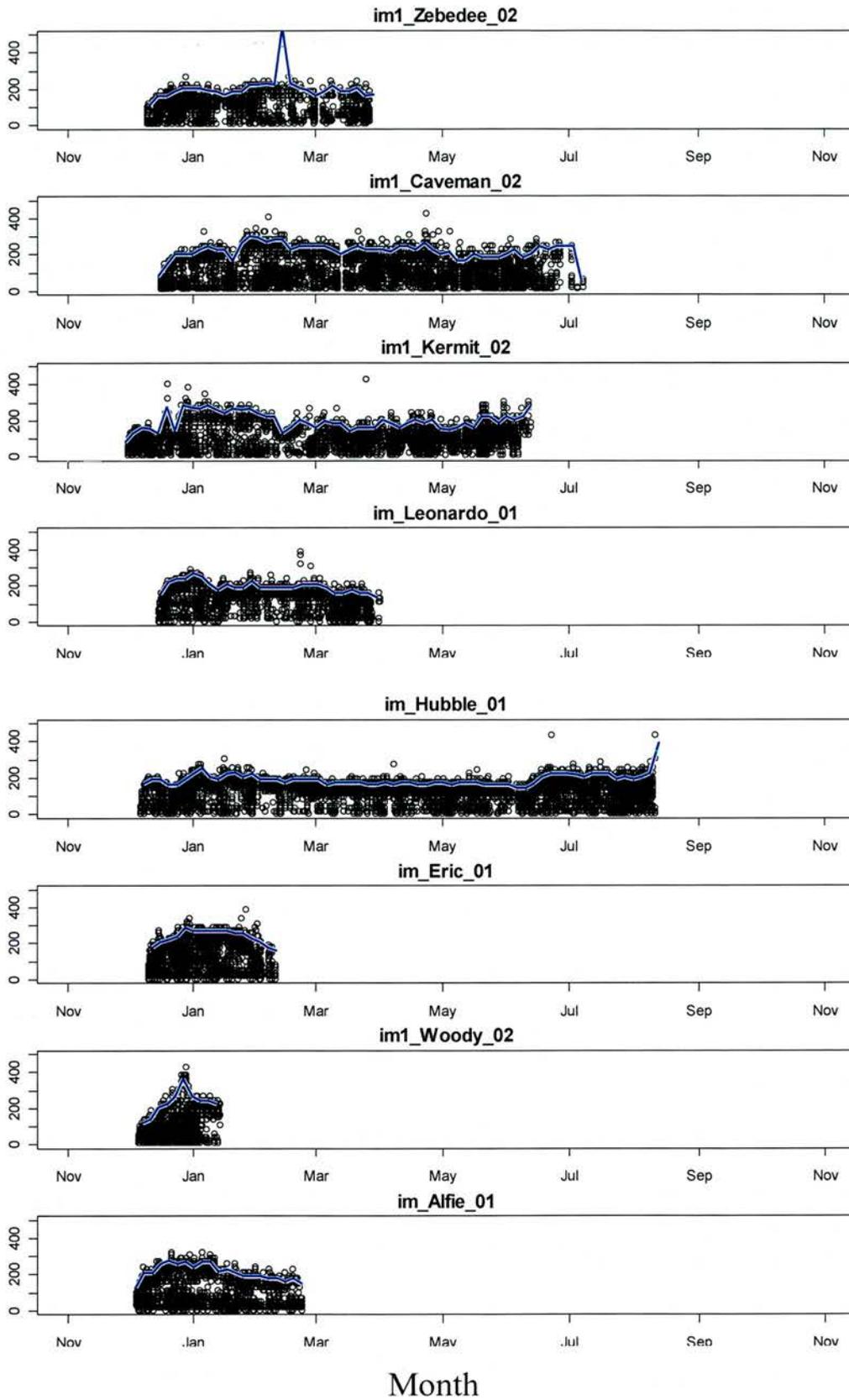
7.3.4.1. Dive duration

The values for $d95_{\text{first}}$, $d95_{\text{max}}$, $day1_{d95}$ and $day90_{d95}$ for each animal for all dives are given in Table 7.5. Dive durations and $d95$ throughout the tracking period are shown for each animal in Figure 7.4. All pups showed an initial increase in $d95$ for all dives between 10 and 85 days after departure and the rate and pattern of the increase varied between animals. In most of the animals, $d95$ then declined and the pattern of decline varied among individuals. In over half the animals the decline continued until the end of the tracking period. In other animals, there was a tendency for $d95$ to stabilise and then to increase in summertime in pups that were tracked for longer. A similar pattern was observed for $d95$ within each depth band (data not shown).

Figure 7.4. Dive durations of all dives for each animal during the tracking period. Blue line shows d_{95} for four-day time bins.

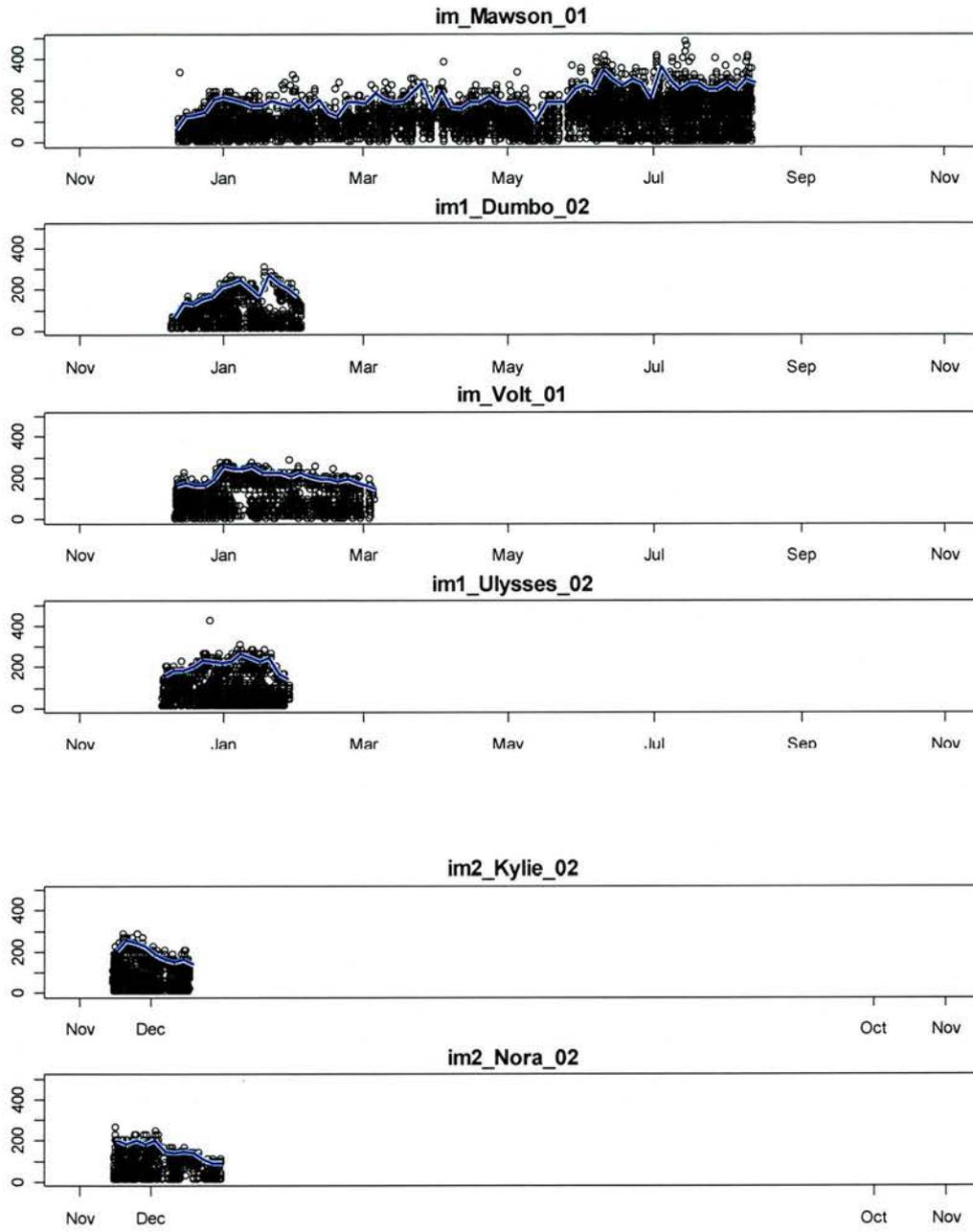


Dive duration (seconds)



Month

Dive duration (seconds)



Month

Table 7.5: Values for $d95_{\text{first}}$, the day after weaning of the mid point of the bin containing the first dives, $d95_{\text{max}}$ and the calendar dates (date) and days after weaning and departure of the midpoints in which $d95_{\text{max}}$ occurred, the absolute increase in $d95$ (increase) and $\text{day}90_{d95}$ for each animal

sex	seal	first		$d95$ (s)	date	max		increase (s)	$\text{day}90_{d95}$
		$d95$ (s)	day after weaning			day after weaning	day after departure		
female	Yeti	144	23	272	02-Feb	72	33	128	33
	Yogi	185	22	285	31-Dec	40	10	100	10
	Queenie	181	32	256	06-Feb	80	52	74	20
	Scooby	100	15	245	19-Dec	31	18	145	14
	Olive oil	82	14	264	27-Dec	42	17	181	17
	Lassie	140	17	305	08-Jan	55	35	165	19
	Ikea	160	29	259	21-Jan	68	36	99	12
	Pascal	186	42	272	05-Jan	49	15	85	11
	Zebedee	115	23	545	13-Feb	84	68	430	68
male	Caveman	80	15	301	28-Jan	65	46	221	46
	Kermit	80	34	285	27-Dec	42	29	205	21
	Leonardo	152	29	272	01-Jan	45	19	120	19
	Hubble	171	28	256	05-Jan	53	32	84	32
	Eric	176	30	288	28-Dec	44	20	112	20
	Woody	120	20	365	27-Dec	36	24	245	24
	Alfie	128	22	273	20-Dec	43	18	145	16
	Mawson	128	39	236	06-Mar	110	85	108	20
	Dumbo	70	15	271	20-Jan	59	47	201	35
	Volt	160	28	256	01-Jan	43	23	96	23
	Ulysses	161	28	265	08-Jan	49	35	103	35
female	Kylie	208	32	254	21-Nov	6	6	46	6
	Nora	205	2	205	17-Nov	2	2	0	2

7.3.4.1.1. All dives irrespective of depth

The LMs that best described the dependent variables related to $d95$ for dives irrespective of depth are shown in Table 7.6. $D95_{\text{first}}$ increased significantly as a function of day after weaning and TBPw. It decreased as a function of DM. $D95_{\text{max}}$ did not change as a function of any of the explanatory variables. $\text{Day}90_{d95}$ was significantly longer in males than in females and increased as a function of $d95_{\text{max}}$.

Table 7.6: LMs that best describe variability in $d95_{\text{first}}$ and $day90_{d95}$ for all dives irrespective of depth. Bold font denotes significant values ($p < 0.05$).

Dependent variable	Independent variable	Individual co-efficients				Model fit			
		co-efficient	s.e.	T	p	F	d.f.	p	r ²
<i>a</i> $d95_{\text{first}}$	Intercept	68.66	24.34	2.82	0.0113	8.41	1,18	0.0095	0.2806
	day after weaning	2.67	0.92	2.90	0.0096				
<i>w</i> $d95_{\text{first}}$	Intercept	91.22	62.00	1.47	0.1651	6.11	3,13	0.0080	0.4895
	day after weaning	2.86	0.83	3.44	0.0044				
	DM	-5.42	1.83	2.96	0.0110				
	TBPw	25.56	10.84	2.36	0.0348				
<i>a</i> $day90_{d95}$	Intercept	4.73	1.51	3.78	0.0015	7.70	2,17	0.0042	0.4134
	$d95_{\text{max}}$	1.00	1.00	3.49	0.0028				
	sex	1.49	1.18	2.35	0.0311				

7.3.4.1.2. Dives within max depth bands

The models that best described variation in $d95_{\text{first}}$, $d95_{\text{max}}$, $day1_{d95}$ or $day90_{d95}$ within depth bands are given in Table 7.7. *Max depth* caused only $d95_{\text{first}}$ to increase but was retained in each model to account for the effect of depth in each case. $d95_{\text{first}}$ increased as a function of day after weaning, but not as a function of day after departure. $d95_{\text{max}}$ increased as a function of day after departure, WM, $d95_{\text{first}}$ and %TBFd, and was significantly shorter in males than in females. $day1_{d95}$ was unrelated to any of the explanatory variables. $day90_{d95}$ increased as a function of $d95_{\text{max}}$ and was significantly longer in males than in females. When body composition information was included, there was a negative relationship between $day90_{d95}$ and %TBFd. Sex could not be substituted for fast duration in any of the models and vice versa.

Table 7.7: LMEs that best describe variability in $d95_{\text{first}}$, $d95_{\text{max}}$, and $day90_{d95}$. Significant parameters are in bold ($p < 0.05$). The number of individuals (seals), number of observations (n) and the AIC is given for each model. a , d and w refer to all the pups or the subset of animals for which body composition information was available at departure and weaning, respectively. Sex comparison is from female to male

Dependent variable	Independent variable	Individual parameters					Model fit		
		Value	s.e.	T	p	d.f.	seals	n	AIC
a $d95_{\text{first}}$	Intercept	53.06	12.85	4.13	0.0001	58			
	Max depth	1.40	0.18	7.82	<0.0001	58	20	80	811.55
	Day after weaning	1.22	0.45	2.68	0.0095	58			
d $d95_{\text{max}}$	Intercept	-176.60	100.07	1.75	0.088	33			
	Max depth	0.05	0.40	0.11	0.9092	33			
	Day after departure	1.39	0.42	3.30	0.0023	33			
	sex	-51.04	17.42	2.93	0.0190	8	12	48	508.33
	WM	2.32	0.86	2.68	0.0278	8			
	$d95_{\text{first}}$	0.41	0.17	2.33	0.0256	33			
	%TBFd	4.91	1.52	3.22	0.0122	8			
a $day90_{d95}$	Intercept	3.21	1.35	3.85	0.0003	58			
	Max depth	1.00	1.00	1.07	0.2889	58			
	$d95_{\text{max}}$	1.00	1.00	3.61	0.0006	58	20	80	13.91
	sex	1.49	1.19	2.30	0.0336	18			
d $day90_{d95}$	Intercept	12.14	2.13	2.39	0.0023	34			
	Max depth	1.00	1.00	0.30	0.7673	34			
	$d95_{\text{max}}$	1.01	1.00	3.47	0.0015	34	12	48	9.61
	sex	2.37	1.20	4.85	0.0009	9			
	%TBFd	-1.04	1.02	2.25	0.0506	9			

7.3.4.2. Post dive surface interval

The models that best described variation in $SI5_{\text{first}}$, $SI5_{\text{min}}$, $day1_{SI5}$ or $day10$ within duration bands are given in Table 7.8. *Duration* was retained in every model and $SI5_{\text{first}}$, $SI5_{\text{min}}$, $day1_{SI5}$ increased significantly as a function of *duration*. The increase in $day10$ with *duration* was almost significant. Given these relationships, $SI5_{\text{first}}$ was longer in males than in females, $SI5_{\text{min}}$ decreased as a function of WM and $day1_{SI5}$ and $day10$ decreased significantly as a function of fast duration. When body

composition information was included, $SI5_{\min}$ increased as a function of TBPw, decreased as a function of both WM and DM and, given these relationships, was longer in males than in females. An alternative model that had a similar fit to the data showed a decrease in $SI5_{\min}$ as a function of TBFw.

Table 7.8: LMEs that best describe variability in $SI5_{\text{first}}$, $SI5_{\min}$, $day1_{SI5}$ and $day10$. Number of seals, total number of observations (obs) and the AIC value for each model are given. a , d and w refer to all the pups or the subset of animals for which body composition information was available at departure and weaning, respectively. Sex comparison is from female to male

	Parameter	Individual parameters					Model fit		
		Value	s.e.	T	p	d.f.	seals	obs	AIC
a $SI5_{\text{first}}$	Intercept	32.04	1.06	57.77	<0.0001	56			
	duration	1.00	1.00	4.51	<0.0001	56	20	77	193.12
	sex	1.10	1.04	2.62	0.0174	18			
a $day1_{SI5}$	Intercept	-0.69	2.08	0.33	0.7393	56			
	duration	0.06	0.01	9.01	<0.0001	56	20	77	441.52
	Fast duration	-0.25	0.06	3.91	0.0010	18			
a $SI5_{\min}$	Intercept	24.08	5.18	4.65	<0.0001	59			
	duration	0.08	0.01	8.20	<0.0001	59	20	80	527.60
	WM	-0.23	0.10	2.33	0.0316	18			
w $SI5_{\min}$	Intercept	22.25	6.96	3.20	0.0024	50			
	duration	0.07	0.01	7.71	<0.0001	50			
	TBPw	7.64	3.19	2.39	0.0340	12	17	68	426.90
	WM	-0.66	0.28	2.32	0.0385	12			
	DM	-0.68	0.21	3.16	0.0082	12			
	sex	4.23	1.63	2.59	0.0235	12			
w $SI5_{\min}$	Intercept	55.03	18.41	2.99	0.0043	50			
	duration	0.07	0.01	7.89	<0.0001	50	17	68	430.46
	TBFw	-0.86	0.40	2.18	0.0459	15			
a $day10$	Intercept	28.29	7.96	3.55	0.0008	59			
	duration	0.05	0.02	1.95	0.0562	59	20	80	666.64
	Fast duration	-0.67	0.27	-2.55	0.0202	18			

7.3.4.3. Proportion of time spent diving

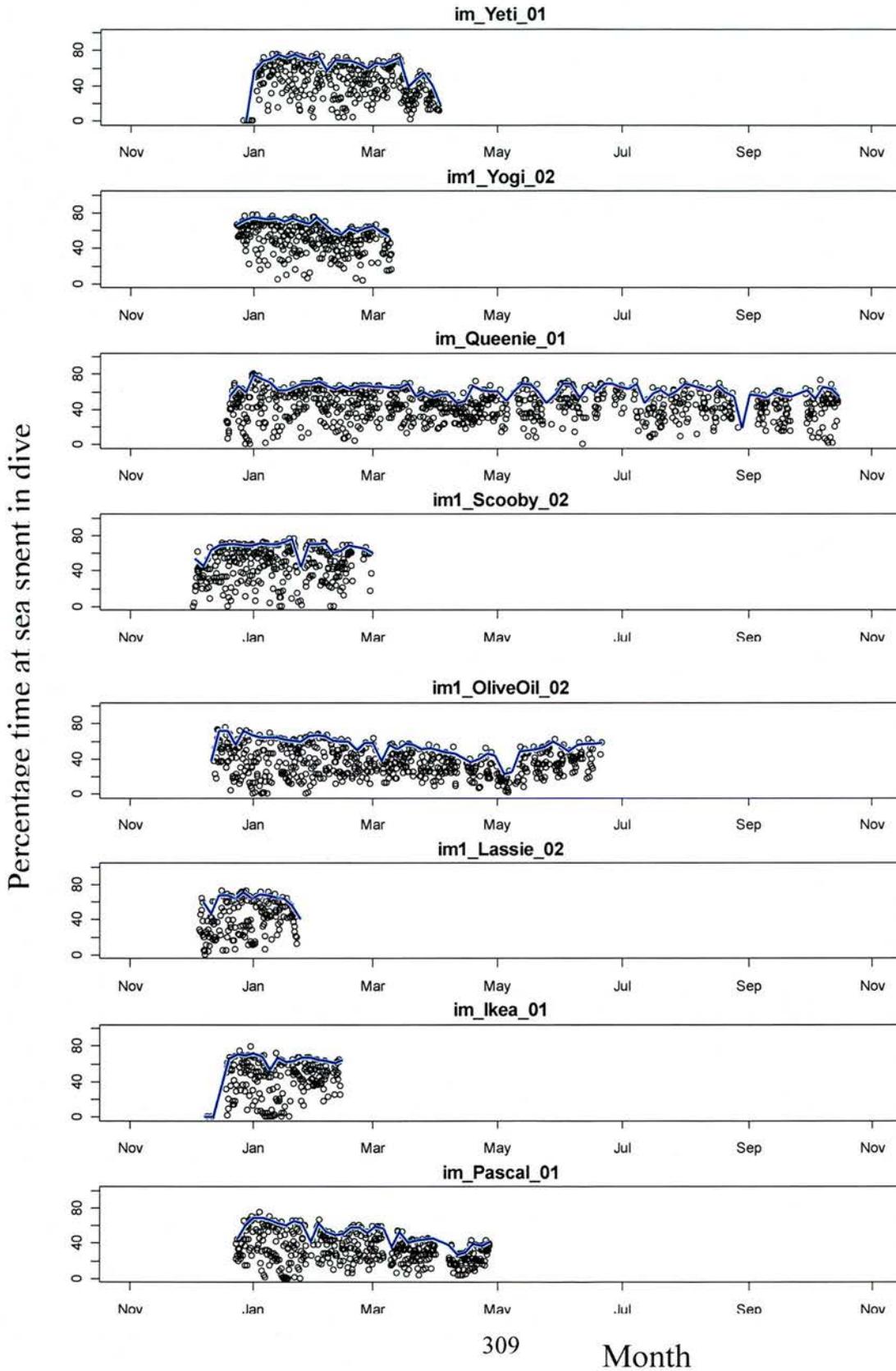
Proportion of time spent diving for 6-hourly periods for each animal is shown in Figure 7.5. % *dive* increased initially and then either remained relatively constant or declined. It increased again later in the tracking period of some of the animals that

were tracked for longer than 4-5months. Values for $\%dive_{\text{first}}$, $\%dive_{\text{max}}$ and $day90_{\%dive}$ are given in Table 7.9.

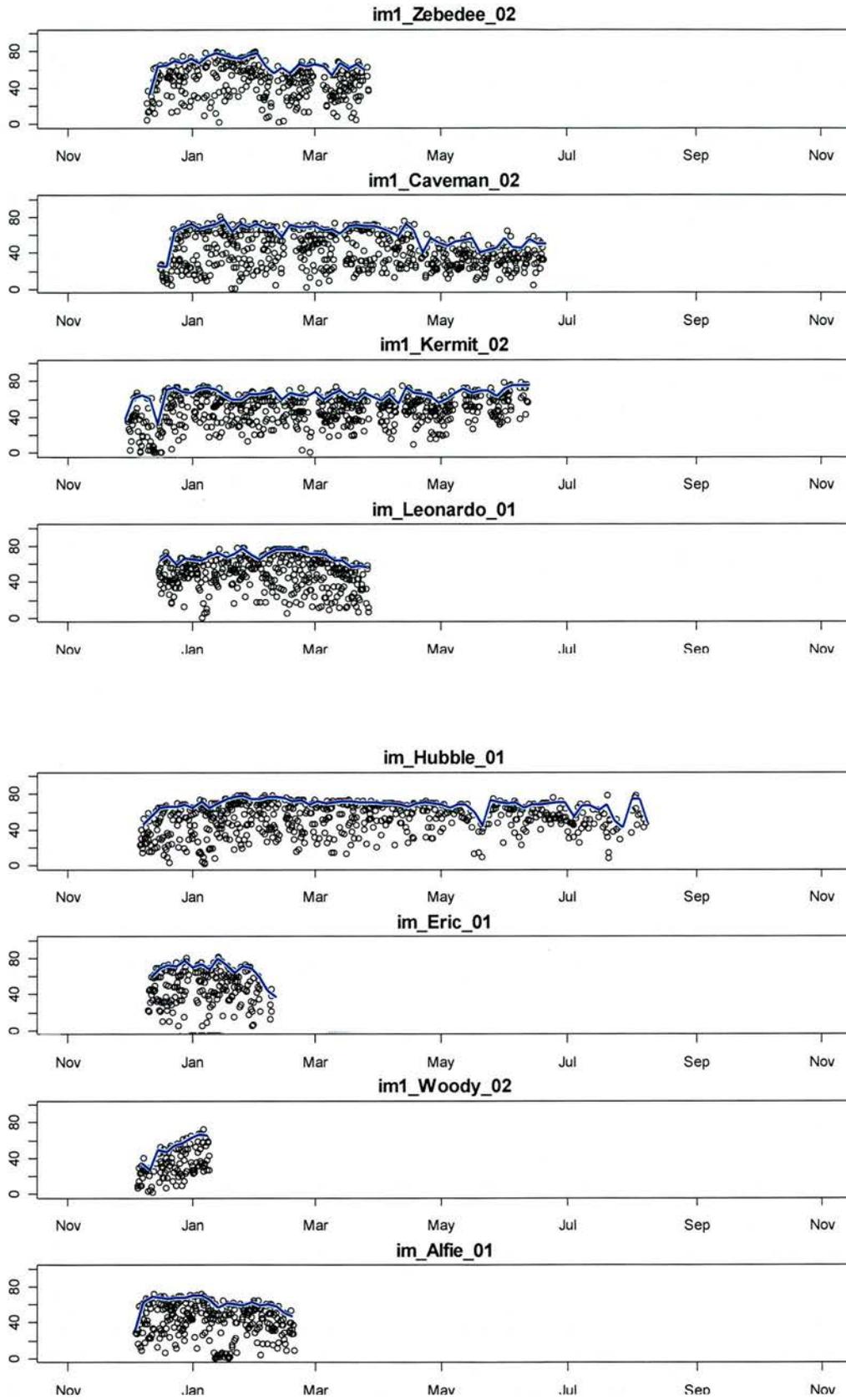
Table 7.9: Values for $\%dive_{\text{first}}$, the day after weaning (weaning) of the mid point of the bin containing the first dives, $\%dive_{\text{max}}$ and the calendar dates and days after weaning and departure of the midpoints in which $\%dive_{\text{max}}$ occurred, the absolute increase in $\%dive$ and $day90_{\%dive}$ for each animal (ranked by weaning mass within each sex)

sex	seal	first		max				increase (%)	$day90_{\%dive}$
		$\%dive$	day after weaning	$\%dive$	date	day after weaning	day after departure		
female	Yeti	57.58	41	76.1	13-Jan	55	14	18.52	6
	Yogi	66.28	32	74.6	02-Jan	44	12	8.32	6
	Queenie	54.54	32	79.62	01-Jan	48	16	25.08	16
	Scooby	54.03	15	73	16-Jan	61	46	18.975	14
	Olive oil	37.21	26	71.88	15-Dec	31	5	34.67	5
	Lassie	60.58	23	71.72	27-Dec	46	23	11.14	11
	Ikea	65.56	36	71.7	09-Jan	70	34	6.14	4
	Pascal	45.20	37	69	05-Jan	52	15	23.8	11
	Zebedee	33.62	20	78.5	01-Feb	76	56	44.88	32
male	Caveman	25.15	20	77.06	16-Jan	53	33	51.915	17
	Kermit	37.85	14	73.48	08-Jan	56	42	35.635	22
	Leonardo	63.77	29	77.7	25-Jan	71	42	13.935	30
	Hubble	46.13	25	77.7	13-Jan	52	27	31.575	19
	Eric	59.98	28	79.78	13-Jan	64	36	19.8	12
	Woody	34.47	16	66.6	04-Jan	48	32	32.135	28
	Alfie	31.38	27	69.96	05-Jan	61	34	38.58	10
	Mawson	16.46	26	73.77	14-Mar	109	93	57.315	21
	Dumbo	49.13	15	67.88	04-Jan	52	27	18.75	27
	Volt	44.80	23	69.16	05-Jan	60	27	24.36	7
Ulysses	45.20	17	68.68	31-Dec	44	27	23.48	15	
female	Kylie	64.68		68.84	21-Nov		6	4.16	2
	Nora	57.42		60.14	15-Dec		31	2.72	2

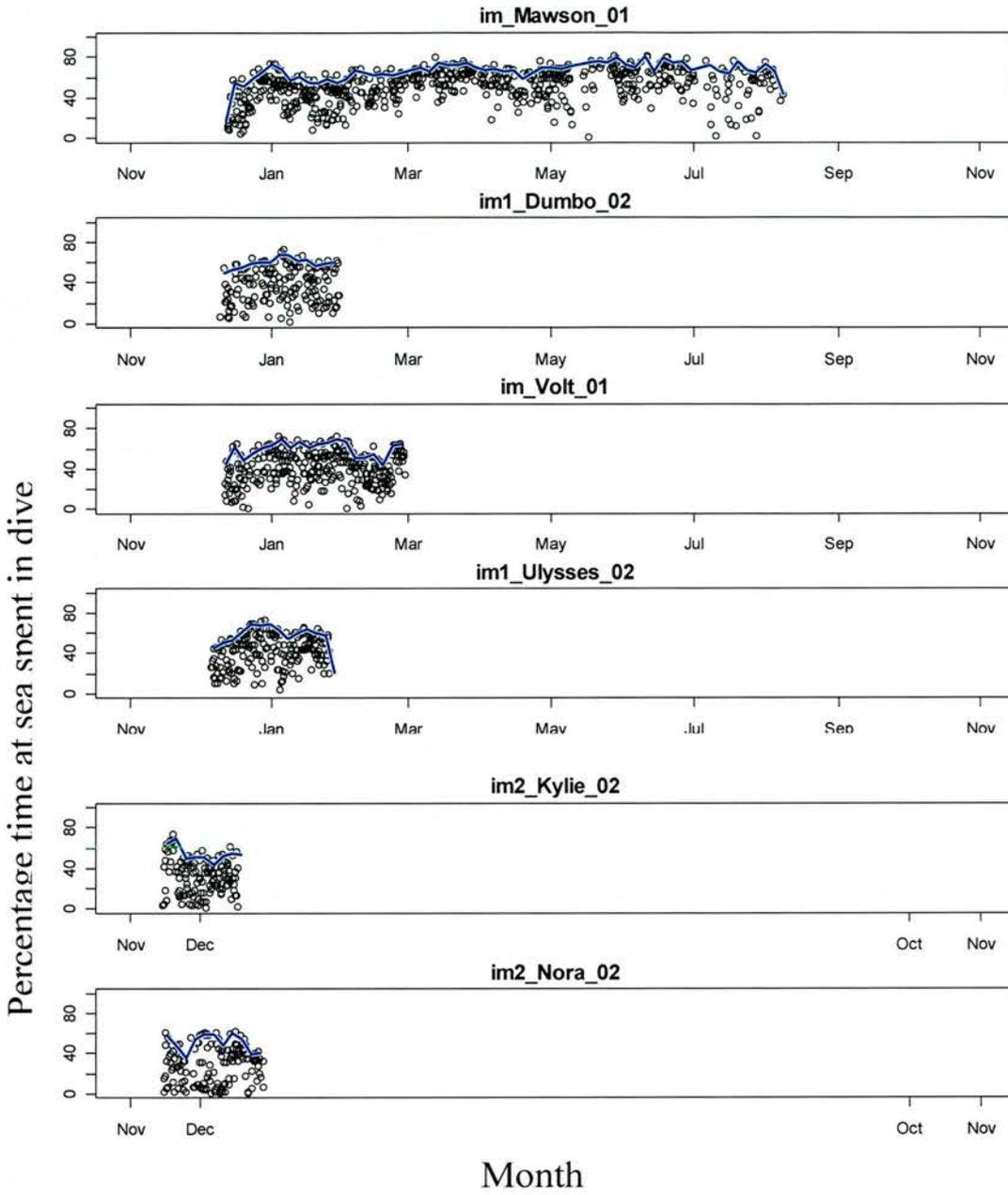
Figure 7.5: Percentage time at sea spent in “dive” throughout the tracking period for each animal. % *dive* is shown in blue.



Percentage time at sea spent in dive



Month



The models that best described changes in $\%dive_{max}$ and are given in Table 7.10. None of the explanatory variables had a significant impact on $\%dive_{first}$. There was a significant increase in $\%dive_{max}$ with DM and fast duration. When body composition data was included in the analysis, $\%dive_{max}$ increased as a function of both $\%TBFd$ and $TBPd$. $day90\%dive$ decreased significantly as a function of fast duration but was not influenced by any other explanatory variables.

Table 7.10: LMs that best describe variability in $\%dive_{max}$, and $day90\%dive$. *a*, *d* and *w* refer to all the pups or the subset of animals for which body composition information was available at departure and weaning, respectively.

dependent variable	independent variable	Individual parameters				Model fit			
		Value	s.e.	T	p	F	d.f.	p	r ²
<i>a</i> $\%dive_{max}$	Intercept	46.10	0.43	11.31	<0.0001				
	DM	<0.0001	<0.0001	4.01	0.0009	9.38	2,17	0.0018	0.4686
	Fast duration	<0.0001	<0.0001	2.57	0.0197				
<i>d</i> $\%dive_{max}$	Intercept	18.58	1.43	3.71	0.0048				
	TBPd	0.55	0.02	5.52	0.0004	15.25	2,9	0.0013	0.7215
	%TBFd	<0.0001	<0.0001	4.12	0.0026				
<i>a</i> $day90\%dive$	Intercept	25.18	0.78	5.94	<0.0001				
	Fast duration	-0.01	<0.0001	3.02	0.0074	9.09	1,18	0.0074	0.2986

7.3.5. Comparison with adults

Mean values for dive duration, post-dive surface interval and proportion of time spent diving for pups, yearlings and sub-adults are given in Table 7.11 Data for sub-adult males is taken from Thompson et al (1991), in which three animals were tracked from the Farne Islands for up to nine days using radio telemetry during August 1986. Since the behaviour of the pups and yearlings was not divided into different activities, the values represent the mean of all dives for these animals.

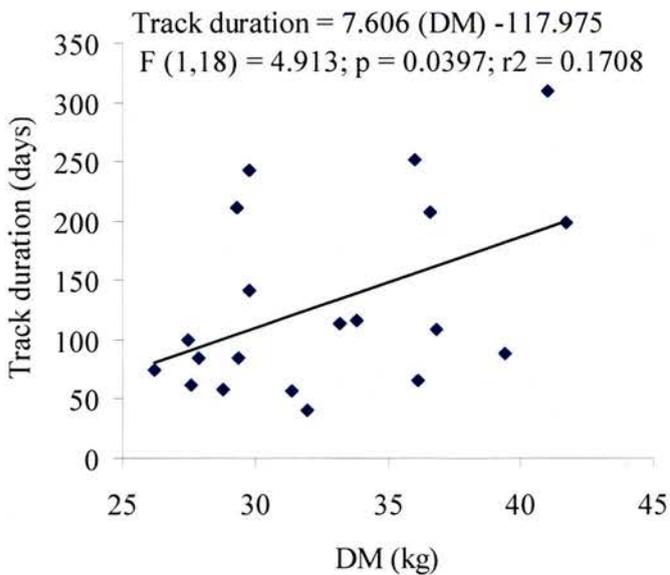
Table 7.11 Mean values \pm standard error of the mean (s.e.) for dive duration (dive), post-dive surface interval (surface) and % time spent diving (% in dive) for pups. Data from sub adult males from Thompson et al, 1991 are shown for comparison.

Animals	Activity	Number of dives	mean (s.e.) dive (s)	mean (s.e.) surface (s)	mean (s.e.) % in dive
Pups	All	77272	115.87 (0.72)	99.68 (0.41)	37.23 (0.21)
	Dives >120 s	37246	174.75 (0.32)	85.29 (0.45)	-
Sub adults	Travelling	399	206 (4.41)	38.5 (0.73)	84.3
	Short trips	161	243 (7.27)	48.0 (1.27)	83.5
	Resting (all)	187	321 (12.1)	58.8 (2.74)	84.5
	Sleeping only	50	491 (7.53)	54.6 (1.73)	90.0

7.3.6. Duration of tracking period

Track duration increased significantly as a function of DM as shown in Figure 7.6. This relationship was relatively weak. There were no relationships between track duration and any other measure of body condition and no difference between sexes or years.

Figure 7.6: Relationship between track duration and DM



7.4. Discussion

7.4.1. Movements of grey seal pups

Grey seal pups are able to reach all parts of the North Sea, in terms of both depth and distance, despite their small body size. Indeed, Mawson, one of the smaller animals at departure, reached depths of over 250m in the Norwegian Trench. Many of the areas utilised by grey seal pups are also used by adults, most notably the known foraging grounds around Wee Bankie and the Marr Bank (McConnell et al, 1999). Since grey seal pups can dive to the same depths in the same areas of the North Sea as adult seals, they are apparently capable of gaining access to the same prey. However, their movement patterns, at least in the first few months after departure from the colony, are markedly different from those of adults.

7.4.1.1. Long distance travel

After an initial phase of relatively coastal movement, most pups undertook one or more trips to sea that lasted a month or more. During these prolonged trips pups often ventured far offshore and their movements were often erratic, with some periods of directed travel interspersed with focussed diving in relatively discrete areas. The duration, frequency and movement patterns of these extended trips was considerably different from both the short duration foraging trips and directed travel between haul out sites exhibited by adults (Thompson et al, 1991; McConnell et al, 1999). This may reflect inexperience in locating prey or exploration of potential foraging sites further afield.

The large scale movements undertaken by many of the pups in the current study, to places as far from the Isle of May as Orkney, Norway and Holland, are similar to those reported from flipper tag recoveries, which demonstrated long distance dispersal of pups in their first year (McConnell et al, 1984). These long distance migrations may not reflect permanent emigration because mature grey seals appear to be philopatric (Pomeroy et al, 2000b).

In several cases these long periods of directed travel were often to places that these naïve animals had never previously visited. For example, Mawson swam rapidly to Norway for the first time within two weeks of leaving the colony, and then returned to the UK several months later to haul out at Donna Nook, a major grey seal haul out on the east coast of England that he had never visited before. This appears to require considerable navigational ability combined with a prior knowledge of the site that the animal did not have. The most likely explanation for this behaviour is that he followed other older, more experienced animals.

The fact that most of the sudden concerted movements out to sea were immediately preceded by a period of time at known grey seal haul-outs suggests the possibility of information flow between animals. In contrast, southern elephant seal pups from Macquarie Island undertake long distance travel in a very directed manner immediately after leaving the colony, without any obvious source of information from older conspecifics. The outward routes from island are to the south east and are often similar between animals, suggesting navigational abilities or use of the same large-scale cues such as sea-bed topography, currents, water temperature and salinity (McConnell et al, 2002).

7.4.1.2. Onset of foraging behaviour

The onset of adult-like behaviour, in terms of short, repeated trips to discrete offshore areas, in some pups later in the tracking period suggests that these pups began to undertake targeted foraging trips, presumably to sites found to be profitable. This may reflect an increase in experience or diving capability that allowed them to begin to utilise prey resources or adopt similar strategies to those of adults. These trips tended to be slightly longer than the foraging trips undertaken by adults. Pups may need to spend longer at sea than adults within individual trips if they are less effective at finding and capturing prey.

Pups are clearly able to survive at sea, even if they are not foraging effectively, before they begin to exhibit movements typically associated with foraging. In adults, foraging behaviour is typified by square-bottom dives, in which 60% of the total dive duration is spent at the maximum depth, usually at or close to the sea-bed. These dives are normally performed during periods of slow, meandering movement in discrete areas at sea (Thompson et al, 1991). The onset of similar dive behaviour in pups during slow sections of the track will be investigated further to indicate when they begin to feed effectively.

Abrupt changes in behaviour have been used to infer foraging in newly weaned southern elephant seals. Three phases of behaviour are identified in newly weaned southern elephant seal pups from Macquarie Island (Hindell et al, 1999; McConnell et al, 2002). They consist of an initial travel phase to distant foraging grounds, marked by directed and rapid movement, followed by an extended period of

slow, undirected movement in discrete patches, after which the animals return rapidly to land, usually at their natal colony (Hindell et al, 1999; McConnell et al, 2002).

In contrast, the onset of foraging in grey seal pups could not be inferred in this study because there were no distinct behavioural transitions in their movement patterns. This may reflect differences in the proximity to feeding grounds between the two species. Southern elephant seal pups must migrate up to 1900km to foraging areas south of the Polar Front from islands in the Subantarctic (McConnell et al, 2002), whereas prey is available locally around the Isle of May and known foraging grounds used by adults are within 50km offshore (McConnell et al, 1999).

Other dive characteristics have also been used to infer long term successful foraging in pinnipeds. Drift dives performed by elephant seals contain a segment of passive drifting in the water column during which the animals are thought to sleep or process food, rather than forage (Crocker et al, 1997). Changes in depth during the drift phase occur as a result of changes in buoyancy (Crocker et al, 1997; Webb et al, 1998). This property has been used to monitor changes in body composition in southern elephant seal pups and an increase in the rate of change in depth has been used to infer the onset of weight gain as fat, which may imply successful foraging (Biuw et al., 2003). Grey seals do not perform drift dives but the rate of descent changes as a result of differences in buoyancy (Beck et al, 2000). Further work will include investigation of the possibility that temporal changes in descent rate in grey seal pups can be used to infer the onset of successful foraging in these animals.

Survival of southern elephant seal pups may depend on their ability to reach foraging grounds before they starve to death. Sex and weaning mass do not influence the timing of the behavioural transition from the travelling phase to the foraging phase in these animals, indicating that food is located in fixed patches and animals had to

reach these specific areas to forage, irrespective of their individual characteristics (McConnell et al, 2002). Large pups are therefore likely to be able to rely on endogenous fuel reserves until they reach the foraging grounds several thousands of kilometres away, whereas small pups may not possess sufficient energy stores to sustain themselves for that long. Ongoing work will include a similar comparison of estimates of the onset of successful foraging with the estimates of days to starvation, provided in chapter 6. This will provide insights into how body size, composition and fasting fuel use strategies affect how closely pups approach fuel depletion and how long they are likely to survive after departure from the colony.

The methods described above will provide useful information about the foraging areas used by grey seal pups and their feeding success in the long term. In the first instance it is likely that pups forage erratically and catch prey sporadically as they learn to forage. Grey seal pups in captivity learn to hunt and kill roach within four days of first exposure to live fish (Kastelein et al, 1995), which demonstrates that they can learn adequate foraging techniques quickly once they have encountered prey. It is these first successful prey encounters that will sustain them until they learn more effective feeding techniques. Dietary intake does not need to meet energetic requirements for pups to survive. Indeed, northern elephant seal pups can gain lean body mass at the expense of fat reserves on only 1% of their daily energy requirements (Condit and Ortiz, 1987). The satellite telemetry data may not contain a strong behavioural signal from these first foraging attempts because they may not be associated with a predictable pattern of behaviour or dive type. When pups first leave the colony, square-bottomed dives and repeated diving in discrete areas need not be associated with successful prey capture, and, conversely, opportunistic feeding may occur during other dive types. Indeed the V-shaped profile dives, more commonly

used during travel phases by adults, are thought to represent opportunistic feeding and allow regular sampling of the sea-bed for potential foraging sites (Thompson et al, 1991; McConnell et al, 1999).

Other ways of estimating when pups first feed effectively could involve the use of stomach temperature loggers and devices that measure gape angle, thereby detecting prey ingestion, and the use of devices that can detect defecation events, which would indicate when the gut becomes active again after fasting. These devices have been used successfully on a variety of sea bird species (Gremillet and Plos, 1994; Wilson et al, 1992; Wilson et al, 1995).

7.4.2. Maximum diving capabilities

7.4.2.1. Time since weaning, fast duration and sex

Grey seal pups that undertake a longer postweaning fast are able to remain submerged for longer during the longest dives performed when they first go to sea than pups that leave the colony earlier. Pups that spent longer fasting on land also exhibit a greater maximum proportion of time at sea underwater and can decrease post dive surface interval to minimum values more rapidly than pups that undertake a shorter fast. This may confer a survival advantage since these animals have a greater chance of early foraging success. Marginal increases in the ability to remain submerged are likely to be especially important for survival during the initial few days or weeks at sea when pups are under considerable pressure to begin to feed before their protein reserves reach critically low levels (chapter 6).

Fast duration is likely to influence diving capabilities as a result of development of oxygen storage capacity and control of oxygen utilisation. An increase in haematocrit, mass specific blood volume, and haemoglobin and myoglobin concentrations results in a 47% increase in overall mass specific oxygen storage capacity during the postweaning fast in northern elephant seal pups (Thorson and Le Boeuf, 1994; Noren et al, 2000). Haemoglobin concentration and haematocrit also increase with time spent fasting in grey seal pups and reach levels similar to those seen in yearlings by 24 days after weaning (Noren et al, 2003b). The duration of sleep apnoea increases during the postweaning fast in northern elephant seals, indicating development of the ability to tolerate and regulate prolonged periods of breath-holding (Blackwell and Le Boeuf, 1993). This is associated with an increase in the ability to regulate cardiorespiratory and vascular responses to breath-holding (Castellini et al, 1986; Castellini et al, 1994). A similar increase in cardiovascular control occurs with age in Weddell seal pups (Burns, 1999) and may also occur in grey seal pups during fasting. In grey seal pups, fast duration varies considerably between animals, from nine (Noren et al, 2003b) to 40 days (current study) and this may substantially impact on the ability to remain submerged if the rate of development of oxygen storage capabilities and control of oxygen usage is relatively consistent between animals. Although the effect of the duration of the postweaning fast on oxygen storage and usage has been demonstrated in grey seal pups (Noren et al, 2003b), this is the first evidence of its impact on early diving capabilities.

Males in this study apparently had reduced diving capabilities compared to females, both when the animals first entered the water and when they achieved their maximum physiological capabilities during their first three months at sea. Since female pups fasted for longer, on average, than males, the sex effects may be due, at

least in part, to differences in the degree of development of diving capabilities on land. An alternative explanation is a difference in foraging strategies used by the two sexes, in which males spend less time submerged than females. These sex differences in diving capabilities or foraging strategy could contribute to the differential first-year survivorship observed between males and females (Hall et al, 2001).

7.4.2.2. Mass and body composition

Pups that were heavier at departure were initially less able to remain submerged during individual dives than lighter pups. This contradicts other results in this study that indicate a larger body size confers greater diving capabilities, and the reason for this contradiction is unclear. It is possible that pups that were heavier at departure had not fasted for as long as pups that were lighter at departure and this finding is a result of less time spent developing on land.

Pups that were larger at weaning were able to achieve greater maximum dive durations and shorter minimum post dive intervals for dives of any given duration than smaller pups. Pups that were heavier at departure also achieved a shorter minimum post dive surface interval and a greater maximum proportion of time spent diving. Animals with greater absolute amounts of body protein and a greater proportion of fat developed better diving capabilities more quickly than smaller leaner animals. Diving capability in general was therefore greater in larger animals in better body condition. This is similar to findings in other phocid pups in their first year of life in which larger animals perform longer dives than their leaner or smaller conspecifics (Burns et al, 1997; Hindell et al, 1999; Irvine et al, 2000).

Pups that were larger with greater lean body mass were able to perform longer dives and spend a greater proportion of time submerged, perhaps as a result of greater oxygen storage capacity. Proportionally fatter animals, which were able to dive for longer and reach their maximum capabilities more quickly than leaner animals, may have lower maintenance and thermoregulatory costs as a result of smaller lean tissue reserves and greater insulation from blubber.

In this study fast duration was artificially extended as a result of holding the animals in pens. However, in completely free ranging pups, fast duration is positively correlated with percentage fat at weaning (Noren et al, 2003b). Pups in better condition at weaning are therefore more likely to have greater oxygen storage capacity at departure from the colony and have had more time to develop cardiovascular responses to breath-holding than leaner individuals (Noren et al, 2003b). The current findings demonstrate that this translates into greater diving capabilities in the initial few days or weeks at sea and provides a potential mechanism by which higher maternal investment in phocid seals confers a possible survival advantage to the offspring.

7.4.2.3. Time since departure

The duration of the longest dives within each depth band increased and the minimum post dive surface interval of dives of any duration decreased, as a function of time since departure. This likely results from continued improvement in the management of oxygen reserves and development of oxygen storage capabilities whilst at sea. This suggestion is supported by findings from other studies. For

instance, mass-specific metabolic rate decreases with age in harp, grey and northern elephant seal pups, despite the energetically costly process of growth (Worthy, 1987; Thorson and Le Boeuf, 1994). Northern elephant seal pups depart from the colony with roughly 75% of the myoglobin concentrations seen in adults (Thorson and Le Boeuf, 1994) but attain adult levels by 300 days of age (Noren et al, 2000). The maturation of myoglobin concentrations is related to the onset of independent foraging in cetaceans, pinnipeds and penguins (Noren et al, 2000). This is likely due to a training effect of diving. Myoglobin concentration increases 31% in the muscles of Barr headed geese as a result of twelve weeks of exercise (Saunders and Fedde, 1991). Hypoxia during apnoea may also contribute to development of myoglobin concentration and blood oxygen stores (Noren et al, 2000).

7.4.3. Comparison between age classes

7.4.3.1. Pups and subadults

Dive durations and percentage time submerged for older, experienced animals far exceeded both the mean and often the maximum values exhibited by pups during their first three months at sea. Surface intervals for dives longer than two minutes performed by pups were almost double those seen after dives of a similar duration in adults, which occurred during travelling and short duration trips. Pups, even when operating at their maximum capabilities, seem unable to achieve the same amount of time underwater as adults, either within individual dives or over longer periods of time. This could reduce their effectiveness at exploiting the same prey resources as

adults if that prey is hard to find or to catch. This is very similar to the findings in Weddell seal and southern elephant seal pups (Burns, 1999; Hindell et al, 1999; Irvine et al, 2000). Southern elephant seal pups perform most of their drift dives during the middle of the day, which is thought to reflect the descent of vertically migrating prey to depths beyond the diving capabilities of these young animals (Hindell et al, 1999). Based on calculated ADLs from body size and expected metabolic rates, Weddell seal pups have only a third of the time that is available to adults to spend diving aerobically to depths of over 100m. Beyond a depth of 150m weaned Weddell seal pups show a reduction in bottom time as a proportion of the duration of the dive and subsequent surface period, termed foraging efficiency (Burns, 1999). It declines in weaned pups in dives longer than eight minutes, which corresponds to 1.25 times the ADL, but only after 16 minutes in yearlings and 34 minutes in adults. Roughly 20% of dives are longer than this critical period of time in weaned pups, compared to 4% in yearlings and less than 0.5% in adults. The inability to remain at depth for very long may account for the low survival probability of grey seals in their first year and has been suggested as a major contributing factor to low juvenile survival in other pinnipeds (Burns, 1999). However, it may be unimportant if the pups find sufficient food to sustain themselves, despite operating at a much reduced proportion of time spent underwater compared to adults.

It should be noted that the sub-adult grey seals used in this comparison were tracked during the summer roughly fifteen years previously (Thompson et al, 1991), when prey distribution and water temperatures were likely to be very different from those experienced by the pups in the current study. Pup behaviour will be compared in more detail with that of adults tracked during winter (McConnell et al, 1999) to investigate the differences between age classes more thoroughly.

7.4.3.2 Pups and inexperienced yearlings

The maximum ability to remain submerged within three months of departure was similar between wild pups-of-the year and yearling animals that had spent their first year in captivity. This demonstrates that within three months of leaving the colony grey seal pups are capable of dive durations comparable with animals that have had experience of diving and are thus adequately equipped to begin foraging.

The increase in the maximum dive duration and percentage time spent submerged did not occur in yearling animals that had experience of diving in captivity but were equally inexperienced in terms of prey capture in the wild. This early increase is therefore unlikely to be a result of learning to find and capture live prey, since the same pattern would be expected in both groups of naïve animals. It likely occurred as a result of development of diving capabilities in pups that had already occurred in the yearling animals in captivity. The early increase in maximum dive durations in pups may also reflect a need to remain submerged for longer and push against physiological limitations more frequently during the early period at sea in order to catch sufficient prey. A comparison between the most frequent dive durations, which represent behavioural choice, and the 95th percentile dive durations will be performed as part of the ongoing analysis of this data to determine how closely grey seal pups approach their physiological limits over time.

Weddell seal pups often exhibit dives that are 25% longer than their ADL. It has been suggested that these animals take advantage of the relatively slow early increase in lactate caused by anaerobic metabolism to extend dive duration beyond their aerobic capabilities, whilst minimising the impact of lactate build up on subsequent dive durations or surface periods (Burns, 1999). However, it is not

possible to sustain this strategy for any length of time because it does not eliminate the lactate, which must still be metabolised aerobically. Instead these findings suggests that an ability to depress cellular metabolism, which allows aerobic metabolism to be maintained at a low level for an extended period and avoids lactate build up, develops early in these animals.

A decline in maximum dive durations over time was seen in both naïve pups and yearlings. This could represent a decrease in oxygen storage capacity, if animals lost considerable amounts of lean body mass. However, northern elephant seal pups preferentially lay down lean body mass at the expense of blubber fat reserves when they first begin to forage (Condit and Ortiz, 1987). An increase in lean body mass is likely to increase metabolic costs. If the blubber layer became depleted through this mechanism in the pups in this study, a further increase in oxygen utilisation may have been required to maintain body temperature. In extreme cases the decline in dive duration and time spent submerged may reflect lack of success in the long term and may be indicative of animals that did not survive. However, there was a decline in maximum dive durations and proportion of time spent underwater of pups that were tracked for the longest periods of time and began to show adult-like movement patterns.

These declines may be due to a reduction in the requirement to remain underwater for extended periods either because the animals began to dive in shallower water, became more proficient at foraging or as a result of seasonal changes in prey type or density that required different foraging strategies. A more detailed comparison with adult data from the same times of year would indicate whether a seasonal change in foraging behaviour occurs in experienced animals.

7.4.4. Survival consequences of size, condition and fast duration

Despite large background variability that likely occurred as a result of tag loss or failure, it was still possible to detect an underlying relationship between departure mass and the duration that animals were tracked. Although this relationship was not strong and cannot be used to determine which animals survived, it is likely that it occurred as a result of greater survivorship in larger pups, similar to that reported previously (Hall et al, 2001). Larger pups have the advantage of a combination of greater energy reserves, that can sustain them for longer as they learn to forage, and larger oxygen stores that allow them to remain submerged for longer and thus increase their opportunities for catching prey.

Track duration increased as a function of departure mass irrespective of fast duration. Although the relationship was relatively weak, these results may suggest that pups should leave the colony when they are larger to increase their chance of survival. However, pups do not leave the colony at weaning, when they are largest, which demonstrates the importance of the postweaning fast as a critical period for development in these animals. Neither do all pups leave the colony after a fixed fast duration or at a fixed body mass. Whilst large pups have the double of advantage of the ability to remain on the colony for longer and depart at a higher mass (Arnbom et al, 1993; Carlini et al, 2001; Biuw, 2003), fast duration and body mass represent a trade off between development, fuel reserves and future survival for pups that are smaller at weaning. These animals can depart earlier at a higher mass, which may compromise their ability to dive but increase time at sea to locate prey before fuel reserves become depleted. Conversely they may choose to remain ashore for longer such that their diving capabilities are greater when they leave but they have less time

in which to find food before they begin to starve. The current results suggest that they may have a greater chance of survival if they choose the former option. Larger fuel reserves appear to be more important than the marginal gains in diving capabilities that occur as a result of longer time spent ashore, provided that pups can dive adequately to find sufficient food during individual dives. This trade off may be more important in years in which prey is scarce, when animals must spend longer underwater to find and capture food.

7.4.5. Summary

Grey seal pups show marked differences in movement patterns between individuals and in comparison to adults. Initially they tend to undertake local and coastal movement, but show wide dispersal from the colony within their first few months at sea. Pups begin to exhibit adult-like repeated trips from known haul-outs to discrete offshore areas 4-5 months after departure. They are able to reach almost all areas and depths available in the North Sea, but appear to be severely constrained in their ability to remain submerged compared with adults. The ability of grey seal pups to remain submerged when they first go to sea is related to the duration of the postweaning fast and thus the degree of development on land. In contrast, maximum diving capabilities achieved during the first three months at sea do not vary substantially between animals and are related to time since departure, and are therefore likely to be a product of diving-induced development of oxygen stores and cardiovascular control. Larger body size and longer fast duration confer increased diving capabilities, which may present a mechanism for increased survivorship in

bigger animals. Higher maternal investment therefore provides pups with considerable advantages during their first year of life.

Chapter 8

General Discussion

8.1 Overview

The main findings of this thesis provide an insight into the mechanism by which maternal investment in grey seals can translate into pup survival.

Chapters 6 and 7 demonstrate that, through the degree of provisioning of pups during suckling, females can influence both fasting fuel allocation and, ultimately, diving capabilities of their offspring, both of which may have a substantial bearing on first year survival. These results help to explain the substantial costs incurred by female seals in producing their pups (Fedak and Anderson, 1982; Anderson and Fedak, 1987; Mellish et al, 1999a; Pomeroy et al, 1999).

Chapters 4, 5 and 6 demonstrate the potential role of cortisol and thyroid hormones (TH) in fuel allocation, and in explaining individual differences in the contribution of fat to energy expenditure. These chapters also demonstrate that repeated handling, at frequencies commonly used in studies on seals on breeding colonies, do not have a substantial impact on levels of TH and cortisol or on energy partitioning.

Chapter 3 highlights the difficulties associated with measurement of protein hormones in non-target species and raised questions about conclusions drawn in previous studies about the role of leptin in seals.

The main findings are discussed below in a wider context, and avenues for further research are suggested.

8.2. Maternal investment and pup survivorship

Previous work on grey seal pups, and other seal species, has focussed on the high average contribution of fat to energetic demands during fasting (Nordoy and Blix 1985; Worthy and Lavigne, 1987; Nordoy et al 1990; Reilly, 1991; Nordoy et al, 1993; Kirby and Ortiz, 1994; Houser and Costa, 2001). This focus may have allowed the importance of protein catabolism in pinnipeds and its potential in limiting fast duration to be overlooked. However, this study has shown that there is substantial variability between grey seal pups in the contribution of fat to energetic needs (chapter 6), as is the case in rats, humans, polar bears and southern elephant seals (Cherel et al, 1992; Atkinson et al, 1996; Dulloo and Jacquet 1999; Biuw, 2003). The contribution of fat to energy expenditure increases with initial adiposity in all these species.

Given that lean tissue is not very energy dense but very metabolically costly, there is little advantage to be gained in possessing excess protein reserves. Female grey seals are likely to have a limited amount of protein that they can afford to transfer to their pups and in turn provide pups only with sufficient reserves to reach very local foraging areas. This study has shown that healthy pups can tolerate greater levels of protein depletion than previously thought, but are more likely reach these critical levels than they are to exhaust fat reserves. This is similar to findings in elephant seals and obese rats (Biuw, 2003; Cherel et al, 1992). This degree of protein depletion may be reached shortly after departure from the colony (chapter 6) and pups are therefore under considerable pressure to begin feeding during their first few days and weeks at sea. It is apparent that a detailed investigation of the timing and location of the onset of foraging, as indicated by characteristics of both movement patterns and

diving behaviour, would be extremely informative in determining how closely pups approach depletion of their fuel reserves.

Variability in the relative contribution of fat and protein to energy expenditure may impact on individuals' chances of survival, since pups that rely less on fat catabolism will deplete their protein reserves more quickly (chapter 6). By provisioning pups with greater fat reserves during suckling, females allow them the possibility of greater reliance on fat as a metabolic fuel whilst fasting, thus sparing crucial protein reserves. This provides fatter pups with the ability to sustain a longer period of fasting after weaning than leaner animals. However, pups with a small lean body mass may not gain from additional fat reserves, since protein limits the ability to sustain a fast. It is vital, therefore, that females provide pups with sufficient protein as well as fat.

In terms of diving capabilities, pups that are larger and fatter at departure, and those that undertake a longer postweaning fast are better equipped to dive, both when they first go to sea and up to three months later (chapter 7). Those pups that are larger at departure are those that appear to survive long enough to develop adult-like movement patterns, which are indicative of repeated foraging trips (Thompson et al, 1991; McConnell et al, 1999). Thus the advantage of being larger at departure persists for at least the first five months of the first year of life.

The benefits of high maternal investment to the pups are therefore two-fold. Firstly, larger pups have the potential to fast for longer on the colony and develop diving capabilities, such as oxygen storage capacity and cardiovascular control, before they go to sea. They are therefore more likely to begin to feed successfully when they first enter the water. Secondly, pups with greater fat stores at weaning have more time

in which to locate prey and learn to feed effectively before their protein reserves become critically depleted.

Clearly, pups that were large with substantial fat reserves at weaning can both undertake a longer postweaning fast on land and depart in good condition at a relatively high body mass. For small, lean pups this option is not available. In these animals there is a trade-off between departing at a higher body mass after a shorter fast, or leaving later, having undergone a greater degree of development but with a shorter time margin in which to find food. The current study indicates that small pups may have a greater chance of success if they adopt the first strategy (chapter 7). This suggests that the benefit in terms of extra time to begin feeding at sea is greater than the benefit in terms of the increment in diving capabilities as a result of a longer fast. Provided that pups have adequate capabilities to catch some food, irrespective of quality or quantity, when they first leave the colony, the ability to remain submerged to maximum capabilities may be relatively unimportant. Those pups with the ability to remain submerged for longer in the first instance are likely to have a considerable advantage only under circumstances when prey density, distribution or type requires a longer time spent underwater to obtain food during individual dives. If animals must spend a long time searching for prey over periods longer than individual dives, pups that have greater energy reserves are more likely to survive. The impact of body size and condition on diving capabilities within individual dives and over longer time-scales of hours and days, deserves further attention to determine to what extent smaller pups or those that have undergone a shorter fast are disadvantaged, relative to larger animals.

Pups from the Isle of May tend to spend a substantial amount of time around the Firth of Forth, Abertay Sands and Farne Islands in the first days to weeks after

departure. Food is relatively abundant in these areas, which are used intensively by adult seals and sea birds (McConnell et al, 1999; Harwood, 2000; Rindorf et al, 2000). However, prey availability for top predators may change from year to year as a result of changes in abundance of food resources for their prey, alterations in populations of other predators utilising the same prey resources, and changes in oceanographic features, such as water temperature, salinity and currents. These factors may be affected by anthropogenic impacts on global or local climate or large-scale fisheries operating in local, inshore foraging grounds. For example, grey seal numbers in the North Sea have grown over the last four decades and for some of this period there was an increase in the catch of the commercial sandeel fishery on the Wee Bankie and Marr Bank (Furness, 2002). Sandeels are a readily accessible prey resource because they are a relatively sedentary species that occurs in large schools in areas of predictable sediment type and they are a major constituent in the grey seal diet (Hammond and Prime, 1990; Hammond et al, 1994 a and b). Seals may benefit from fisheries that target piscivorous fish, such as mackerel, whiting and cod, if they decrease competition for this resource (Furness, 2002). However, the sandeel fishery reduced the availability of sandeels to predators in 1998 (Harwood, 2000). Grey seals are generalist predators and are not reliant solely on one prey species. The reduction in sandeel numbers forced a switch to greater reliance on cod and whiting in these animals, and had a large impact on the foraging strategies and reproductive success of many sea bird species (Harwood, 2000; Rindorf et al, 2000; Furness, 2002). Grey seal pups, especially individuals with less developed diving capabilities, may not be able to utilise alternative prey types as readily as larger and older animals if those prey are harder to catch. Differences in success of sea birds between years also resulted from interannual changes in timing of life history events of sandeels, driven by climatic and

oceanographic factors (Rindorf et al, 2000). This raises the possibility that survival of pups could also be compromised by further reductions in sandeel numbers caused by the fishery in years when recruitment is poor as a result of other factors.

8.3. Involvement of cortisol and TH during the postweaning fast

Chapter 6 demonstrated the importance of the availability of fuel reserves in energy partitioning during fasting in grey seal pups, by investigating both the effect of endogenous reserves and supplementary feeding. The similarity in body composition between weaning and departure, despite substantial mass loss of both fat and protein, demonstrates tight regulation of fuel use. The mechanism that controls the timing of departure is also likely to be linked to energy reserves because protein stores limit the amount of time that animals are able to fast (chapter 6). Although the detailed mechanism of fuel regulation remains unknown, the results in chapters 4 and 6 indicate that both cortisol and TH may be involved. Changes in the levels of these hormones are associated with natural and feeding-induced alterations in mass loss rate (chapter 4). Feeding slowed or stopped mass loss and in some cases caused an increase in body mass (chapter 4). Artificially elevated glucocorticoid (GC) concentrations also stimulated an increase in mass loss (chapter 6).

It remains unclear whether these hormones act on fat or protein catabolism, or elevate the utilisation of both components in concert in fasting grey seal pups. Given the large inter-individual variability in energy metabolism and the short term nature of the effects of the hormones on whole body mass loss rate, these possibilities could be distinguished by further direct manipulations of both cortisol and TH levels on a

greater number of animals, over shorter periods of time using techniques that are more sensitive to small, short term changes in body composition.

The role of both TH and cortisol in the decision to leave the colony is unclear. Neither hormone showed a large change towards the end of the fasting period and artificially elevated GC levels did not induce departure. Both hormones could act indirectly on timing of departure through an impact on fuel use, but did not seem to provide a proximate signal to terminate fasting.

8.3.1. Cortisol and departure

The current findings (chapter 6) are consistent with the proposed role of cortisol in fat mobilisation in fasting southern elephant seal pups (Ortiz et al, 2001) and lactating adult female sub-Antarctic fur seals (Guinet et al, 2004), but do not provide evidence for the suggestion that it promotes foraging behaviour in pinnipeds (Chapter 4 and chapter 6). In other animals cortisol causes an increase in the motivation to seek food in other animals and an elevation in blood concentrations is concurrent with the onset of phase III of fasting (Debons et al, 1986; Cherel et al, 1988a, b and c; Cherel et al, 1992; Green et al, 1992; Chen and Romsos, 1996; Robin et al, 1998). An elevation in cortisol, along with other hormonal and metabolic changes typically associated with phase III of fasting, can be caused by artificial blockade of fatty acid metabolism. (Bernard et al 2002). Increased cortisol levels associated with reduced fuel use seem to be inextricably linked to a metabolic shift away from fatty acid metabolism. The absence of involvement of cortisol in the departure from the colony in seal pups seems to be fundamentally different from its role in terrestrial mammals (Cherel et al, 1992; Challet et al, 1995), and other species

that undergo prolonged periods of fasting, such as geese and penguins (Cherel et al, 1988a, b and c; Robin et al, 1998). Cortisol may not play a significant role in departure from the colony in healthy weaned pups because these animals do not enter phase III whilst fasting on land (Nordoy et al, 1990; Nordoy et al, 1991). Indeed this would be maladaptive because increased cortisol at the onset of phase III acts to increase protein catabolism (Koubi, 1991; Challet et al, 1995), and seal pups must minimise further protein depletion when they leave the colony, because they may already be approaching critically low levels. Cortisol may only become a significant factor in the termination of the land-based fast in starving pups, which have severely limited fat reserves.

One caveat to these conclusions is the limited ability of dexamethasone to enter the central nervous system. To allow elevated GC levels to be completely ruled out from a role in timing of departure in healthy pups requires manipulation of levels using another cortisol analogue that can cross the blood-brain barrier more easily.

8.3.2. TH and development

One possible role for thyroid hormone changes in the postweaning fast may be in erythropoiesis. TH potentiate red blood cell production in humans and mice (Dainiak et al, 1978; Sullivan and McDonald, 1992) and a dramatic increase in blood oxygen storage capacity is known to occur during the postweaning fast (Thorson and Le Boeuf, 1994; Noren et al, 2003b). TH treatment of weaned pups could be used to investigate this possibility further.

8.3.3. Cortisol, TH and endocrine disrupters

Some pollutants, such as polychlorinated biphenyls (PCBs), are capable of binding to intracellular receptors and serum carrier proteins for steroid and thyroid hormones (Hall et al, 1998; Jenssen et al, 1995). These pollutants occur in the marine environment and are highly persistent and lipophilic. As a result, they become concentrated up the food chain and accumulate in the blubber of marine mammals, and can be transferred from females to offspring in maternal milk (Jenssen et al, 1995). Alterations in the ratio of T3 to T4 (Hall et al, 1998) and the ratio of free and bound hormone (Jenssen et al, 1995) are caused by PCB exposure in seals, even at very low levels. T3 is more active than T4 and free TH is more available to tissues than TH bound to transthyretin and other carrier proteins in the blood. Alterations in the ratios of circulating forms of TH can therefore have profound effects on energy balance. Exposure to high levels of PCBs has been suggested as one reason for the decline in the Baltic population of grey seals, perhaps mediated through effects on TH and steroids (Jenssen, 1996). Young seals are less capable of metabolising PCBs and are more vulnerable to the effects of toxic compounds (Jenssen et al, 1995). Since cortisol and TH seem likely to be important in fuel use and/ or development during fasting in grey seal pups, exposure to these endocrine disrupters during this critical life history stage could have severe detrimental effects on survivorship and may have an impact at the population level.

8.3.4. Impact of handling

Although cortisol and thyroid hormone levels showed rapid, short lived changes in response to handling stress, hormone concentrations and fuel use during fasting were not affected by repeated handling at the frequencies that were used in this study. Daily blood sampling, however, can cause local inflammation and changes in hormone levels. This highlights the need to obtain blood samples rapidly, not only to minimise stress, but to reduce the potential noise in hormone measurements, even those that are not directly involved in the stress response. Grey seal pups seem relatively resilient to repeated exposure to short-lived stressors. However, stress caused by more frequent handling, or by other anthropogenic factors, such as repeated disturbance of breeding colonies, could cause chronic alterations to the endocrine system and regulation of fuel use. Any such changes may be detrimental to survival of grey seal pups since fuel economy is crucial, at least until they can forage effectively.

8.4. Potential involvement of other hormones in fasting fuel use

Prolactin was not detectable in grey seal pups and was thus eliminated as a possible endocrine mediator of fuel regulation and timing of departure from the colony in these animals (chapter 3). It is possible that hormones not considered here, such as insulin, glucagon, growth hormone, ghrelin, melatonin and others, play an important part in the control of fuel use and the onset of foraging behaviour in grey seals.

Insulin is involved in carbohydrate metabolism. It promotes glucose uptake, either for utilisation or storage as glycogen and it stimulates fat storage. Levels tend to

decrease with the nutritional status of the animal (Strack et al, 1995; Remesar et al., 1997) and decline in fasting penguins and geese (Cherel et al, 1988a b and c). Low levels of insulin allow glucagon and cortisol, which become elevated at the onset of phase III, to promote foraging and increased protein use (Cherel et al, 1988a b and c).

Glucagon antagonises the actions of insulin by stimulating the release and utilisation of stored fuel. It is a potent gluconeogenic hormone and increases progressively throughout phase II and III of fasting in penguins to maintain the supply of gluconeogenic precursors for metabolism (Cherel et al, 1988a, b and c). The substantial increase in this hormone at the onset of phase III is thought to maintain FFA availability despite diminishing fat stores (Bernard et al, 2002).

Growth hormone promotes growth and development (Nilsson et al, 1994; Wester et al, 1998) and is a powerful stimulant of lipolysis (Berle et al, 1974; Tsipoura et al, 1999; Ottoson et al, 2000; Djurhuus et al, 2004) and protein synthesis (Wester et al, 1998; Beaufriere, 1999; Bush et al, 2003). Growth hormone increases during fasting in northern elephant seal pups (Ortiz et al, 2003c). Circulating levels correlate with an increase in FFA and a decline in BUN, suggesting that it promotes fat catabolism and is involved in protein sparing in seals, as in other animals (Ortiz et al, 2003c).

Ghrelin is a growth hormone receptor agonist involved in growth regulation, feeding behaviour and energy homeostasis (Kojima et al, 1999; Kamegai et al, 2000; Wren et al, 2000; Nakazato et al, 2001). Levels increase during fasting in northern elephant seal pups and have been implicated in the control of metabolism in these animals (Ortiz et al., 2003c).

Melatonin secretion is regulated by photoperiod. It is an endocrinological clock and calendar that entrains intrinsic biological rhythms to the external light-dark

cycle. It thus ensures the appropriate diurnal and seasonal adjustments in behaviour and physiology occur (Reiter, 1981; Wehr, 1998), including energy intake and expenditure (Nelson et al, 1992; Saarela and Reiter, 1993; Basco et al, 1996; Le Gouic et al, 1996), and may be involved in diurnal and seasonal rhythmicity in diving behaviour in seals (Bennett et al, 2001).

8.5. The role of leptin in seals

Chapter 3 highlighted the inability of current techniques to measure leptin accurately in seal serum, despite the presence of a leptin-like protein in the blood of grey seals. Other researchers may therefore have ruled out a role for leptin in fat regulation in pinnipeds prematurely, based on results from an unreliable assay (Ortiz et al, 2001; Arnould et al, 2002). A relationship between leptin levels and body fat reserves may be obscured by leptin secretion by sites other than the fat stores (Hammond et al, in press). Alternatively, the role of leptin may be divorced from body fat regulation in seals because these animals must allow radical changes in body composition to occur as a result of intensive foraging bouts followed by extended periods of fasting during moult and breeding seasons, or as a result of the dual role of blubber as a fat reserve and as insulation.

However, seal pups appear to regulate relative fat content very closely (Nordoy and Blix, 1985; Rea and Costa, 1992; Carlini et al, 2001; Biuw, 2003; Noren et al, 2003a; chapter 6) and this requires considerable co-ordination between fat deposition and accretion in blubber stores, energy expenditure and feeding. The presence of leptin mRNA in blubber suggests that it performs a similar function in fat

regulation in seals as it does in other mammals. Low leptin concentrations (Ortiz et al, 2001; Arnould et al, 2002) may be a consequence of the high fat and low carbohydrate diet of seals. However, a largely fat-based metabolism should not result in a lower flux through the hexosamine pathway, which controls leptin secretion in other mammals (Wang et al, 1998; Considine et al, 2000; McClain et al, 2000; Rosetti, 2000), and should not therefore result in decreased leptin secretion through this mechanism. Low circulating leptin concentrations are seen in dogs, for which a species-specific leptin assay exists (Iwase et al, 2000) and could be a consequence of carnivory.

Development of a seal specific leptin assay is necessary to measure blood levels accurately. This is now possible with the sequencing of the phocine leptin protein and production of a seal-specific anti-seal leptin antibody (Hammond et al, in press). Investigation of leptin levels in the blood and expression in the blubber during fasting and feeding is required to investigate its role in seals at the whole-animal level. Studies on the ability of leptin to increase fat metabolism in isolated adipocytes would also confirm whether phocine leptin is biologically active in the same way as in terrestrial mammals.

One exciting possibility is that leptin is involved in respiratory physiology in seals. Grey and harbour seals are the only adult mammals investigated so far to express leptin in the lung (Hammond et al, in press). This may reflect the particular physiological challenges faced by mammals that repeatedly dive to depth. To prevent tissue nitrogen accumulation they exhale prior to diving and allow the lungs to collapse whilst at depth (Kooyman, 1989). For the lungs to re-inflate as the animals return to the surface may require copious surfactant production, which reduces surface

tension at the air-water interface to allow the lungs to inflate. Weddell seals cough up large amounts of surfactant at the surface after deep dives (Miller et al, 2003).

Surfactant production is caused by mechanostimulation of the lungs in California sea lions, *Zalophus californianus* (Miller et al, 2003). In terrestrial animals, a paracrine feedback loop involving both leptin and parathyroid hormone-related protein (PTHrP) is required for the dramatic stretch-induced increase in surfactant production (Torday and Rehan, 2001; Torday et al, 2002). Mechanostimulation of the lungs induces leptin expression in lung lipofibroblasts (LFs) and triglyceride (TG) trafficking in response to stretch-induced secretion of PTHrP from neighbouring epithelial alveolar type II (ATII) cells (Torday et al, 2002). The increase in TG availability and leptin promote surfactant phospholipid synthesis and the expression of surfactant protein by the ATII cells (Torday and Rehan, 2001).

Leptin is normally only expressed in lung in the foetus at a critical stage in gestation during fluid distension of the lungs. This promotes surfactant production in the foetus to allow the lungs to inflate for the first time at birth (Torday et al, 2002). Since the lungs never naturally collapse in most mammals after birth, there is no requirement for continued leptin secretion in lung and leptin is not expressed in lung in adult terrestrial mammals (Iwase et al, 2000). In contrast, the large changes in lung volume experienced by phocids during diving may provide the stimulus for leptin secretion, which in turn may mediate surfactant production on a dive-by-dive basis and allow the lungs to re-inflate as the animals approach the surface. A comparison of leptin expression in lung tissue between marine mammals that dive with full, partially full and collapsed lungs is planned and will provide a test of this hypothesis. Studies on the level of expression of leptin in seal lungs and production of surfactant in

response to mechanostimulation and leptin would provide direct evidence of the role of leptin in this process.

8.6. Summary

This thesis has furthered our understanding of pinniped biology by demonstrating potential mechanisms through which maternal investment can influence pup survival. Rather than focussing on the heavy reliance on fat as a metabolic fuel by fasting pups, the importance of variability in energy partitioning has been highlighted and has emphasised the role of protein as a limiting fuel resource for these animals. By providing pups with greater fat reserves females provide them with the opportunity to fast for longer to develop diving capabilities, and the capacity to survive for longer on endogenous reserves at sea before they find food.

This study has used feeding and hormonal manipulation intervention studies for the first time in wild, fasting seals to try to elucidate the mechanism underlying individual differences in fuel allocation strategies. A link has been demonstrated between energy availability, GC levels and fuel utilisation. More information about hormonal regulation of energy use in these animals is required for our understanding of how the size of initial energy causes observed individual variability in energy partitioning.

To identify mechanisms behind the signal to leave the colony we need better understanding of the complex interactions between energy availability and developmental requirements. Reliable hormone assays are needed, along with *in vivo* and *in vitro* hormonal manipulations.

The importance of body size and condition and the duration of the postweaning fast in the early diving capabilities of grey seals have been demonstrated for the first time. Further examination of ontogeny of behaviour at sea will provide more details about the impact of body condition in terms of diving characteristics and long-term success.

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Appendix 1

Immunoassay design

Assay design and protocols are provided by the manufacturers and are outlined briefly below.

Leptin and cortisol RIAs

A fixed volume of sample (unknown concentration) or Ag standard (known concentration) competes with a fixed quantity of ^{125}I -labelled Ag tracer for limited binding sites on an anti-Ag Ab. In the cortisol assay, this is a polyclonal rabbit anti-cortisol Ab bound to the assay tubes (Orion-Diagnostica, 2001). In the leptin assay, the Ab is a guinea-pig anti-human leptin Ab in solution, and separation of Ab bound leptin is achieved by incubation of the solution with precipitating reagent, followed by centrifugation (Linco Research Inc., 2000). After decanting and washing the reaction tubes, the amount of radioactivity bound to Ab in the tubes is counted in a gamma counter. The amount of radioactivity (counts per minute) present is inversely related to the amount of unlabelled Ag in the original sample or standard.

Leptin IEMA

Leptin, in the sample or standard, binds simultaneously to an Ab pre-coated to the wells of a microplate and a guinea pig anti-canine leptin Ab in solution. A horseradish peroxidase (HRPO) labelled anti-guinea pig IgG Ab is then added to bind

to the IgG in the Ab-leptin-Ab complex. Excess HRPO-labelled Ab is removed by washing and the bound HRPO-labelled Ab is detected by the addition of the HRPO substrate, 3,3',5,5'-tetramethylbenzidine (TMB), to produce a change from colourless to blue. The reaction is stopped using sulphuric acid, causing a change from blue to yellow, and the absorbance is read at 450nm, with 630nm as reference. Absorbance is directly related to the amount of HRPO-labelled Ab present and therefore to the concentration of leptin ([leptin]) in the original sample, standard or control.

TH and PRL

In the TH assays, Ag in the samples, standards and controls is detected by a mouse anti-Ag monoclonal Ab labelled with alkaline phosphatase (BioChemImmunoSystems, b and c). Unlabelled Ag competes with fluorescein-conjugated Ag-derivative for access to binding sites on the Ab. The Ag-derivative contains 8-anilo-1-naphthalene sulphonate, which displaces Ag in serum from carrier proteins to allow the total amount of the hormone to be measured.

For the PRL IEMA, two mouse anti-PRL monoclonal antibodies bind to two distinct epitopes on PRL in the samples, standards and controls, forming a "sandwich"(BioChemImmunoSystems a). One antibody is labelled with alkaline phosphatase and the other is conjugated to fluorescein.

In both the EIAs and the IEMA, the Ag-label-anti-Ag complex is detected by a sheep anti-fluorescein Ab, added in excess. This Ab is covalently bound to magnetic particles, which allows the whole complex to be sedimented by exposure to a magnetic field. Unbound Ag and/or Ab in the supernatant is decanted and washed away. Addition of alkaline phosphatase substrate, phenolphthalein monophosphate

(PMP), results in a change from colourless to pink. The reaction is stopped using sodium hydroxide and the intensity of the developed colour is measured photometrically. The amount of Ag-alkaline phosphatase conjugate present is directly related to absorbance. Absorbance is inversely related to unlabelled [TH] (EIAs) and directly related to [PRL] (IEMA) in the original sample, standard or control.

Appendix 2

Details of LMEs in chapter 4

Table A2.1: Result of LME comparing [cortisol] at *S1*. AIC = 759.683; n (individuals) = 28; n (observations) = 82. Group is abbreviated to the appropriate letter (F, H, L and U).

a. Within *timepoint* (df=20). * denotes a significant difference between males and females within the FED group within *timepoint*. Red font denotes a significant sex by group interaction.

b. Between *timepoint* (df = 37). Bold font indicates a significant difference between *timepoint* within each sex within each group. * denotes a significant difference between males and females within group. Red font indicates a significant difference in the change in [cortisol] between *timepoint* between the FED group and the group highlighted. ~ denotes a significant interaction between sex, *timepoint* and the FED group with the group indicated.

a.

<i>Timepoint</i>	Group comparison	Females		Males	
		T	p value	T	p value
*early	F→H	1.57	0.1314	0.97	0.3456
	F→L	1.79	0.0894	1.66	0.1132
	F→U	1.61	0.1224	0.11	0.9166
	H→L	0.21	0.8336	0.69	0.4991
	H→U	0.14	0.8890	0.90	0.3814
	L→U	0.38	0.7101	1.45	0.1621
mid	F→H	1.68	0.1091	0.77	0.4490
	F→L	1.44	0.1645	0.47	0.6400
	F→U	1.69	0.1062	0.21	0.8346
	H→L	0.23	0.8173	0.30	0.1045
	H→U	0.19	0.8536	0.84	0.4102
	L→U	0.07	0.9415	0.60	0.5559
late	F→H	0.41	0.6869	0.39	0.7007
	F→L	1.02	0.3218	0.22	0.8288
	F→U	0.27	0.7919	0.13	0.9015
	H→L	1.43	0.1689	0.15	0.8845
	H→U	0.71	0.4869	0.44	0.6623
	L→U	0.83	0.4168	0.30	0.7651

b.

Group	Timepoint comparison	Females		Males	
		T	p value	T	p value
F	*early→mid	1.70	0.0984	1.81	0.0600
	mid→late	1.49	0.1455	0.28	0.7826
	early→late	0.29	0.7712	1.94	0.0786
H	~*early→mid	2.52	0.0160	0.38	0.7091
	mid→late	0.06	0.9535	0.69	0.4951
	early→late	2.37	0.0230	0.47	0.6411
L	~*early→mid	2.42	0.0208	0.64	0.5293
	mid→late	1.42	0.1639	0.49	0.6248
	early→late	0.45	0.6547	0.06	0.9493
U	early→mid	2.75	0.0090	1.65	0.1083
	mid→late	0.67	0.5093	0.12	0.9045
	early→late	1.47	0.1495	1.64	0.1085

Table A2.2. Results of comparison of Δ [cortisol] (AIC = 763.791; df = 52) and rate of Δ [cortisol] (AIC = 481.364; df = 51) between *timepoint*. Direction of change is indicated by \rightarrow . Bold font indicates a significant difference between *timepoints* ($p < 0.05$). n (individuals) = 28; n (observations) = 82.

	Timepoint comparison	T	p value
Δ [cortisol]	early→mid	1.26	0.2129
	mid→late	0.86	0.3934
	early→late	2.09	0.0411
rate of Δ [cortisol]	early→mid	2.59	0.0123
	mid→late	1.30	0.2005
	early→late	3.44	0.0012

Table A2.3. Results of comparison of [TT4] between *timepoint* (AIC = 49.618; df = 52). Direction of change is indicated by \rightarrow . Bold font indicates a significant difference between *timepoints* ($p < 0.05$). n (individuals) = 28; n (observations) = 82.

Timepoint comparison	T	p value
early→mid	2.81	0.0070
mid→late	0.58	0.5639
early→late	3.33	0.0016

Table A2.4: Result of LME comparing [cortisol] within and between FED and HIGH groups within and between *day*. AIC = 48.422; n (individuals) = 14; n (observations) = 117.

a. Comparison of [cortisol] within each *day*. Bold font indicates a significant difference ($p < 0.05$) in [cortisol] between group within *day* within each sex ($df = 68$). * denotes a significant difference between males and females within each *day* and group ($df = 10$) and red font indicates a significant interaction between sex and group within *day* ($df = 10$).

b. Comparison of the change in [cortisol] between *day* ($df = 68$). Bold font indicates a significant difference ($p < 0.05$) in the change in [cortisol] between *day* within each sex and group. Red font denotes a significant difference in the change in [cortisol] between *day* between group within each sex. * and # denote significant differences in the change in [cortisol] between *day* between the two sexes within the FED and HIGH group respectively. ~ indicates a significant interaction between group, *day* and sex.

a.

<i>Day</i>	Females		Males	
	T	p value	T	p value
* 1	2.23	0.0498	0.71	0.4946
4	0.57	0.5800	0.48	0.6407
7	0.42	0.6804	2.54	0.0294
10	3.09	0.0115	3.37	0.0072
13	1.21	0.2559	1.17	0.2691
16	0.40	0.6971	0.10	0.9244
19	0.53	0.6095	0.79	0.4506
* 22	0.99	0.3443	0.71	0.4926
25	0.47	0.6840	0.70	0.5034

b.

Day comparison	Females				Males			
	FED		HIGH		FED		HIGH	
	T	p value	T	p value	T	p value	T	p value
~1 → 4	2.49	0.0154	0.54	0.5892	0.26	0.7987	1.55	0.1267
1 → 7	2.57	0.0124	0.31	0.7617	1.90	0.0618	0.01	0.9907
1 → 10	3.84	0.0003	1.38	0.1716	2.30	0.0245	0.56	0.5809
~* 1 → 13	2.53	0.0137	0.99	0.3271	0.29	0.7704	1.58	0.1197
1 → 16	1.94	0.0562	0.86	0.3954	0.44	0.6584	1.04	0.3021
~1 → 19	2.13	0.0368	0.66	0.5088	0.34	0.7331	1.78	0.0801
1 → 22	1.12	0.2688	0.24	0.8111	1.40	0.1672	1.36	0.1793
1 → 25	0.78	0.4389	1.20	0.2358	0.05	0.9616	0.21	0.8349
4 → 7	0.19	0.9064	0.28	0.7813	1.48	0.1449	1.53	0.1300
4 → 10	1.69	0.0954	0.94	0.3527	1.83	0.0710	2.11	0.0389
4 → 13	0.15	0.8778	0.46	0.6491	0.52	0.6085	0.10	0.9184
4 → 16	0.52	0.6023	0.33	0.7396	0.16	0.8767	0.44	0.6624
4 → 19	0.11	0.9131	0.12	0.9043	0.06	0.9488	0.33	0.7441
4 → 22	1.31	0.9154	0.30	0.7654	1.03	0.3091	0.07	0.9472
4 → 25	1.69	0.0956	0.74	0.4620	0.20	0.8447	1.15	0.2542
7 → 10	1.58	0.1194	1.23	0.2223	0.42	0.6769	0.54	0.5923
~7 → 13	0.04	0.9702	0.75	0.4553	2.20	0.0310	1.62	0.1101
7 → 16	0.64	0.5227	0.62	0.5368	1.44	0.1538	1.07	0.2866
7 → 19	0.21	0.8309	0.40	0.6876	1.55	0.1269	1.85	0.0681
7 → 22	1.41	0.1626	0.06	0.9563	0.42	0.6795	1.42	0.1605
7 → 25	1.79	0.0785	1.04	0.3038	1.82	0.0738	0.21	0.8377
10 → 13	1.55	0.1271	0.45	0.6570	2.62	0.0108	2.04	0.0451
10 → 16	2.22	0.0299	0.60	0.5486	1.87	0.0664	1.55	0.1269
10 → 19	1.62	0.1090	0.81	0.4235	1.97	0.0532	2.25	0.0276
10 → 22	2.82	0.0064	1.20	0.2339	0.83	0.4097	1.83	0.0715
10 → 25	3.14	0.0025	0.19	0.8529	2.24	0.0284	0.13	0.8936
13 → 16	0.69	0.4955	0.15	0.8845	0.75	0.4583	0.57	0.5686
13 → 19	0.25	0.8043	0.36	0.7184	0.65	0.5188	0.25	0.8074
~* 13 → 22	1.44	0.1535	0.78	0.4371	1.75	0.0856	0.18	0.8558
13 → 25	1.80	0.0766	0.26	0.7931	0.34	0.7317	1.24	0.2178
16 → 19	0.36	0.7193	0.21	0.8314	0.10	0.9189	0.82	0.4170
16 → 22	0.83	0.4086	0.64	0.5266	1.02	0.3127	0.39	0.6996
16 → 25	1.20	0.2353	0.42	0.6788	0.40	0.6905	0.89	0.3759
19 → 22	1.10	0.2768	0.45	0.6542	1.12	0.2661	0.43	0.6693
19 → 25	1.43	0.1576	0.62	0.5348	0.30	0.7648	1.40	0.1652
22 → 25	0.36	0.7178	1.04	0.3036	1.43	0.1577	1.33	0.2609

Table A2.5: Result of LME comparing [cortisol] within and between FED and HIGH groups within and between *prop*. AIC = 56.852; n (individuals) = 13; n (observations) = 105.

a. Differences in [cortisol] between group within *prop* (df = 10). Bold font indicates a significant difference between group.

b. Differences in [cortisol] between *prop* categories within and between group (df = 75). Bold font indicates a significant difference within group between *prop* categories.

* denotes a significant difference between group between *prop* categories.

<i>Prop</i>	T	p value
0.01-0.1	1.13	0.2850
0.11-0.2	0.46	0.6589
0.21-0.3	0.98	0.3517
0.31-0.4	3.19	0.0097
0.41-0.5	0.36	0.7293
0.61-0.7	0.34	0.7386
0.71-0.8	0.43	0.6740
0.81-0.9	0.53	0.6047
0.91-1	0.65	0.5320

b.

<i>Prop comparison</i>	FED		HIGH	
	T	p value	T	p value
0.01-0.1 → 0.11-0.2	1.80	0.0758	1.33	0.1879
0.01-0.1 → 0.21-0.3	3.08	0.0029	0.82	0.4158
* 0.01-0.1 → 0.31-0.4	3.01	0.0036	1.59	0.1167
0.01-0.1 → 0.41-0.5	1.08	0.2847	0.52	0.6076
0.01-0.1 → 0.61-0.7	1.56	0.1231	0.75	0.4545
0.01-0.1 → 0.71-0.8	0.97	0.3338	0.62	0.5369
0.01-0.1 → 0.81-0.9	1.67	0.0983	0.04	0.9720
0.01-0.1 → 0.91-1	1.94	0.0556	1.94	0.0563
0.11-0.2 → 0.21-0.3	1.12	0.2672	0.40	0.6874
* 0.11-0.2 → 0.31-0.4	1.30	0.1971	2.75	0.0076
0.11-0.2 → 0.41-0.5	0.87	0.3848	1.85	0.0678
0.11-0.2 → 0.61-0.7	0.39	0.6950	0.56	0.5809
0.11-0.2 → 0.71-0.8	0.72	0.4731	1.91	0.0597
0.11-0.2 → 0.81-0.9	0.13	0.8975	1.24	0.2174
0.11-0.2 → 0.91-1	0.25	0.8063	0.55	0.5844
0.21-0.3 → 0.31-0.4	0.33	0.7390	2.20	0.0310
0.21-0.3 → 0.41-0.5	2.20	0.0312	1.30	0.1970
0.21-0.3 → 0.61-0.7	1.67	0.0991	0.12	0.9069
0.21-0.3 → 0.71-0.8	1.83	0.0709	0.42	0.1612
0.21-0.3 → 0.81-0.9	1.27	0.2073	0.77	0.4457
0.21-0.3 → 0.91-1	0.78	0.4394	0.95	0.3473
* 0.31-0.4 → 0.41-0.5	2.20	0.0306	1.16	0.2480
* 0.31-0.4 → 0.61-0.7	1.76	0.0832	2.21	0.0300
* 0.31-0.4 → 0.71-0.8	1.92	0.0590	0.99	0.3248
* 0.31-0.4 → 0.81-0.9	1.41	0.1621	1.57	0.1204
* 0.31-0.4 → 0.91-1	0.98	0.3311	3.35	0.0013
0.41-0.5 → 0.61-0.7	0.53	0.5978	1.27	0.2093
0.41-0.5 → 0.71-0.8	0.03	0.9745	0.14	0.8896
0.41-0.5 → 0.81-0.9	0.73	0.4682	0.53	0.5983
0.41-0.5 → 0.91-1	1.08	0.2851	2.52	0.0140
0.61-0.7 → 0.71-0.8	0.41	0.6812	1.32	0.1901
0.61-0.7 → 0.81-0.9	0.25	0.8024	0.69	0.4950
0.61-0.7 → 0.91-1	0.63	0.5289	1.11	0.2690
0.71-0.8 → 0.81-0.9	0.61	0.5436	0.64	0.5247
0.71-0.8 → 0.91-1	0.93	0.3541	2.60	0.0113
0.81-0.9 → 0.91-1	0.38	0.7071	1.84	0.0696

Table A2.6: Result of LME comparing [TT4] within and between FED and HIGH groups within and between *day*. AIC = 80.685; n (individuals) = 14; n (observations) = 117.

a. Differences in [TT4] between group within *day* within each sex (df = 10). Bold font indicates a significant difference between group.

b. Differences in [TT4] between *day* categories within and between group (df = 87). Bold font indicates a significant difference within group between *prop* categories. * denotes a significant difference between group between *prop* categories

a.

<i>Day</i>	Females		Males	
	T	p value	T	p value
1	1.28	0.2308	0.53	0.6054
4	0.03	0.9789	1.92	0.0837
7	1.57	0.1477	3.70	0.0041
10	0.39	0.7059	2.43	0.0354
13	0.67	0.5200	1.30	0.2222
16	2.47	0.0329	0.63	0.5407
19	0.37	0.7195	1.57	0.1468
22	0.72	0.4871	1.19	0.2614
25	0.65	0.5333	1.07	0.3107

b.

<i>Day</i> comparison	FED		HIGH	
	T	p value	T	p value
1 → 4	0.42	0.6786	1.28	0.2047
*1 → 7	2.19	0.0314	1.57	0.1204
1 → 10	1.13	0.2624	1.12	0.2676
1 → 13	1.31	0.1931	0.42	0.6749
1 → 16	0.48	0.6347	1.90	0.0606
1 → 19	1.78	0.0794	0.58	0.5643
1 → 22	1.41	0.1612	0.67	0.5033
1 → 25	1.27	0.2084	0.41	0.6802
4 → 7	1.76	0.0825	0.30	0.7620
4 → 10	0.70	0.4887	0.17	0.8660
4 → 13	0.88	0.3817	1.77	0.0795
* 4 → 16	0.04	0.9656	3.32	0.0013
4 → 19	1.34	0.1833	1.94	0.0557
4 → 22	0.98	0.3283	2.04	0.0447
4 → 25	0.84	0.4039	1.55	0.1246
7 → 10	1.11	0.2708	0.47	0.6374
* 7 → 13	0.92	0.3621	2.08	0.0406
* 7 → 16	1.79	0.0770	3.63	0.0005
7 → 19	0.36	0.7238	2.24	0.0274
* 7 → 22	0.73	0.4683	2.34	0.0215
7 → 25	0.88	0.3819	1.81	0.0744
10 → 13	0.19	0.8481	1.61	0.1211
* 10 → 16	0.68	0.4976	3.15	0.0022
10 → 19	0.71	0.4828	1.77	0.0802
10 → 22	0.33	0.7414	1.87	0.0652
10 → 25	0.18	0.8571	1.41	0.1625
13 → 16	0.87	0.3849	1.55	0.1256
13 → 19	0.52	0.6036	0.17	0.8694
13 → 22	0.15	0.8832	0.26	0.7935
13 → 25	0.00	0.9975	0.06	0.9526
16 → 19	1.37	0.1786	1.38	0.1706
16 → 22	0.98	0.3287	1.28	0.2026
16 → 25	0.83	0.4078	1.24	0.2183
19 → 22	0.36	0.7182	0.10	0.9224
19 → 25	0.51	0.6129	0.08	0.9372
22 → 25	0.15	0.8845	0.02	0.9830

Appendix 3

Details of LMEs in chapter 5

Table A3.1. Result of LME comparing change in [cortisol] with *day* a. within (df= 131) and b. between (df= 26) group. Bold font highlights significant differences (p<0.05). *, # and ~ denote a significant difference between CONTROL and SALINE, CONTROL and DEX and SALINE and DEX, respectively (p<0.05). AIC = 15.95; n (individuals) =29; n (observations) = 179.

a.

Within group between <i>day</i>						
<i>Day</i>	CONTROL		SALINE		DEX	
	T	p	T	p	T	p
1-4	0.20	0.8447	0.07	0.9426	0.36	0.7177
1-7	1.85	0.0659	2.10	0.0375	0.96	0.3377
1-10	1.65	0.1008	1.92	0.0576	1.25	0.2119
#,~ 1-11	0.83	0.4053	0.86	0.3913	16.80	<0.0001
1-14	1.87	0.0636	0.63	0.5274	1.45	0.1499
1-17	1.36	0.1760	1.70	0.0907	0.16	0.8759
1-20	0.87	0.3843	1.14	0.2558	1.13	0.2624
4-7	1.78	0.0771	2.24	0.0269	0.62	0.5351
4-10	1.56	0.1218	2.05	0.0427	0.91	0.3619
#,~ 4-11	0.69	0.4938	0.96	0.3389	17.00	<0.0001
4-14	1.79	0.0757	0.72	0.4724	1.13	0.2587
4-17	1.26	0.2097	1.80	0.0742	0.15	0.8799
4-20	0.79	0.4298	1.20	0.2309	1.40	0.1636
7-10	0.17	0.8674	0.19	0.8483	0.28	0.7836
#,~ 7-11	1.10	0.2753	1.28	0.2033	16.36	<0.0001
7-14	0.21	0.8341	1.38	0.1699	0.54	0.5933
7-17	0.12	0.9077	0.12	0.9047	0.67	0.5011
7-20	0.02	0.9865	0.29	0.7737	1.84	0.0687
#,~ 10-11	0.89	0.3735	1.09	0.2791	16.56	<0.0001
10-14	0.35	0.7246	1.20	0.2322	0.28	0.7789
10-17	0.02	0.9865	0.04	0.9646	0.91	0.3624
10-20	0.09	0.9271	0.16	0.8728	2.04	0.0429
#,~ 11-14	1.18	0.2394	0.18	0.8573	15.26	<0.0001
#,~ 11-17	0.73	0.4665	0.98	0.3306	14.26	<0.0001
#,~ 11-20	0.49	0.6224	0.56	0.5737	13.08	<0.0001
14-17	0.28	0.7795	1.10	0.2751	1.11	0.2669
14-20	0.08	0.9331	0.67	0.5009	2.19	0.0303
17-20	0.08	0.9378	0.18	0.8552	1.17	0.2424

b.

Within *day* between group

<i>Day</i>	CONTROL - SALINE		CONTROL - DEX		SALINE - DEX	
	T	p	T	p	T	p
1	0.36	0.7238	1.03	0.3111	0.73	0.4728
4	0.12	0.9059	1.28	0.2107	1.20	0.2421
7	0.45	0.6577	0.20	0.8455	0.25	0.8071
10	0.43	0.6734	0.61	0.5485	0.19	0.8490
11	0.34	0.7401	16.6 3	<0.0001	16.7 4	<0.0001
14	0.96	0.3455	0.43	0.6682	1.50	0.2751
17	0.34	0.7401	0.30	0.7676	0.69	0.4979
20	0.01	0.9949	0.97	0.3409	1.37	0.1809

Table A3.2. Result of LME comparing change in [TT4] with *day* a. within (df= 123) and b. between (df= 25) group. Bold font highlights significant differences (p<0.05). *, # and ~ denote a significant difference in the change in [TT4] between CONTROL and SALINE, CONTROL and DEX and SALINE and DEX, respectively (p<0.05). AIC = 1629.828; n (individuals) = 29; n (observations) = 170.

a.

<i>Day</i>	Within group between <i>day</i>					
	CONTROL		SALINE		DEX	
	T	p	T	p	T	p
1-4	0.41	0.6831	0.96	0.3366	0.17	0.8651
* 1-7	2.26	0.0257	0.53	0.5957	0.38	0.7058
1-10	1.32	0.1906	0.49	0.6266	0.84	0.4048
1-11	0.76	0.4487	2.58	0.0110	2.16	0.0331
1-14	0.02	0.9870	0.88	0.3794	1.21	0.2278
1-17	1.57	0.1185	0.73	0.4666	1.63	0.1066
4-7	1.99	0.0485	0.42	0.6774	0.22	0.8288
4-10	0.98	0.3306	1.50	0.1369	1.05	0.2976
* 4-11	0.38	0.7060	3.66	0.0004	2.05	0.0422
4-14	0.41	0.6848	0.02	0.9812	1.08	0.2805
4-17	1.31	0.1924	0.08	0.9343	1.51	0.1331
7-10	0.95	0.3445	1.04	0.3024	1.27	0.2068
*# 7-11	1.61	0.1090	3.13	0.0022	1.83	0.0698
# 7-14	2.17	0.0322	0.37	0.7132	0.88	0.3796
7-17	0.21	0.8302	0.36	0.7206	1.35	0.1809
# 10-11	0.61	0.5421	2.16	0.0328	3.19	0.0018
*# 10-14	1.26	0.2099	1.38	0.1706	2.12	0.0356
~ 10-17	0.52	0.6022	1.07	0.2871	2.41	0.0173
11-14	0.74	0.4603	3.40	0.0009	0.87	0.3886
* 11-17	1.02	0.3092	2.49	0.0141	0.14	0.8857
14-17	1.57	0.1196	0.10	0.9929	0.58	0.5654

b.

<i>Day</i>	Within <i>day</i> between group					
	CONTROL - SALINE		CONTROL - DEX		SALINE - DEX	
	T	p	T	p	T	p
1	0.97	0.3392	0.23	0.8184	0.77	0.4511
4	0.18	0.8613	0.02	0.9386	0.20	0.8437
7	1.55	0.1330	1.52	0.1410	0.03	0.9770
10	0.18	0.8565	1.74	0.0941	2.04	0.0525
11	2.57	0.0165	1.47	0.1540	1.13	0.2674
14	0.22	0.8629	1.25	0.2236	1.11	0.2792
17	1.14	0.2650	0.09	0.9255	1.27	0.2099

Table A3.3. Result of LME comparing change in [TT3] with *day* within each sex within group. Bold font highlights significant differences ($p < 0.05$). Blue font denotes a significant difference in the change in [T3] between days between males and females ($p < 0.05$). * indicates a significant difference between CONTROL and SALINE groups. # indicates a significant difference between CONTROL and DEX groups. AIC = 326.2151; n (individuals) = 29; n (observations) = 170.

Day	CONTROL				SALINE				DEX			
	Males		Females		Males		Females		Males		Females	
	T	p	T	p	T	p	T	p	T	p	T	p
1-4	1.25	0.2149	1.73	0.0870	3.53	0.0006	0.60	0.5494	2.60	0.0106	0.39	0.6974
* 1-7	0.08	0.9391	1.76	0.0814	3.55	0.0006	1.74	0.0843	1.77	0.0801	0.07	0.9455
* 1-10	1.26	0.2101	0.09	0.9261	3.72	0.0003	2.58	0.0110	1.93	0.0555	0.64	0.5231
* 1-11	2.03	0.0450	1.21	0.2273	3.21	0.0017	0.21	0.8313	2.17	0.0323	0.95	0.3464
1-14	2.94	0.0039	0.58	0.5646	3.79	0.0002	1.63	0.1054	2.66	0.0090	0.45	0.6502
*# 1-17	0.24	0.8102	1.20	0.2336	3.77	0.0003	2.97	0.0036	2.46	0.0155	1.24	0.2188
4-7	1.29	0.2005	0.05	0.9596	0.18	0.8544	1.32	0.1910	0.78	0.4386	0.35	0.7267
4-10	0.07	0.9435	1.68	0.0965	0.44	0.6642	2.29	0.0240	0.67	0.5071	1.13	0.2604
4-11	0.92	0.3596	0.51	0.6113	0.09	0.9261	0.41	0.6801	0.33	0.7415	0.63	0.5302
4-14	2.05	0.0431	2.17	0.0319	0.80	0.4225	1.28	0.2032	0.47	0.6416	0.90	0.3679
* 4-17	1.32	0.1895	0.21	0.8341	1.69	0.0944	2.80	0.0059	0.73	0.4645	1.70	0.0926
7-10	1.31	0.1922	1.72	0.0878	0.25	0.8032	0.88	0.3828	0.14	0.8882	0.80	0.4237
7-11	2.22	0.0287	0.56	0.5765	0.28	0.7820	1.71	0.0892	0.46	0.6466	1.02	0.3119
* 7-14	3.20	0.0018	2.23	0.0279	0.64	0.5261	0.02	0.9877	1.20	0.2338	0.60	0.5464
7-17	0.33	0.7402	0.18	0.8562	1.58	0.1174	1.81	0.0725	1.28	0.2020	1.48	0.1420
10-11	0.83	0.4091	1.18	0.2424	0.53	0.5941	2.72	0.0076	0.34	0.7372	1.84	0.0680
10-14	1.96	0.0529	0.69	0.4898	0.41	0.6834	0.83	0.4061	1.10	0.2724	0.13	0.8959
* 10-17	1.36	0.1749	1.17	0.2428	1.44	0.1526	1.25	0.2151	1.21	0.2283	0.88	0.3821
11-14	1.28	0.2025	1.76	0.0806	0.91	0.3655	1.69	0.0934	0.80	0.4259	1.58	0.1179
*# 11-17	2.11	0.0371	0.51	0.6128	1.76	0.0811	3.13	0.0022	0.99	0.3254	2.31	0.0228
*# 14-17	3.10	0.0024	1.61	0.1111	1.16	0.2491	1.83	0.0698	0.39	0.6996	0.99	0.3292

Table A3.4. Result of LME comparing change in TT3: TT4 with *day* a. within (df = 119) and b. between (df = 26) group. Bold font highlights significant differences (p<0.05). *, # and ~ denote a significant difference in the change in TT3: TT4 between CONTROL and SALINE, CONTROL and DEX and SALINE and DEX, respectively (p<0.05). AIC = 836.3589; n (individuals) = 29; n (observations) = 167.

a.

<i>Day</i>	Within group between <i>day</i>					
	CONTROL		SALINE		DEX	
	T	p	T	p	T	p
1-4	0.56	0.5745	0.43	0.6644	0.62	0.5364
1-7	1.10	0.2717	0.84	0.4001	0.42	0.6761
~ 1-10	0.57	0.5683	2.97	0.0036	0.14	0.8856
1-11	0.63	0.5319	3.25	0.0015	1.26	0.2086
1-14	1.63	0.1056	0.97	0.3337	2.04	0.0441
1-17	0.45	0.6533	1.94	0.0549	1.82	0.0711
4-7	0.64	0.5263	0.48	0.6334	0.21	0.8379
~ 4-10	0.03	0.9769	2.88	0.0047	0.52	0.6044
* 4-11	0.10	0.9190	3.24	0.0015	0.74	0.4590
4-14	1.27	0.2070	0.65	0.5162	1.66	0.1002
4-17	0.97	0.3334	1.78	0.0782	1.50	0.1375
* 7-10	0.59	0.5579	2.33	0.0217	0.31	0.7573
* 7-11	0.51	0.6076	2.71	0.0078	0.95	0.3433
7-14	0.69	0.4903	0.19	0.8508	1.88	0.0623
*# 7-17	1.45	0.1484	1.34	0.1821	1.68	0.0961
10-11	0.07	0.9433	0.47	0.6426	1.29	0.1990
*~ 10-14	1.22	0.2237	2.05	0.0425	2.23	0.0279
# 10-17	0.98	0.3278	0.66	0.5137	1.94	0.0549
*~ 11-14	1.17	0.2428	2.43	0.0164	0.97	0.3337
11-17	1.06	0.2934	1.04	0.3003	0.96	0.3379
14-17	2.00	0.0479	1.16	0.2475	0.20	0.8422

b.

<i>Day</i>	Within <i>day</i> between group					
	CONTROL - SALINE		CONTROL - DEX		SALINE - DEX	
	T	p	T	p	T	p
1	0.23	0.8174	0.21	0.8391	0.03	0.9799
4	0.09	0.9283	0.25	0.8022	0.17	0.8686
7	0.07	0.9417	0.50	0.6212	0.44	0.6643
10	2.42	0.0226	0.22	0.8245	2.87	0.0080
11	2.70	0.0120	0.81	0.4275	2.02	0.0538
14	0.55	0.5885	0.46	0.6487	1.10	0.2826
17	2.27	0.0317	2.21	0.0360	0.14	0.8902

Table A3.5: Result of LMEs assessing short-term changes in total WBC number and individual cell types in response to treatment, within (df = 39) and between (df = 24) group. AIC for each model is given. Bold font highlights significant differences ($p < 0.05$). *, #, and ~ denote a significant difference in the change in cell number between *times* between CONTROL and SALINE, CONTROL and DEX and SALINE and DEX respectively ($p < 0.05$). n (individuals) = 27; n (observations) = 72.

Cell type	Time	Within group between time (df=24)						Between group within time (df =39)					
		CONTROL		SALINE		DEX		0		1		4	
		T	P	T	P	T	P	T	P	T	P	T	P
Total AIC = 55.90	0-1	3.55	0.0010	2.93	0.0056	5.38	< 0.0001	0.08	0.9376	0.74	0.4676	0.28	0.7798
	~1-4	1.14	0.2613	0.04	0.9675	4.27	0.0001	0.12	0.9067	0.59	0.5612	1.18	0.2490
	0-4	1.75	0.0874	2.62	0.0123	0.46	0.6459	0.22	0.8294	1.46	0.1565	1.69	0.1044
Neutrophils AIC = 17.41	~0-1	2.77	0.0086	2.64	0.0119	5.50	< 0.0001	0.17	0.8658	0.58	0.5659	0.23	0.8168
	~1-4	1.02	0.3153	0.09	0.9304	3.82	0.0005	0.49	0.6290	0.86	0.3970	0.76	0.4562
	0-4	1.24	0.2232	2.24	0.0311	1.02	0.3118	0.35	0.7286	1.59	0.1245	1.14	0.2646
Monocytes AIC = 1.52	0-1	3.26	0.0023	2.36	0.0232	4.29	0.0001	0.33	0.7447	0.84	0.4105	1.10	0.2822
	~1-4	2.10	0.0422	0.12	0.9051	3.20	0.0027	0.11	0.9147	0.40	0.6921	0.02	0.9808
	0-4	0.60	0.5519	1.98	0.0546	0.61	0.5459	0.24	0.8095	1.36	0.1850	1.31	0.2024
Eosinophils AIC = 182.87	#~0-1	0.49	0.6258	0.81	0.4209	3.98	0.0003	0.06	0.9557	0.24	0.8156	0.53	0.6022
	1-4	0.84	0.4070	2.08	0.0440	2.49	0.0172	1.62	0.1183	1.08	0.2915	0.15	0.8858
	0-4	0.43	0.6701	1.36	0.1832	1.06	0.2940	1.85	0.0771	0.93	0.3624	0.45	0.6585
Lymphocytes AIC = 38.31	0-1	2.30	0.0268	0.62	0.5373	0.04	0.9663	0.83	0.4167	0.90	0.3794	0.44	0.6624
	1-4	0.68	0.5029	0.74	0.4663	0.46	0.6464	0.36	0.7223	1.83	0.0794	0.68	0.2543
	0-4	1.24	0.2216	1.29	0.2039	0.42	0.6737	0.51	0.6117	1.03	0.3126	1.88	0.0720

Appendix 4

Details of LMEs in chapter 6

Table A4.1: Change in DML (AIC = 87.938) and DML, including mass as a covariate (DML + mass), (AIC = 90.522) a. within group from early to late portions of the fast (df = 22 and df=21) and b. between group (df=24) (abbreviated) within early and late portions of the fast. Bold font indicates a significant ($p < 0.05$) difference. * and # respectively denote a significant difference between groups in the change in DML and DML + mass from the early to late portions of the fast. n (individuals) = 28, n (observations) = 54.

a.

Group	DML		DML + mass	
	T	p	T	P
FED	2.848	0.0094	3.233	0.0040
HIGH	3.992	0.0006	3.622	0.0016
LOW	2.800	0.0104	2.461	0.0226
UNKNOWN	3.050	0.0059	2.767	0.0115

b.

Group comparison	DML				DML + mass			
	EARLY		LATE		EARLY		LATE	
	T	p	T	p	T	p	T	p
F → H*#	4.922	0.0001	0.834	0.4123	5.294	<0.0001	0.811	0.4250
F → L*#	4.019	0.0005	0.826	0.4165	4.336	0.0002	0.823	0.4186
F → U*#	3.913	0.0007	1.142	0.2646	4.022	0.0005	1.384	0.1791
H → L	0.903	0.3754	0.023	0.9821	0.968	0.3428	0.041	0.9673
H → U	1.009	0.3230	0.338	0.7382	1.287	0.2105	0.608	0.5492
L → U	0.106	0.9165	0.305	0.7631	0.326	0.7469	0.549	0.5882

Table A4.2: Change in DML (AIC = 12.286) and DML including mass as a covariate (DML + mass) (AIC = 10.781) between *day* within and between FED and HIGH groups. Bold font indicates a significant difference ($p < 0.05$) in DML between *day*. * and # respectively denote where the change in DML and DML + mass between *day* is significantly different between groups. n (individuals) = 14, n (observations) = 103.

<i>Day</i> comparison	DML				DML + mass			
	FED		HIGH		FED		HIGH	
	T	p	T	p	T	p	T	p
4 → 7	1.972	0.0523	0.781	0.4374	2.011	0.0480	0.829	0.4095
4 → 10	1.326	0.1890	1.901	0.0612	1.376	0.1729	1.959	0.0539
4 → 13	0.998	0.3216	1.408	0.1634	0.897	0.3725	1.490	0.1406
4 → 16*#	0.207	0.8366	3.134	0.0025	0.099	0.9213	3.189	0.0021
4 → 19	0.247	0.8058	1.675	0.0982	0.337	0.7371	1.771	0.0807
4 → 22*#	1.441	0.1539	2.918	0.0046	1.286	0.2025	2.977	0.0039
4 → 25*	0.225	0.8228	2.665	0.0094	0.356	0.7230	2.733	0.0078
7 → 10	0.708	0.4812	1.166	0.2475	0.696	0.4887	1.187	0.2392
7 → 13*#	3.253	0.0017	0.652	0.5162	3.196	0.0020	0.703	0.4843
7 → 16*#	2.387	0.0195	2.449	0.0167	2.311	0.0236	2.497	0.0147
7 → 19	1.807	0.0748	0.930	0.3552	1.748	0.0846	1.013	0.3142
7 → 22*#	3.643	0.0005	2.224	0.0291	3.522	0.0007	2.296	0.0245
7 → 25*#	1.831	0.0711	2.052	0.0437	1.708	0.0919	2.122	0.0271
10 → 13	2.545	0.0130	0.513	0.6092	2.504	0.0145	0.481	0.6316
10 → 16*#	1.679	0.0973	1.283	0.2033	1.620	0.1095	1.322	0.1903
10 → 19	1.127	0.2634	0.235	0.8146	1.082	0.2826	0.162	0.8721
10 → 22*#	2.963	0.0041	1.059	0.2931	2.863	0.0055	1.134	0.2603
10 → 25	1.151	0.2535	1.058	0.2935	1.049	0.2977	1.129	0.2626
13 → 16	0.866	0.3891	1.797	0.0764	0.882	0.3808	1.807	0.0749
13 → 19	1.319	0.1913	0.278	0.7817	1.324	0.1897	0.319	0.7508
13 → 22	0.518	0.6061	1.572	0.1202	0.471	0.6385	1.620	0.1094
13 → 25	1.295	0.1994	1.496	0.1390	1.343	0.1831	1.543	0.1270
16 → 19	0.486	0.6282	1.519	0.1331	0.476	0.6352	1.487	0.1414
16 → 22	1.350	0.1811	0.225	0.8229	1.319	0.1911	0.179	0.8582
16 → 25	0.462	0.6452	0.037	0.9710	0.501	0.6178	0.008	0.9933
19 → 22*#	1.770	0.0809	1.294	0.1996	1.729	0.0879	1.308	0.1950
19 → 25	0.023	0.9816	1.258	0.2121	0.025	0.9804	1.276	0.2058
22 → 25	1.746	0.0848	1.069	0.2884	1.756	0.0833	0.162	0.8720

Table A4.3: Change in DML (AIC = 4.882) and DML including mass as a covariate (DML +mass) (AIC = 3.00) between *day* within and between CONTROL, SALINE and DEX groups. Bold font indicates a significant difference in DML or DML + mass ($p < 0.05$) between *days*. n (individuals) = 29, n (observations) = 144.

Day comparison	DML				DML + mass					
	CONTROL		SALINE		CONTROL		SALINE		DEX	
	T	p	T	p	T	p	T	p	T	p
4 → 7	1.563	0.1212	1.778	0.0785	1.548	0.1249	1.736	0.0858	0.727	0.4688
4 → 10	2.084	0.0397	3.302	0.0013	2.025	0.0455	3.219	0.0017	2.653	0.0093
4 → 11	2.934	0.0041	2.984	0.0036	2.884	0.0048	2.895	0.0047	2.489	0.0145
4 → 14	3.179	0.0020	3.513	0.0007	3.135	0.0023	3.367	0.0011	1.214	0.2277
4 → 17	3.312	0.0013	2.849	0.0053	3.286	0.0014	2.713	0.0079	2.971	0.0037
7 → 10	0.598	0.5510	1.616	0.1092	0.564	0.5744	1.595	0.1140	1.991	0.0493
7 → 11	1.466	0.1458	1.280	0.2036	1.437	0.1539	1.253	0.2131	1.823	0.0713
7 → 14	1.861	0.0656	1.978	0.0507	1.836	0.0694	1.915	0.0583	0.542	0.5888
7 → 17	2.144	0.0345	1.346	0.1812	2.131	0.0356	1.286	0.2013	2.443	0.0163
10 → 11	0.823	0.4120	0.337	0.7370	0.830	0.4083	0.341	0.7340	0.172	0.8640
10 → 14	1.278	0.2042	0.511	0.6106	1.284	0.2022	0.480	0.6321	1.365	0.1753
10 → 17	1.629	0.1064	0.053	0.9575	1.639	0.1043	0.084	0.9334	0.824	0.4121
11 → 14	0.550	0.5835	0.816	0.4162	0.550	0.5834	0.789	0.4319	1.205	0.2311
11 → 17	0.994	0.3226	0.238	0.8123	1.001	0.3193	0.210	0.8339	0.964	0.3373
14 → 17	0.476	0.6349	0.502	0.6167	0.483	0.6301	0.505	0.6147	1.934	0.0560