THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

The Role Of Nutrient Sensing Pathways In Delaying Ageing

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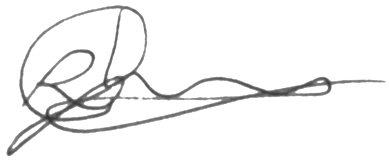
Declaration

I hereby declare that the intellectual content, analyses and results presented in this thesis are my own work, performed under the supervision of Professor David G. Le Couteur, Associate Professor Victoria C. Cogger, Dr. Aisling C. McMahon, and Professor Stephen J. Simpson unless otherwise attributed, and that all sources, and assistance received in preparing this thesis have been acknowledged.



Rahul Gokarn

Acknowledgements

The completion of this thesis would not have been possible without the ongoing support of a great number of individuals, and as much as I am drawn to procrastination, I don't think I will have the ability to name them all – save to say an enormous thank you to everyone involved. I would however like to dedicate this thesis to my family, in particular, my grandmother, who has been a tremendous source of inspiration in my life. Through her hard work and determination, she has made our family what it is today, and I am eternally grateful for having her in my life. Special mention to my girlfriend Chan, who has put up with my irritation and multiple changes in career over the years – I would not be where I am today if it wasn't for you, so thank you from the bottom of my heart. I would also certainly not be forgiven if I didn't mention my cats, Soups and PJ, whose love and affection is matched only by their relentless pursuit of food.

"If I have seen further it is by standing on the shoulders of giants." — *Isaac Newton*

If I were to describe my life, I would say there have been three main phases to date; my formative years, where I stared to learn about the world and develop passions; my tertiary educational years, where I studied how the world works, through science and medicine, and realised what I want to do with my life; and I am currently entering the third stage, where I hope to start achieving the goals and aspirations that I have for my career. At each of these stages, I was fortunate enough to find mentors who were willing to dedicate their time and energy to help me develop as both personally as well as professionally. I know I will never be able to repay all of you for what you have done for me, so I instead to use this fact as motivation to be the best person I can be to make you all proud.

The first were my parents; my mother who was always cool, calm and collected, and had the perfect balance of being supportive while still allowing me to grow in my own way; and my father, who has, in many ways, shaped my perspective of the world through our long and often philosophical discussions (read: arguments) about the world. I have an immense appreciation for you both, and value you not only as parents, but also as some of my closest friends.

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Finally, as I enter this latest phase in my life; transitioning to a career (or the real world, as it is also known), I am fortunate enough to have met Shehan Dissanayake. Over the short time that I have known you, you have inspired me to push myself beyond what I thought was possible.

"Impossible is just a big word thrown around by small men who find it easier to live in the world they've been given than to explore the power they have to change it. Impossible is not a fact. It's an opinion. Impossible is not a declaration. It's a dare. Impossible is potential. Impossible is temporary. Impossible is nothing." — *Muhammad Ali*

Finally, collaboration has been one of the most important aspects in making this thesis possible. From the animal work performed by the biogerontology lab at the ANZAC Research Institute and Charles Perkins Centre, to the various experiments performed on a variety of tissue samples collected from our large scale dietary study; collaboration has formed long lasting relationships between people and labs across the world, and produced some incredible scientific findings. I would therefore like to thank the following people for their contributions to this thesis:

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Publications and Presentations

Publications

Gokarn, R., Solon-Biet, S.M., Cogger, V.C., Cooney, G.J., Wahl, D., McMahon, A.C., Mitchell, J.R., Mitchell, S.J., Hine, C., de Cabo, R., Raubenheimer, D., Simpson, S.J., Le Couteur, D.G. Long-term dietary macronutrients and hepatic gene expression in aging mice. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 2018. **(A version of this paper is presented in Chapter 4)**

Gokarn, R., Solon-Biet, S.M., Cogger, V.C., Cooney, G.J., Wahl, D., McMahon, A.C., de Cabo, R., Raubenheimer, D., Simpson, S.J., Le Couteur, D.G**.** Hypothalamic gene expression is influenced by macronutrient intake in aging mice. Under review for submission, 2018. **(A version of this paper is presented in Chapter 4)**

Wahl, D., **Gokarn, R.**, Mitchell, S.J., Solon-Biet, S.M., Cogger, V.C., Simpson, S.J., Le Couteur, D.G., de Cabo, R. Central nervous system SIRT1 expression is required for cued and contextual fear conditioning memory responses in aging mice. Under review for publication in *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 2018. **(Contributions made to this paper are not directly related to this thesis)**

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Solon-Biet S.M., Cogger V.C., Pulpitel T., Heblinski M., Wahl D., McMahon A.C., Warren A., Durrant-Whyte J., Walters K.A., Krycer J.R., Ponton F., **Gokarn R.**, Wali J.A., Ruohonen K., Conigrave A.D., James D.E., Raubenheimer D., Morrison C.D., Le Couteur D.G., Simpson S.J. Defining the Nutritional and Metabolic Context of FGF21 Using the Geometric Framework. *Cell Metabolism*, 24(4), pp.555–565, 2016. **(Contributions made to this paper are not directly related to this thesis)**

Wahl, D., Cogger, V.C., Solon-Biet, S.M., Waern, R.V., **Gokarn, R.**, Pulpitel, T., Cabo, R., Mattson, M.P., Raubenheimer, D., Simpson, S.J., Le Couteur, D.G. Nutritional strategies to optimise cognitive function in the aging brain. *Ageing Research Reviews*, 31:80-92, 2016. **(Contributions to this paper are not directly related to this thesis)**

Solon-Biet S.M., Mitchell, S.J., Coogan S.C., Cogger V.C., **Gokarn R.**, McMahon, A.C., Raubenheimer D., de Cabo R., Simpson S.J., Le Couteur D.G. Dietary Protein to Carbohydrate Ratio and Caloric Restriction: Comparing Metabolic Outcomes in Mice. *Cell Reports*, 11(10), pp.1529–1534, 2015. **(Contributions made to this paper related to hepatic and pancreatic histology are presented in Chapter 5.3)**

Solon-Biet S.M., McMahon, A.C., Ballard J.W., Ruohonen K., Wu L.E., Cogger V.C., Warren A., Huang X., Pichaud N., Melvin R.G., **Gokarn R.**, Khalil M., Turner N., Cooney G.J., Sinclair D.A., Raubenheimer D., Le Couteur D.G., Simpson S.J. The Ratio of Macronutrients, Not Caloric Intake, Dictates Cardiometabolic Health, Aging, and Longevity in Ad Libitum-Fed Mice. *Cell Metabolism*, 19(3), pp.418–430, 2014. **(Contributions made to this paper related to mTOR activation are presented in Chapter 4, and contributions related to mitochondrial function are presented in Chapter 5.2)**

Presentations

Oral abstract presentations:

Royal Prince Alfred Hospital President's Prize 2016 (Sydney) – The correlation between macronutrient intake, telomere length and longevity (Won prize for best research by a clinician)

18th International Neuroscience Winter Conference 2016 (Sölden, Austria) – How does diet influence ageing? Altered hypothalamic gene expression profiles may be the key

International Association of Gerontology and Geriatrics (IAGG) Conference 2015 (Chiang Mai, Thailand) – High dietary P:C ratios are linked with shortened liver telomere lengths in mice

Sydney Lifespan Symposium 2015 (Sydney) – The link between diet, mitochondria and longevity

Sydney Interuniversity Neuroscience Conference 2015 (Sydney) – Gene regulation in the hypothalamus

Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (ASCEPT) Conference 2015 (Hong Kong) – SIRT1 expression in the CNS is associated with fear and anxiety responses in mice

Emerging Research in Ageing 2013 (Sydney) – Low dietary protein intake protects against age-related hepatic changes and provides a link between caloric restriction and inflamm-ageing

Poster presentations:

ASCEPT Conference 2013 (Melbourne) – Macronutrient related hepatotoxic changes, a link between CR and inflamm-ageing

Gerontological Society of America (GSA) Conference 2015 (USA) – Low dietary protein to carbohydrate ratios are linked with longer telomere lengths in mice

Gerontological Society of America (GSA) Conference 2015 (USA) – Diet significantly influences hypothalamic gene expression, a mechanism linking diet and ageing

List of Experimental Techniques Performed

Experimental Technique
DNA, RNA, Protein Extraction
Protein Quantification (BCA, Bradford)
DNA, RNA Quantification (Spectrophotometry)
Western Blots (mTOR, phosphor-mTOR)
Mitochondrial Seahorse (Mitochondrial function)
Polymerase chain reaction (PCR)
Paraffin embedding and sectioning
Frozen block embedding and sectioning (OCT)
Histology (H&E, SR, Oil Red O, PAS)
Immunohistochemistry (Insulin, Glucagon)
Immunoflourescent staining (H2Ax)
Confocal Microscopy
Mouse handling (Scruffing, Injecting, Husbandry, Dietary Measurement and Manipulation)
Mouse culling and tissue collection (Anaesthesia, cervical dislocation, dissection)
Blood collection (Eye, Tail, IVC)
Evan's Blue BBB Leakage Assay (Peritoneal injection)
Cell culture (Growth, Splitting and Media Change)
NMR (Body composition analysis)
Metabolic cage analysis
Insulin Tolerance Test (ITT) and Oral Glucose Tolerance Test (OGTT)
Behavioural experiments (Fear Conditioning, Elevated Plus Maze, Morris Water Maze)
Exercise, coordination and performance experiments (Rotarod, Inverted Cage Hang)

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List of Abbreviations

AA's	Amino acids
ADP	Adenosine diphosphate
AgRP	Agouti-related peptide
AICAR	Aminomidazole carboxamide ribonucleotide
Akt	Akt, also known as protein kinase B - a serine/threonine-specific protein kinase
AL	Ad libitum
ALT	Alanine transaminase
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AST	Aspartate aminotransferase
ATLR	Average telomere length ratio
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BAX	BCL2-associated X protein
BCA	Bicinchoninic acid
BCAA	Branch chain amino acid
BCL2	B-cell leukaemia/lymphoma 2 protein
Bdnf	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
BSKO	Brain specific knockout
BSOE	Brain specific overexpressor
C:F	Carbohydrate-to-Fat
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CR	Caloric restriction
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
DR	Dietary restriction
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum

ERK1-2	Extracellular signal related kinases 1/2
ETC	Electron transport chain
FDA	Food and Drug Administration
FFA	Free fatty acids
FGF	Fibroblastic growth factor
FOXO1	Forkhead box protein O1
FOXO3	Forkhead box protein O3
GAM	Generalised additive model
GF	Geometric framework
GFN	Geometric framework for nutrition
GGT	Gamma-glutamyl transpeptidase
GH	Growth hormone
GHR	Growth hormone receptor
GHRH	Growth hormone releasing hormone
GLUT1-4	Glucose transporter 1-4
GO	Gene ontology
GOI's	Genes of interest
GTP	Guanosine triphosphate
GTT	Glucose tolerance test
GUI	Graphical user interface
GWAS	Genome-wide association studies
H&E	Hematoxylin and eosin stain
HDAC	Histone deacetylase
HDLc	High-density lipoprotein cholesterol
HOAD	3-hydroxyacyl-CoA dehydrogenase
HOMA-IR	Homeostatic model assessment of insulin resistance
HPLC	High-protein low-carbohydrate
IF	Immunofluorescence
IGF-1	Insulin-like growth factor 1
IGF-1R	IGF-1 receptor
IHC	Immunohistochemistry
IIS	Insulin/IGF-like signalling
IL1-8	Interleukin 1-8
IR	Insulin receptor
IRS	Insulin receptor substrate

ITT	Insulin tolerance test
IVC	Inferior vena cava
JAK	Janus kinase
JNK	c-Jun n-terminal kinase
kJ	Kilojoules
LDLc	Low-density lipoprotein cholesterol
LPHC	Low-protein high-carbohydrate
mAB	Monoclonal antibody
MC4R	Melanocortin-4-receptor
miRNA	Micro-RNA
mtDNA	Mitochondrial DNA
mTOR	Mechanistic target of rapamycin, formerly Mammalian target of rapamycin
mTORC1-2	mTOR Complex 1/2
NAD	Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance scan
NPY	Neuropeptide Y
OCT	Optimal cutting temperature compound
P:C	Protein to carbohydrate
P:C:F	Protein to carbohydrate to fat
P:F	Protein to fat
PAS	Periodic acid–Schiff
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
PIKK	PI3K-related protein kinases
PKC	Protein kinase C
POMC	Pro-opiomelanocortin
QTL	Quantitative trait loci
Rag-GTPase	Ras-related GTP-binding hydrolase enzyme
RAPTOR	Regulatory-associated protein of mTOR
RCR's	Respiratory control ratios
REML	Restricted maximum likelihood
RICTOR	Rapamycin-insensitive companion of mTOR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S6K	Ribosomal protein S6 kinase

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sir2	Silent information regulator 2
siRNA	Small interfering RNA
SIRT1-7	Sirtuin 1-7
SNPs	Single nucleotide polymorphisms
SR	Sirius red stain
STAC	Sirtuin activating compound
STAT	Signal transducer and activator of transcription
TBS	Tris-buffered saline
TNF-α	Tumour necrosis factor-alpha
TOR	Target of rapamycin
UV	Ultraviolet radiation
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue

Abstract

Caloric restriction (CR) without malnutrition is a robust dietary intervention that extends lifespan and improves health in virtually every species studied. Recently, some of the mechanisms for the effects of CR on ageing have been elucidated, including several cellular master switches collectively called "nutrient sensing pathways". CR is not sustainable in humans, therefore research has begun to focus on developing ad libitum-fed diets that maximise lifespan and healthspan. Recently, it has been shown that ad libitum-fed mice on low protein, high carbohydrate diets have a longer lifespan compared to diets with other macronutrient ratios. These low protein, high carbohydrate diets therefore seem to recapitulate some of the benefits of CR.

This thesis presents research on the effects of diets with differing ratios of macronutrients on nutrient sensing pathways, and their impact on ageing and age-related end points. This is achieved using the paradigm of the Geometric Framework (GF), an analytical tool used to disentangle the effects of various nutritional components such as macronutrient ratios and energy intakes on outcomes. Using the GF, the relationship between measured outcomes and experimental conditions is mapped on an n-dimensional nutritional space, where each dimension represents an intake axis; in this thesis, protein, carbohydrates and fat. The power of the GF is in its ability to both visually represent the data, as well as perform statistical analyses to uncouple the complexities of nutrition, through the creation of these response topologies. Using the GF, a large-scale nutritional study is used to examine the aging process through genotypic and phenotypic changes in nutrient sensing pathways, and their relationship with parameters of health and lifespan.

This thesis contains three research chapters, each corresponding to a major set of work, with the overarching aim of better understanding the mechanisms involved in the relationship between nutrition and the ageing process:

Chapter 3 – Methods: The Geometric Framework

There have been challenges with the statistical tools used to analyse experimental data according to the GF model. These include visualisation of the data in multiple dimensions, and the use of GF to interpret very large datasets. The R statistical language was used to develop algorithmic bioinformatic techniques to automate data handling, processing, analysis and visualisation, including three-dimensional models. Improvements in algorithmic efficiency were also studied to allow the automation of the GF across very large datasets, supporting, for example, the analysis of an entire transcriptome. These statistical advances underpinned the analysis of experimental data presented in Chapters 4 and 5.

Chapter 4 – Nutrient Sensing Pathways

This chapter involved investigation into the impact of macronutrient intake on nutrient sensing pathways and correlation with health and lifespan. The majority of experimental work was carried out on mouse tissue from a large-scale dietary intervention and longevity study in which 858 mice were fed one of 25 diets varying in protein, carbohydrate, fat or total energy content. Several experimental approaches were undertaken including primary analysis of gene expression and quantitative PCR in liver and hypothalamus. Analysis of gene expression was performed using both a univariate analysis and the GF multivariate approach developed in Chapter 3. By studying gene expression in the liver and hypothalamus, this section shows that low protein, high carbohydrate diets, which were associated with the best health outcomes and lifespan in the cohort, also significantly influenced nutrient sensing pathways, thereby providing a mechanism linking dietary macronutrients and ageing. In the liver, gene expression was mostly influenced by dietary protein intake. Biological pathways linked with protein intake included mitochondrial function, metabolic signaling (PI3K-Akt, AMPK, mTOR) and metabolism of protein and amino acids. Among the genes of interest with expression linked with dietary protein were *Cth, Gls2, Igf1* and *Nnmt*, which were

increased with high protein intake, and *Igf2bp2, Fgf21, Prkab2* and *Mtor*, linked with low protein intake. In the hypothalamus, gene expression was most influenced by fat and total energy intake. Using the GF analytical approach, there were a number of genes of interest: carbohydrate intake influenced expression of *Igfbp2, Igf1* and *Sirt2*; protein intake significantly influenced *Npy, Akt3, Sirt1*; and the interaction between protein and carbohydrates *Sirt5, Rps6a4/5, Igf2r, Igf1r* and *Sirt3*.

Chapter 5 – Phenotypic End Points Associated with Ageing

This set of work focused on three phenotypic endpoints associated with ageing, namely hepatic telomere length (measured using a PCR method), mitochondrial function (measured using Seahorse technology and gene expression), and inflammation (determined by microscopy). Here, it is shown that low protein, high carbohydrate diets are associated with longer telomere lengths, improved mitochondrial function, and lower levels of organ level inflammation, even in short-term dietary studies. For each outcome, diets that were low in protein and high in carbohydrates were associated with results that are consistent with biological changes supportive of a longer lifespan.

Taken together, the results presented here form a case that manipulation of the nutrient sensing pathways are a mechanism for the beneficial effects of dietary interventions on ageing and health. Further investigation of these growth pathways could help to develop guidelines for optimal nutritional intake, or form the basis for pharmacological interventions targeted at prolonging lifespan, healthspan, and promoting health in old age, without the use of long-term dietary manipulation. The algorithmic tools developed here, also have the potential to be applied to a wider array of experimental fields, supporting the overarching experimental model of comparing multi-dimensional inputs on responses, as opposed to the more commonly used control versus treatment model that is largely seen in scientific research.

Chapter 1: General Introduction

Background

In the ancient Indian epic, the Mahabharata, both heaven and earth combined forces to churn the ocean in order to find the Amrut, an elixir preventing ageing and granting immortality. Similar myths such as the Holy Grail, the philosopher's stone, and many others, are found across virtually all cultures around the world. While ageing and immortality have fascinated humans since the beginning through the ages, there is no doubt that the 'churning of the scientific ocean' in the last few decades has produced a plethora of advances in the fields of biogerontology and geroscience.

Despite ageing being one of the most fundamentally defining features of life, a universally accepted definition of ageing has been elusive (Medvedev 2008). Most definitions of ageing describe specific aspects such as phenotypic and physiological ageing, chronological ageing, or the change in likelihood of mortality over time [Figure 1.1, Table 1.1] (Le Couteur et al. 2014).

In recent times, major advances have been made in terms of understanding the biological processes involved in ageing, largely through the use of model organisms such as yeast, worms, flies and mice, with many of these advances stemming from the discovery of the beneficial effects of caloric restriction on longevity (Heilbronn & Ravussin 2003). While the effects of caloric restriction on ageing have been described anecdotally for centuries, it was not until 1935 that the first formal publication in the scientific literature was reported (McCay et al. 1935). Since then, dietary interventions such as caloric restriction have been shown to be the most robust methods of prolonging lifespan across virtually all species, and have become one of the most important paradigms in biogerontological research (Solon-Biet et al. 2015b).

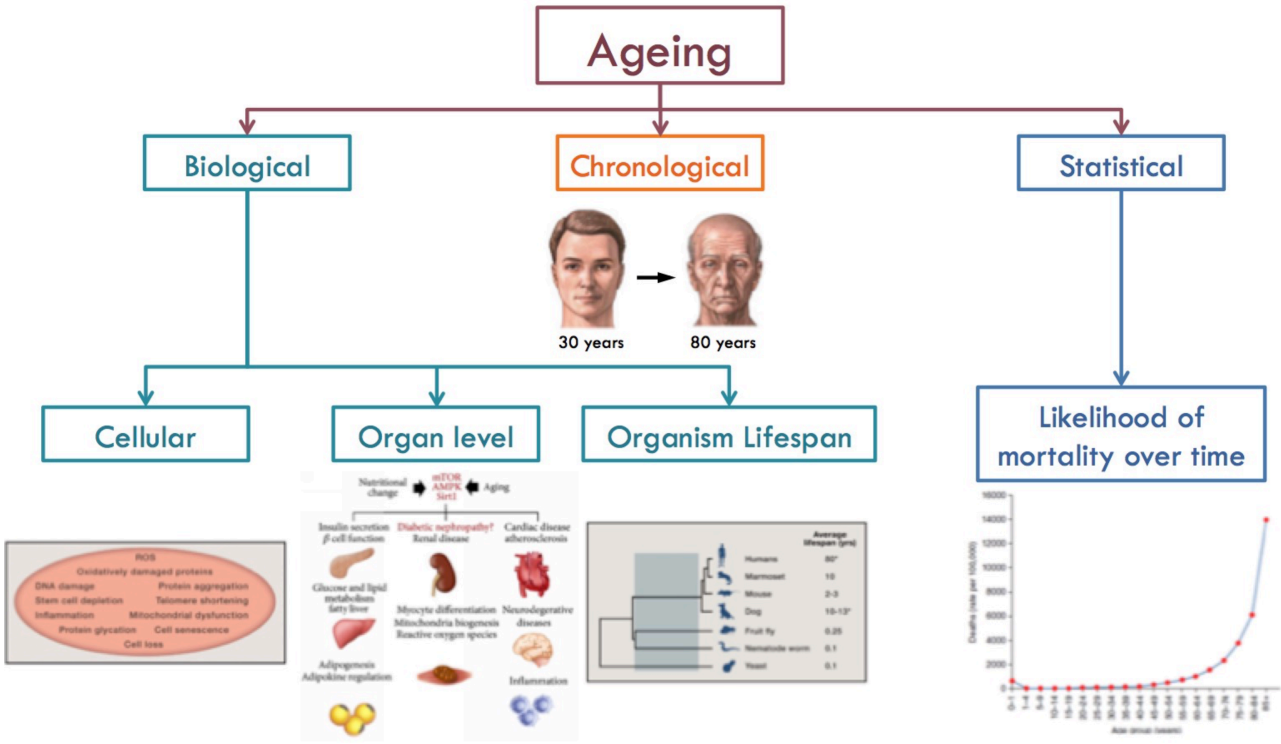


Figure 1.1 Various definitions of ageing.

In humans however, the use of life-long, or long-term CR interventions to delay ageing have not been performed. This is partly because of the difficulty that humans have in adhering to low calorie diets, and partly because it is not feasible to continue human clinical trials over a lifetime. Additionally, long-term studies on the scale of human lifespan are more likely to be influenced by confounding factors and thus are prone to develop experimental artifacts, making results difficult to interpret or reproduce (de Cabo et al. 2014). Instead trials have been undertaken with surrogate outcomes such as cardiometabolic parameters or biomarkers of ageing over shorter periods (Heilbronn et al. 2006). For example, intermediate length trials, such as the 2-year CALERIE (Comprehensive Assessment of Long-Term Effects of Reducing Intake of Energy) study have been performed to assess the impact of caloric restriction on age-related biomarkers (Ravussin et al. 2015). Trials such as these have provided support for the beneficial effects of dietary restriction in humans, despite not directly assessing their effects on lifespan. However, a widely used therapeutic approach to directly combat ageing in human populations has not been developed or implemented (Redman et al. 2018).

This is not to say that no change has been made to improve human lifespan over the course of history. In fact, over the last two centuries alone, global median life expectancy has increased from 30 years to nearly 71 years [Figure 1.2], and in the case of Okinawan women, the group with the highest life expectancy at birth, this value has exceeded 89 years (Riley 2005; Vijg & Campisi 2008; D. C. Willcox et al. 2006). These results however, have stemmed through medical and technological improvements, especially over the late 19th and 20th centuries, with antibiotics, access to food and clean water, healthcare and infrastructure (Vaiserman et al. 2016). Such interventions have impacted primarily on infant mortality and the management of disease in adults, rather than influencing the ageing process directly per se. Even so, it should be pointed out that the increase in human lifespan in the last century equals or exceeds the increases in lifespan that have been achieved in the laboratory by manipulating the ageing process in animal models (Le Couteur et al. 2014).

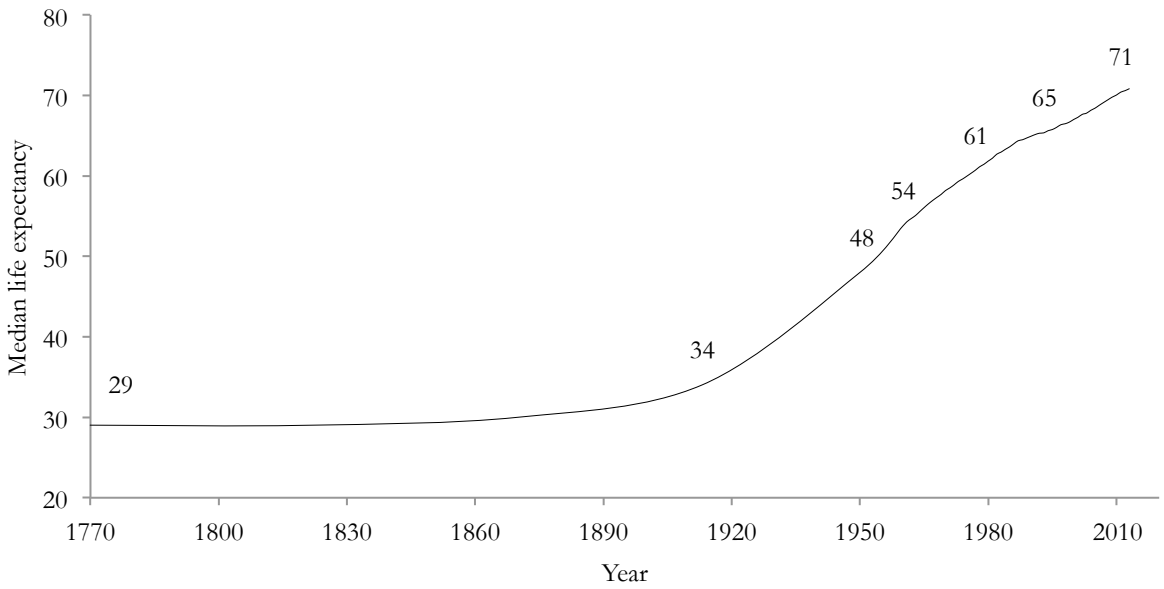


Figure 1.2. Life expectancy at birth over the ages. Adapted from (Riley 2005), and World Bank & Max Planck Research Institute websites.

Phenotype		Change with age	*H. sapiens*	*M. musculus*	*D. melanogaster*	*C. elegans*
Physical/cognitive	Bone Density	Decline	Yes	Yes	NA	NA
	Changes in fat	Varied	Yes	Yes	Yes	Yes
	Muscle mass	Decline	Yes	Yes	Yes	Yes
	Dermatological changes	Varied	Yes	Yes	Yes	Yes
	Cognitive function	Decline	Yes	Yes	Yes	Yes
	Fitness	Decline	Yes	Yes	Yes	Yes
	Balance	Decline	Yes	Yes	Yes	N/A
Metabolic changes	Insulin resistance	Increase	Yes	Yes	Yes	Yes
	Cardiac function	Decline	Yes	Yes	Yes	N/A
	Basal metabolic rate	Decline	Yes	Yes	Yes	Yes
	Mitochondrial function	Decline	Yes	Yes	Yes	Yes
	Cancer, hyperplasia	Increase	Yes	Yes	No	No
	Apoptosis, senescence	Increase	Yes	Yes	Yes	Yes
	Genome instability	Increase	Yes	Yes	Yes	Yes
	Protein aggregation	Increase	Yes	Yes	Yes	Yes
Cellular changes	Immune system function	Decline	Yes	Yes	Yes	Yes
	Inflammation	Increase	Yes	Yes	No	No
	Neurodegenerative changes/atrophy	Increase	Yes	Yes	Yes	Yes

Table 1.1. Phenotypic changes associated with ageing, classified by physical, metabolic, and cellular levels. Table adapted from (Margolick & Ferrucci 2015; Vijg & Campisi 2008).

The relationship between ageing and disease

While there is semantic debate whether ageing is a disease, or simply a consequence of cellular degeneration over time, there is no doubt that disease and ageing are inextricably linked; old age being one of the most significant risk factors for most chronic diseases in the Western world (Le Couteur et al. 2011). Some diseases, such as diabetes mellitus and infection with the Human Immunodeficiency Virus are also claimed to accelerate the ageing process, however most only mimic some of the phenotypic changes seen in ageing, rather than accelerating the ageing process as a whole (Margolick & Ferrucci 2015). Despite the controversy over this classification, the consensus certainly exists that it is possible to distinguish between individuals and organisms of different age on the basis of biological changes; which are remarkably similar across taxa (Rattan 2006; Vijg & Campisi 2008).

Old age is the most powerful risk factor for disease, especially chronic diseases, and increases the risk of mortality and morbidity associated with most diseases (Tacutu et al. 2011). While mortality is known to increase exponentially with age (until ~95 years), a function known as the Gompertz-Makeham law (Missov & Lenart 2013), death itself is generally considered to be caused by diseases such as infections, diabetes, dementia, cancer, cerebrovascular stroke and heart disease [Figure 1.3]. A complementary pattern is also seen in late life, where the longest-lived groups display delayed manifestations of age-related diseases (Willcox et al. 2008). Targeting the ageing process therefore has the potential to reduce the incidence of all these diseases through one intervention, rather than targeting each pathological process directly; a strategy known as the compression of morbidity [Figure 1.4] (Fries 1980; Guarente 2014; Seals & Melov 2014). Thus, understanding the ageing process is one of the most important challenges faced by the scientific community, and will be critical in alleviating the burden on healthcare systems predicted to occur in the coming century given the rapidly growing ageing population around the world (Tarry-Adkins & Ozanne 2016).

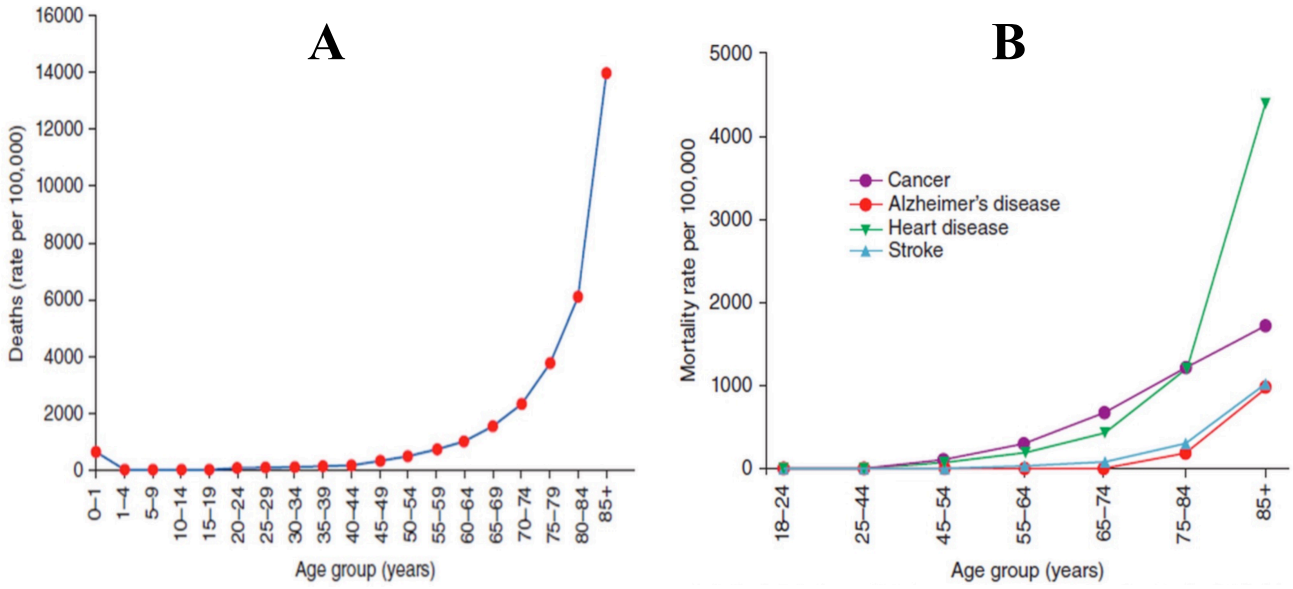


Figure 1.3. Age related morality. A) Chronological age group vs. death rate in the United States (2010). B) Age vs. rates of common chronic disease related mortality in the United States (2010-2012) Figures adapted from Harrison's Principles of Internal Medicine (Le Couteur et al. 2012a).

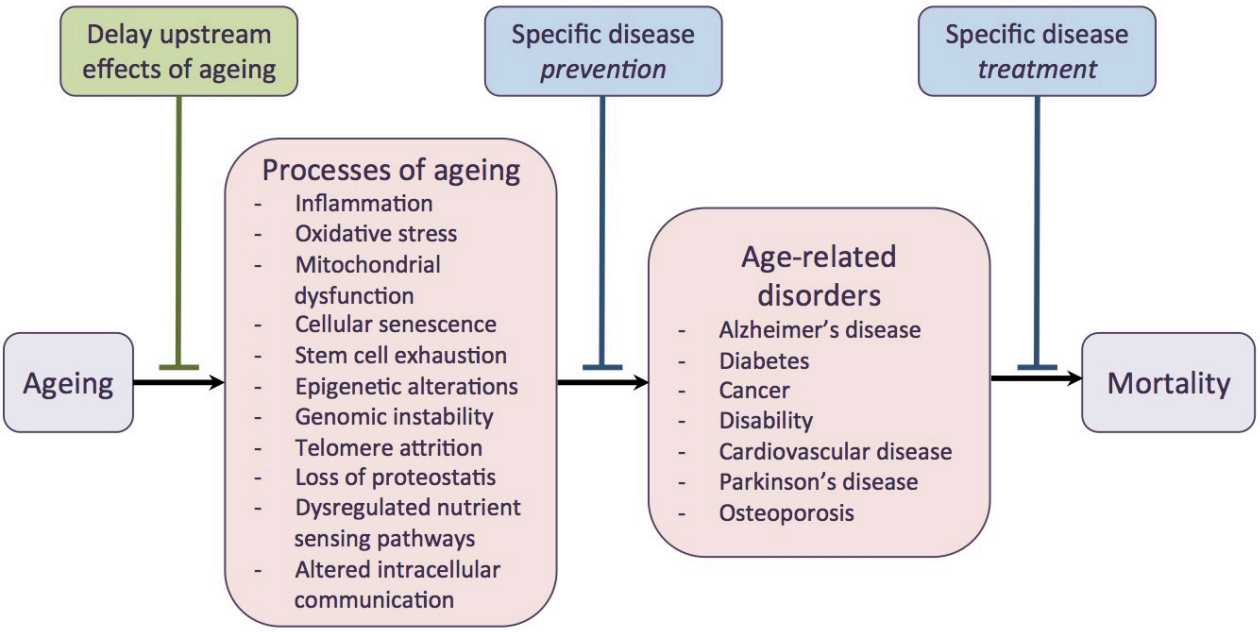


Figure 1.4 Compression of morbidity to decrease mortality. Slowing the processes of aging might prove to be the most effective method of reducing morbidly and mortality compared to disease prevention or treatment. Figure adapted from (Seals & Melov 2014).

It can be argued that ageing is an inevitable process, as the second law of thermodynamics necessitates increases in entropy over time. This definition however, does not directly correlate with biological ageing, where in many cases the entropy may remain unchanged or even decrease with age (Takahashi et al. 2012; Humeau et al. 2008). Furthermore, the timeframe in which the second law of thermodynamics applies is not limited by the lifespan of organisms, making it a weak argument for the basis of biological ageing (Le Couteur et al. 2012a).

There is no reason why biological ageing must occur; and indeed, there are some species, which are biologically immortal such certain types of jellyfish and plants, and Hydra, which display negligible senescence perhaps related to their high numbers of stem cells. There are also species that die without typical features of ageing such as annual plants and semelparous animals (Le Couteur et al. 2011). Despite this, the evolutionarily dominant strategy for survival of a species is achieved through reproduction rather than immortality (Seals & Melov 2014). Thus there appears to be a trade-off between longevity and reproduction that might be the evolutionary foundation of ageing and mortality.

In 1990, Zhores Medvedev published a widely cited paper attempting to classify the theories of ageing. In this paper, he cited over 300 theories, however concluded that it was unrealistic to expect any single theory to encompass all aspects of ageing (Medvedev 2008). This is primarily due to the fact that biological ageing is highly variable, not only between species, but even within a single species, organ, cell or organic molecule (Rattan 2006). Interestingly, most of the evolutionary theories of ageing seem to be connected by their attempts to explain trade-offs between reproduction versus longevity. Some of the main theories are briefly reviewed below.

Programmed death theory

One of the first evolutionary theories of ageing was the programmed death theory, stating that natural selection, which was known to occur at multiple levels, favoured the removal of older individuals so resources could be allocated to younger individuals in order to optimise reproductive capacity (Weismann 1882; Longo et al. 2005). Therefore, death was hypothesised to be a programmed event that had evolved to benefit the species. Several lines of evidence have made this theory fall out of favour, especially given the fact that selection pressure at the group level is far less dominant than at the individual level (Shanahan 1998), however given the fact that no single theory can explain ageing, there is still debate about the merit of a programmed theory of age (Longo et al. 2005; Kirkwood & Melov 2011; Kowald & Kirkwood 2016).

Some have suggested that the immune system, endocrine system, senescence pathways, and the Hayflick limit on cell divisions may provide potential mechanisms mediating programmed death of cells (apoptosis), or the entire organism (phenoptosis) (Jin 2010). Studies in the yeast, *S. cerevisiae* showed that the proteins BCL2 (B-cell leukaemia/lymphoma 2 protein) and BAX (BCL2-associated X protein) stimulated anti- and pro-apoptotic pathways respectively, rescuing the organism from death, or causing it (Longo et al. 1997).

While this may be considered support for the programmed death theory, no correlate of this has been found to program phenoptosis in higher eukaryotes. Furthermore, given that ageing is seen to be more dominant in iterparous species, it was proposed that rather than being programmed per se, ageing was a response to an accumulation of damage over time that eventually overcomes an organism (Medawar 1952).

Mutation accumulation theory

This theory focuses on the idea that natural selection is most powerful for traits favouring reproduction in early life (Darwin 1859). Therefore, the inability of natural selection to weed out mutations that are deleterious in later life would result in accumulation of these mutations in a species over time, leading to age-related decline (Medawar 1952; Kowald & Kirkwood 2016). This theory however would imply an increase in genetic variance in mortality rate in later life, and while some evidence has be circumstantially used to support this theory, most findings are better explain by the more contemporary theories discussed below (Kirkwood & Melov 2011).

Table 1.2. Trade-offs between longevity, early fecundity, and resistance to stressors. Populations selected for increased longevity show increased resistance to stressors in later life, however most studies show a decrease in early fecundity as a result. Table adapted from (Kirkwood & Austad 2000).

Organism	Population selected	Traits affected			Resistance to stressors		
		Longevity	Early fecundity	Early viability	ROS	Heat	UV
C. elegans	Dauer larvae	↑	↓	↑	↑	↑	↑
C. elegans	Various mutants	↑	↓	↓	↑	↑	↑
D. melanogaster	Artificial selection	↑	↓	↓	↑	↑	↑
D. melanogaster	Methuselah mutant	↑			↑	↑	
M. musculus	Caloric restriction	↑	↓		↑	↑	
M. musculus	p66shc mutant	↑			↑		↑

Antagonistic pleiotropy

An extension of the mutation accumulation, this theory goes one step further to propose that genes selected through natural selection which may be beneficial in early life may have negative effects in late life (Williams 1957). Some lines of evidence for this theory include the fact that sex hormone genes, which are important for fertility in early life can contribute to the risk of cancer in old age (Gann et al. 1996), the findings that mutations promoting lifespan have been shown to exhibit a fitness cost in *C. elegans* (Jenkins et al. 2004; Walker et al. 2000), and the increase in longevity of fruit flies selected for late-life reproductive success (Sgro & Partridge 1999) [Table 1.2]. Once again however, given the scarcity of data in individual alleles supporting this theory, most of the evidence initially brought forward for antagonistic pleiotropy is now thought to be better explained by the disposable soma theory, which is a special case of the antagonistic pleiotropy theory which is conceptually similar to a redundancy model (Boonekamp et al. 2015; Kirkwood & Austad 2000).

Disposable soma theory

One of the most influential recent theories of ageing is the disposable soma theory of ageing, which hypothesises that ageing results from a trade-off in distribution of limited resources towards either reproduction (germ cells) or survival of the organism (somatic cells) (Kirkwood & Holliday 1979). Compared to the complexities involved in the creation of life, the ongoing maintenance of somatic cells is relatively straightforward (Gladyshev 2016; Kirkwood 2010). Freshwater hydra have the ability, not only to be immortal, but also to sustain regenerative abilities. This is due to the fact that the structure of the hydra is almost entirely comprised of germ cells and shows no signs of senescence (Martinez 1998). Germ cells are able to largely avoid the ageing process but they are unable to form significantly complex organisms, as this requires differentiation into neural, muscular, and other types of cells (de Cabo et al. 2014).

This provides a potential evolutionary basis for why caloric restriction (CR) can enhance lifespan, since limiting nutrient availability would cause the balance to shift towards survival over growth and reproduction [Figure 1.5]. This is thought to be an evolutionary response to periods of famine, where switching from reproduction to survival would increase the chance of an organism surviving until food is available, and reproduction can occur in an environment where the offspring is most likely to survive given the availability of nutrients after birth. An adjunct to the disposable soma theory suggests that this trade off influences the cellular growth pathways which mediate survival and longevity (Le Couteur et al. 2011). This theory is also congruous to data seen in CR animal models, since CR seems to alter the activation of cellular growth pathways (Longo et al. 2015). As discussed, many evolutionary theories of aging surmise that there is a trade-off between reproduction and survival, and the diminishing ability of natural selection to apply selection pressure post-reproduction that causes ageing.

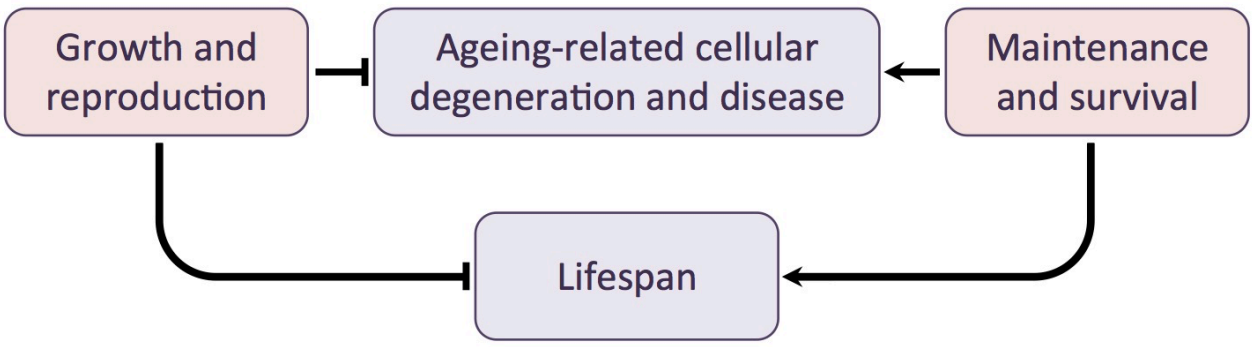


Figure 1.5. The disposable soma theory. Figure adapted from (Vijg & Campisi 2008).

Nutrition and ageing

While the concept of caloric restriction has been around for centuries [Philipus Paracelcus (1493-1541), Luigi Conaro (1466-1566)], it was McCay and colleagues from Cornell University, whose seminal work in 1935 provided scientific proof of the beneficial effects of CR on ageing and lifespan (McCay et al. 1935). Since then, dietary interventions such caloric restriction and intermittent fasting have been shown to be some of the most robust non-genetic interventions to delay the onset of age-related disease and improve lifespan (Solon-Biet et al. 2015b).

Table 1.3. The effects of caloric restriction and mutations/drugs influencing nutrient sensing pathways on lifespan and health in a variety of species. Table adapted from (Fontana et al. 2010).

	Lifespan increase		Beneficial health effects	
	CR	Mutations/drugs	CR	Mutations/drugs
Yeast	3-fold	10-fold	Extended reproductive period	Extended reproductive period, decreased DNA damage/mutations
Worms	2- to 3-fold	10-fold	Resistance to misexpressed toxic proteins	Extended motility, resistance to misexpressed toxic proteins and germ-line cancer
Flies	2-fold	60-70%	Increased activity	Resistance to bacterial infection, extended ability to fly
Mice	30-50%	30-50% (100% in combination with CR)	Protection against cancer, diabetes, atherosclerosis, cardiomyopathy, autoimmune, kidney, and respiratory diseases; reduced neurodegeneration	Reduced tumour incidence; protection against age-dependent cognitive decline, cardiomyopathy, fatty liver and renal lesions. Extended insulin sensitivity
Humans	Not determined	Not determined	Prevention of obesity, diabetes, hypertension; reduced risk factors for cancer and cardiovascular disease	Possible reduction in cancer, metabolic and neurodegenerative diseases

Caloric restriction has yielded beneficial effects on metabolic, cardiovascular, and neurological outcomes in virtually all species studied, and is typically accompanied by an extension of lifespan of up to 30-50% [Table 1.3] (Fontana et al. 2010). Growing evidence indicates beneficial effects in humans, on outcomes such as inflammation, hypertension, and cardiovascular and metabolic diseases (Cava & Fontana 2013). Compared to other interventions, by extrapolation of results from animal models to humans, CR, or interventions targeting the underlying mechanisms of CR could be estimated to produce one of the, if not the, most impact on longevity [Figure 1.6] (Baur et al. 2012).

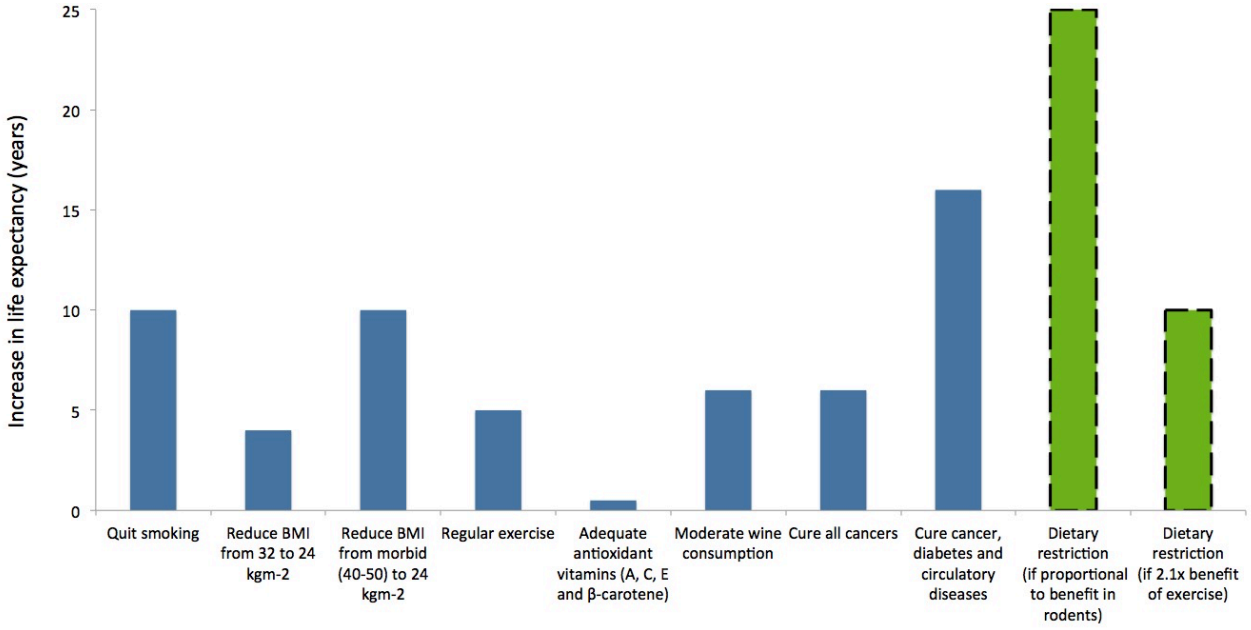


Figure 1.6. Estimated effects of a variety of interventions on human life expectancy. Figure adapted from (Baur et al. 2012).

One of the best pieces of evidence for this is from Okinawan population in south Japan, which boasts the highest rates of centenarians in the world (D. C. Willcox et al. 2006). This may be partly due to genetics, but seems to be primarily due to the higher than average levels of daily exercise, lower than average food intake, and the composition of dietary macro- and micronutrients (Le Couteur et al. 2016). In order to disentangle the effects of genetics, a sub-population of Okinawans who had moved to Brazil was compared to the native population. On adopting a Western diet, this sub-population experienced a significant drop in life expectancy, by approximately 17 years compared to their counterparts who remained in Japan, providing evidence that the basis for their longevity is driven more by environment than by genetics (Mizushima et al. 1997). That being said, there is debate as to which dietary regimens are optimal, and the impracticality of human trials means that long-term dietary interventions are not likely to ever be a therapeutic methodology applied to humans (de Cabo et al. 2014). Therefore, it is critically important to understand the mechanisms by which diet mediates its beneficial effects on health and ageing, in order to directly target pathways through optimal short-term interventions, or pharmaceutical agents such as CR mimetics [Table 1.4]. Although lifespan is an important outcome of interest, criticism towards ageing research has been generated by the notion that increasing lifespan may be accompanied by an increase in burden of disease carried by the elderly population (Seals & Melov 2014). While this is a valid concern related to the ageing population that is accruing throughout the modern world, this brings up the concept of optimal longevity, where lifespan increases are complemented by increased healthspan, or portion of life which is morbidity-free (Longo et al. 2015). Research has also consistently shown that the increase in lifespan that occurs due to dietary interventions is accompanied by an increase in health outcomes through life, a term which is known as healthspan (Fontana et al. 2010), although there is some evidence that lifespan and healthspan can be dissociated depending on the intervention used (Hansen & Kennedy 2016). In humans, while median lifespan has increased drastically over the last century, this has not been matched by a proportional increase in healthspan (Mercken et al. 2012). However, as discussed earlier,

this is likely due to the fact that the changes in human longevity are primarily due to advances in medicine and development of treatments to diseases rather than interventions targeting the ageing process such as caloric restriction (de Cabo et al. 2014).

Table 1.4. Effects of various interventions on lifespan and health in humans, or in mice where data in humans was not available. Table adapted from (de Cabo et al. 2014).

Treatment	**Positive effects**	**Negative effects**
Caloric restriction	Decreased body fat, blood pressure, resting heart rate and improved lipid profile	Danger of malnutrition (e.g. neurologic deficits, lowered fertility and libido, wound healing problems, amenorrhea, osteoporosis, decreased potential to combat infections)
Fasting strategies	Longer lifespan; decreased hypertension and of other features of metabolic syndrome; improved verbal memory loss in the aged and overweight; weight loss in the obese	Limited if not integrated with health-associated diets; might be harmful in children, underweight people and during pregnancy as well as in some disease states
Exercise	Prevents cardiovascular diseases, diabetes, osteoporosis, sarcopenia and depression; prolongs independent living by the elderly	Excessive exercise in the elderly is correlated to mortality
Resveratrol	(in mice) prevents oxidative stress in the aging heart, neurodegeneration, vascular disease and diabetes; increases lifespan under metabolic stress conditions (high-fat diet or every other day feeding)	(in humans) at high doses, nausea, gastrointestinal discomfort; (in mice) at high doses, nephrotoxicity
Rapamycin	(in mice) extends lifespan; exerts antiproliferative effects	Potent immunosuppressive properties; long-term administration has adverse effects (e.g. impaired wound healing, proteinuria, or pneumonitis)
Spermidine	(in mice) extends lifespan; inhibits neurodegeneration, induces cardiac autophagy	(in mice) high doses can cause emaciation, aggressiveness, convulsions and paralysis
Metformin	(in humans) decreases hepatic gluconeogenesis; (in mice) increases insulin sensitivity; lifespan extension	Gastrointestinal disturbances; at high doses, can cause tachycardia, hypoglycaemia; very small chance of lactic acidosis

Nutrient sensing pathways

Recently, research has been undertaken to elucidate which components of dietary interventions are most beneficial and to determine the cellular pathways that mediate the response of ageing to changes in dietary composition. It has generally been concluded that it is simply the reduction of energy intake that increases lifespan, although recent research has focused on each of the macronutrients (protein, carbohydrates, fat) or the ratio of macronutrients, or even the effects of short periods of fasting and hunger (Mair et al. 2005; Solon-Biet et al. 2014; Solon-Biet et al. 2015c; Le Couteur et al. 2015).

A recent meta-analysis of nearly 150 animal studies of CR concluded that protein restriction was more important for life extension than the degree of caloric restriction (Nakagawa et al. 2012). The benefits of dietary interventions seem to therefore be derived from some combination of CR and reduced macronutrient intake, and more recently the ratio of dietary protein-to-carbohydrates (P:C) (Le Couteur et al. 2015; Fontana et al. 2016). Interestingly, with the long-lived Okinawan population, the average ratio of P:C in the traditional diet was about 1:10, similar to that found to optimise lifespan in animal models (Le Couteur et al. 2015).

While low-protein to high-carbohydrate (LPHC) diets are associated with longevity, high-protein to low-carbohydrate (HPLC) diets have been seen to be associated with higher rates of reproduction, in line with the evolutionary theories indicating there is a trade-off between reproduction and longevity (Le Couteur et al. 2015). Comparing LPHC diets to caloric restriction, studies have showed that many common pathways are involved, including downregulation of mechanistic target of rapamycin (MTOR), alteration to mitochondrial function and changes in metabolic regulation [Table 1.5] (Solon-Biet et al. 2015a).

Table 1.5. Outcomes of studies in animals on LPHC ad libitum diets versus CR diets. Table from (Solon-Biet et al. 2015a; Solon-Biet et al. 2015b).

Outcome	**LPHC ad libitum diets**	**CR diets**
Food intake	↑	↓
Body weight	↑	↓
Body fat	↑	↓
Temperature	↑	↓
Insulin	↓	↓
LDLc	↓	↓
HDLc	↑	↑
Mitochondrial number	↓	↑
Mitochondrial free radicals	↑	↓
PGC-1α	↓	↓
Uncoupling protein	↓	-
mTOR phosphorylation	↓	↓
AMPK phosphorylation	?↓	↑
Reproductive fitness	↓	↓
Lifespan	↓	↑

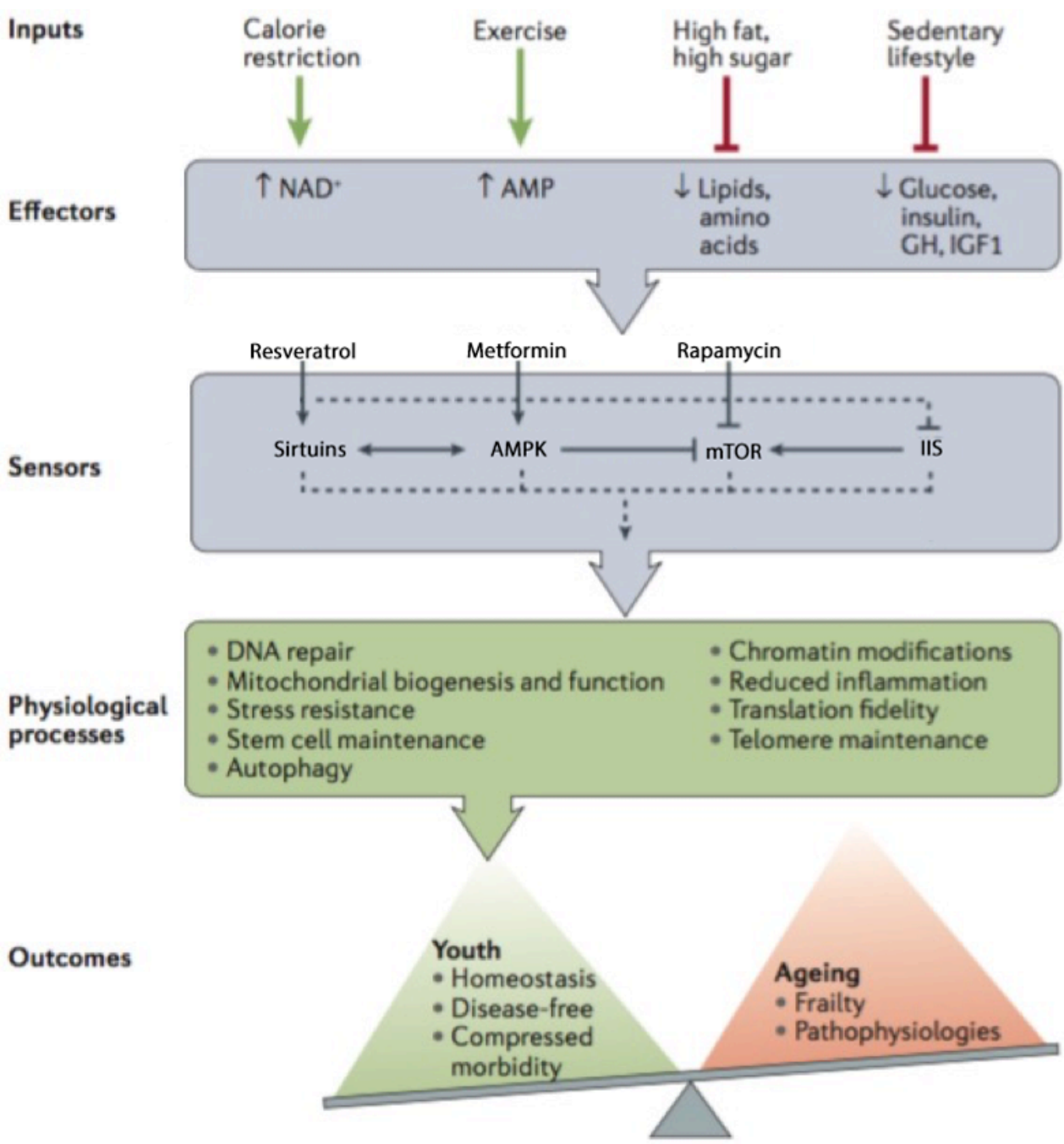


Figure 1.7. Proposed mechanisms mediating the beneficial effects of dietary restriction on ageing. Figure adapted from (Bonkowski & Sinclair 2016).

Dietary studies over the years have also looked at mechanistic factors such as endocrine function, oxidative stress, inflammation, genetic and epigenetic changes. Despite this, no single mechanism has been found to be solely, or even mostly responsible for the lifespan extension seen in dietary studies

(de Cabo et al. 2014). Given however, a great deal of work has focused on studying nutrient sensing pathways, as these provide a potential mechanism linking diet with ageing (Finkel 2015). As these nutrient sensing pathways share common downstream targets influencing cellular processes such as mitochondrial function, metabolism, anabolic/catabolic balance and protein synthesis, they are able to control the balance between growth and reproduction versus survival and ageing [Figure 1.7] (Solon-Biet et al. 2015b).

In 2013, a workshop was held in Erice, Italy to bring together ageing research experts from a variety of disciplines in order to discuss potential interventions to delay ageing and improve healthspan in humans. While many pharmacological and gene targets were discussed, consensus was reached that the most promising interventions were primarily focused on modifying nutrient sensing pathways (Longo et al. 2015):

1. Interventions mimicking dietary restriction (caloric, macronutrient, intermittent fasting, etc.)
2. Inhibition of the Target Of Rapamycin - S6-Kinase (TOR-S6K) pathway
3. Activation of the Sirtuin histone deacetylase pathways
4. Inhibition of the Insulin-like Growth Factor 1 (IGF-1)/Growth Hormone (GH) axis
5. Activation of the AMP-activated protein kinase (AMPK) pathway

Given that long-term studies of caloric restriction are not feasible in humans, interest has shifted towards the drug and nutritional interventions that act on these nutrition sensing pathways but don't involve reduced food intake (de Cabo et al. 2014). These signaling pathways are largely evolutionarily conserved, facilitating the study of diets, genes and pharmaceutical agents in simpler laboratory models such as yeast, worms and mice (Vaiserman et al. 2016).

MTOR-S6K pathway

Formerly known as mammalian target of rapamycin, mTOR has been renamed as mechanistic target of rapamycin, after the discovery that it is highly conserved amongst eukaryotic cells (Wei et al. 2015). Related to the phosphoinositide 3-kinase (PI3K)-related protein kinases (PIKK) family, mTOR is the catalytic subunit of two individual complexes, mTOR complex 1 (mTORC1) and mTORC2. These form unique complexes; mTORC1 with Regulatory-associated protein of mTOR (RAPTOR), and mTORC2 with Rapamycin-insensitive companion of mTOR (RICTOR) (Zoncu et al. 2010). The mTOR pathway is activated by circulating amino acids through Rag-GTPase complexes, in addition to being responsive to insulin/IGF-1 signaling, circulating glucose, Wnt ligands, oxygen, and cAMP (Laplante & Sabatini 2009; Efeyan et al. 2015). Activation of mTOR is linked with anabolic cell growth through protein and lipid synthesis, while inhibition of mTOR is linked with cellular stress responses such as autophagy (Jewell et al. 2013). The mTOR pathway is one of the leading candidates thought to mechanistically link diet and ageing, as it is sensitive to circulating amino acids, and modification of this pathway has been shown to produce lifespan extension in many species [Table 1.6] (Lamming et al. 2013; Johnson et al. 2013; Jia 2004; Kapahi et al. 2004). Caloric and protein restriction has also been shown to be associated with a decrease in mTOR activity in mice (Solon-Biet et al. 2014; Xiao et al. 2011). The mTORC1 complex has two major substrates, S6 kinase (S6K) and 4E-BP1, both of which have been shown to be related to protein and lipid synthesis, and mitochondrial metabolism (Longo et al. 2015). The mTORC2 complex on the other hand is involved in the phosphorylation and activation of Akt and protein kinase C (PKC), which is involved in anabolism, cell survival and cell cycle progression, and inhibition of forkhead box protein O1 (FOXO1), a transcription factor that regulates many cellular processes including apoptosis, metabolism and proliferation [Figure 1.8] (Zoncu et al. 2010).

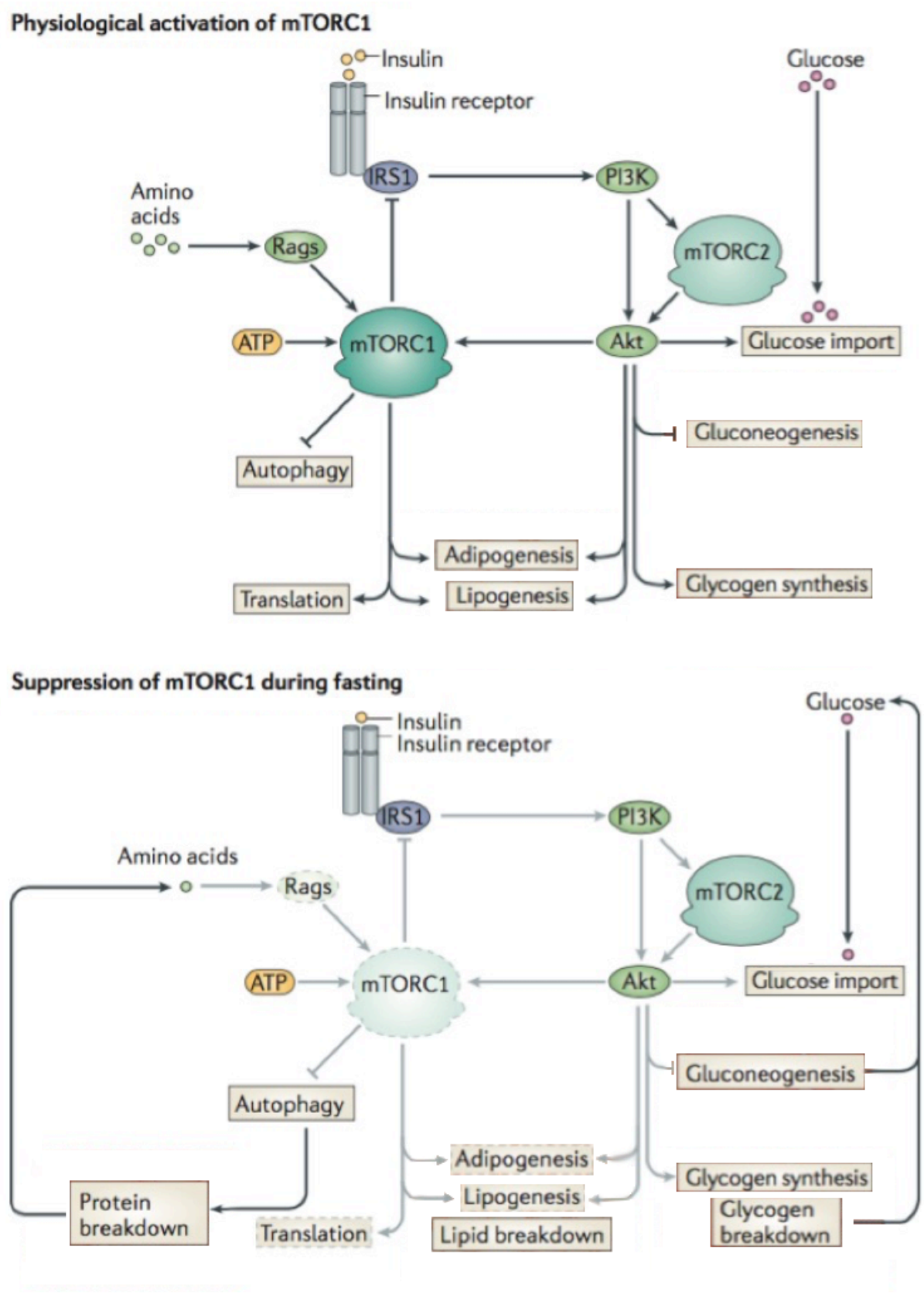


Figure 1.8. The role of mTOR in metabolism. Figure adapted from (Zoncu et al. 2010).

Together, these mTOR complexes are a key target in ageing research, especially given the availability of the pharmacological agent rapamycin, which inhibits the complexes, and has been shown to produce a lifespan extension of up to 26% in mice (Harrison et al. 2009; Vaiserman et al. 2016). Rapamycin, despite being shown to increase longevity in a subset of animals [Table 1.6], has significant adverse effects, particularly through metabolic dysregulation (Lamming et al. 2012; Deblon et al. 2012), immunosuppression (Blagosklonny 2013) and haematopoetic proliferative disorders (Soefje et al. 2011). While rapamycin has been given FDA-approved status for immunosuppression following organ transplantation in humans, these side effects have precluded its use as an anti-ageing drug (Arriola Apelo & Lamming 2016). For this reason, despite the immense appeal of mTOR as a target for improving human longevity, robust safety studies must be performed to further understand the role and function of mTOR and its substrates in the ageing process (Longo et al. 2015).

In animal studies, inhibition of mTORC1, mTORC2 and S6K and activation of 4E-BP1 and FOXO has displayed longevity benefits (Seo et al. 2013; Johnson et al. 2013; Kennedy & Pennypacker 2014; Kenyon et al. 1993; Melendez 2003). In fact, *4EBP* deletion has been shown to block lifespan extension mediated by caloric restriction in *D. melanogaster* (Kapahi et al. 2004; Zid et al. 2009). Gene mutations of the mTOR signaling pathway in many model organisms have consistently shown increases in mean lifespan, thereby demonstrating the importance of understanding the interaction between diet, mTOR and ageing [Table 1.6] (Lamming et al. 2013; Evans et al. 2011). Reduced mTORC1 signaling has also been shown to be linked with upregulated autophagy (Laplante & Sabatini 2009; Salminen & Kaarniranta 2009), mitochondrial oxidative phosphorylation (Guarente 2008; Bonawitz et al. 2007; Zid et al. 2009), and resistance to stress due to reactive oxidative species (Wei et al. 2009), all of which are potential mechanisms linking mTOR with ageing (Evans et al. 2011). The role of mTOR, particularly due to its function as a dietary protein sensor is of significant interest, and is investigated throughout this thesis.

Table 1.6. The impact of mTOR-related genes and rapamycin on longevity in model organisms. Compiled from (Evans et al. 2011; Lamming et al. 2013; Vaiserman et al. 2016).

Species	Gene or drug	Change in lifespan
S. cerevisiae	*SCH9* (*Akt*/*S6K* homolog) mutant	Increase in mean chronological lifespan (30%)
	SCH9 (*Akt*/*S6K* homolog) deletion	Increase in mean chronological lifespan (three-fold)
	SCH9 (Akt/*S6K* homolog) mutant	Increase in mean replicative lifespan (18%)
	SCH9 (Akt/*S6K* homolog) deletion	Non-significant increase in mean replicative lifespan
	TOR1 deletion	Increase in mean and maximum replicative lifespan (20%) Increase in median chronological lifespan (2.7-fold)
	Rapamycin	Increase in chronological lifespan (54%) Increase in replicative lifespan (15-19%)
C. elegans	TOR (*let-363*) RNAi	Increase in mean lifespan (2.5-fold)
	Raptor (*daf-15*) heterozygous	Increase in mean lifespan (30%) and maximum lifespan (19%)
	S6K (rsks-1) RNAi	Increase in mean lifespan (12%–47%)
	S6K (*rsks-1*) deletion mutant	Increase in mean lifespan (15%)
	TOR (let-363) RNAi	Increase in mean lifespan (15%–44%)
	S6K (*rsks-1*) RNAi	Increase in mean lifespan (22%)
	S6K (*rsks-1*) deletion mutant	Increase in mean lifespan (9%)
	TOR (let-363) RNAi	Increase in mean lifespan (10%)
	Rictor (*rict-1*) deletion mutant	Decrease in median lifespan (24%–43%) and maximum lifespan (21%–32%; normal diet, 25°C) Increase in mean lifespan (4%–34%) and maximum lifespan (7%–39%; nutrient-rich diet, 25°C)
	Rictor (*rict-1*) RNAi	Increase in mean lifespan (12%–33%; normal diet, 20°C)
	Raptor (*daf-15*) RNAi	Increase in mean lifespan (7%–21%)
	Rag-GTPase (*raga-1*) RNAi	Increase in mean lifespan (9%–35%)

	Rag-GTPase (*ragc-1*) RNAi	Increase in mean lifespan (13%–33%)
	Rheb (*rheb-1*) RNAi	Increase in mean lifespan (18%–25%)
	Rapamycin	Increase in mean lifespan (19%)
D. melanogaster	*dTSC1* overexpression	Increase in mean lifespan (14%; males)
	dTSC2 overexpression	Increase in mean lifespan (12% at 29°C, 20% at 25°C; males)
	dTOR overexpression	Prevention of eclosion
	dTOR FRB domain (dominant negative)	Increase in mean lifespan (24% at 29°C, 26% at 25°C; males)
	dS6K dominant negative	Increase in mean lifespan (22%; males)
	dS6K constitutive active	Decrease in mean lifespan (34%; males)
	dTOR mutant (hypomorph)	Increase in median lifespan (20%)
	d4E-BP null	Decrease in mean lifespan (males, 30%; females, 17%; yeast extract, 5%)
	d4E-BP overexpression	No effect on lifespan (males or females)
	d4E-BP weak activated	No effect (males) and increase in mean lifespan (females, 14%)
	d4E-BP strong activated	Increase in mean lifespan (males, 11%; females, 22%)
	dS6K constitutive active	Slight, significant decrease in mean lifespan (females)
	d4E-BP null	Decrease in mean lifespan (39%; females)
	Rapamycin	Increases in mean and median lifespans (17%, 23%; rich media diet; females only), (54%, 36%; starvation diet; females only)
M. musculus	Loss of S6K1	Increases in mean, median, and maximum lifespans (20%, 18%, and 10%, respectively; females only)
	Mtor+/–*Mlst8*+/– genotype	Increases in mean, median, and maximum lifespans (14%, 13%, and 18%, respectively; females only)
	Rapamycin	Increase in mean lifespan (8%–26%)

Sirtuin pathway

Since the discovery of the Silent information regulator 2 (Sir2) (Kennedy et al. 1995), genome-wide association studies (GWAS) have shown that the *SIR2* locus one of the most important regulators of replicative lifespan in yeast (Stumpferl et al. 2012). Sir2 homologues in other taxa are collectively known as sirtuins, and are a family of NAD^+-dependent histone deacetylases (HDAC) and ADP-ribosyltransferases, which have since been shown to modulate stress responses, disease susceptibility and longevity in a variety of species (Giblin & Lombard 2016). In mammals, the sirtuin family comprises of seven proteins (SIRT1-7), has become one of the most widely studied nutrient sensing pathways (Houtkooper et al. 2012). The various sirtuin proteins localise to different parts of the cell and have functions including nutrient sensing, regulation of glucose and lipid metabolism, mitochondrial biogenesis and transcription regulation (Michán & Sinclair 2007). SIRT1 and 6 are primarily found in the nucleus, SIRT2 is mainly cytosolic, SIRT3-5 localise to the mitochondria, and SIRT7 is found in the nucleolus (Houtkooper et al. 2012). The activity of each sirtuin protein is dependent on specific substrates based on its cellular localisation, but in general, regulation is controlled by gene expression in response to energy status; caloric restriction upregulates SIRT1 expression (Nemoto 2004), while high dietary fat downregulates expression (Coste et al. 2008). SIRT1 is also known to act through its multiple downstream targets, notably FOXO1 and p53, which are involved in metabolism and apoptosis respectively (Bonkowski & Sinclair 2016). In mice, SIRT1 overexpression has also been shown to display a caloric restriction phenotype (Bordone et al. 2007), while downregulation has been shown to display an accelerated ageing phenotype (Sommer et al. 2014). While SIRT1 rose to fame due to its relationship with longevity, studies in other species have showed mixed results, with many pro-longevity results being derived in metabolically stressed conditions with exposure to high fat diets [Table 1.7] (Bass et al. 2007; Scheibye-Knudsen et al. 2014; Kaeberlein & Powers 2007). In humans, gene association studies have found no polymorphisms in *Sirt1* to be associated with longevity (Razi et al. 2017; Flachsbart et al. 2006). Therefore, while sirtuins,

have been shown impact the ageing process, the focus is now on their role in ameliorating metabolic disease processes and affecting lifespan through their impact on health (Baur 2010).

Table 1.7. The impact of Sirtuins and Sirtuin Activating Compounds (STACs) on longevity and caloric restriction. Table compiled from (Bonkowski & Sinclair 2016; Bhullar & Hubbard 2015).

Species	**Sirtuin knockout shortens longevity**	**Sirtuin overexpression extends longevity**	**STACs extend longevity (% change in lifespan)**	**Sirtuins mediate the effects of CR on longevity**	**Sirtuin expression altered by CR**
S. cerevisiae	Yes (20–50%)	Yes (30–50%)	Resveratrol (30–70%; failed in other studies)	Mixed (Sir2, Hst2)	Yes; however other studies showed NAD+ levels unchanged
C. *elegans*	Yes (~5–10%)	High expression (2.1 line 50%); low gene copy number (10-25%), eleven independent low copy overexpressing lines (~5-15%)	Resveratrol (9–65%; failed in other studies); SRT1720 failed	Mixed	Not tested
D. melanogaster	Yes (~50%); No change in other studies	Yes (~18–29%; dose dependant; fat-body type specific); No effect in other studies	Resveratrol (8–29%; failed in other studies)	Yes; Fat-body type specific	Yes
A. mellifera	Not tested	Not tested	Resveratrol (33-38%)	Not tested	Not tested
N. fuzeri	Not tested	Not tested	Resveratrol (27-59%)	Not tested	Not tested
N. guentheri	Not tested	Not tested	Resveratrol (19%)	Not tested	Not tested
M. musculus	Yes (~20–50%) Elevated embryonic/ postnatal mortality	Brain specific *Sirt1* (9–16%); *Sirt6* (9-17%; males)	Resveratrol (31%; high fat diets; failed in other studies); SRT1720 (~8–22%); SIRT2104 (~10%; males)	Yes	Yes

With regards to lifespan, SIRT1 in particular has been well studied, after its activator resveratrol was identified (Baur & Sinclair 2006). Resveratrol, a polyphenol found in red wine, has also been shown to improve insulin sensitivity, decrease inflammation, protect against neurodegenerative disorders and carcinogenesis, and promote cardiovascular health in both animal models and humans (Cottart et al. 2013; Smoliga et al. 2013; Liu et al. 2014; Hausenblas et al. 2014). While the effects of resveratrol have been shown to mimic the effects of caloric restriction in mammals, the benefits to lifespan have only been seen in yeast, worms and flies, and in mice on high fat diets [Table 1.7] (Lagouge et al. 2006; Baur et al. 2006). Other sirtuin family members such as SIRT3 and SIRT6 have also been implicated in promoting longevity, however the bulk of evidence suggests that sirtuins act mainly to improve health outcomes rather than directly on lifespan (Guarente 2011). Furthermore, many Sirtuin Activating Compounds (STACs), including resveratrol, have been found to have multiple effects not directly related to SIRT1, raising the question whether the effects of these molecules on lifespan is dependent on SIRT1 (Pacholec et al. 2010).

In humans, resveratrol is known to be safe in doses of 1-2mg/day, as these doses can be found in dietary sources consumed by the general population (Smoliga et al. 2012). On the other hand, while studies some have concluded that doses up to 450mg/day may be safe in 60kg individuals (Williams et al. 2009), the optimal dose remains unclear for healthy individuals (Brown et al. 2010). This is because, while resveratrol has been shown to ameliorate metabolic (Bhatt et al. 2012; Magyar et al. 2012; Crandall et al. 2012) and neurodegenerative disease processes (Sun et al. 2010), human clinical trials have not shown the same degree of positive outcomes (Yoshino et al. 2012; Bo et al. 2013). These clinical trials have been thoroughly reviewed by (Novelle et al. 2015). Further studies are needed to evaluate optimal dosage, safety and efficacy of STACs prior to their use as a pro-longevity agent. The role of the sirtuin pathway in ageing, and its relationship with diet is therefore a key area of interest of this thesis.

Insulin, IGF-1 and growth hormone axis

One of the first pro-longevity gene mutations discovered was a PI3K (*age*-1) mutation in *C. elegans* (Friedman & Johnson 1988), and subsequently the knockout of insulin receptor (*daf-2*), which is upstream of PI3K, was also shown double lifespan in the nematode (Kenyon et al. 1993). Similar results have been demonstrated in a variety of species, through various alterations to the insulin, insulin-like growth factor-1 (IGF-1) and growth hormone (GH) pathway; collectively known as the Insulin and IGF-1 Signaling (IIS)/GH axis (Tatar 2001; Svensson et al. 2011; Holzenberger et al. 2003; Fontana et al. 2010). GH is secreted by the anterior pituitary gland in response to hypothalamic release of Growth Hormone Releasing Hormone (GHRH), and in turn induces production of IGF-1, primarily in the liver (Milman et al. 2016). Both GH and IGF-1 have pleiotropic effects on cells via GH receptors (GHR), and IGF-1R and insulin receptor (IR) respectively (Junnila et al. 2013). In humans, IGF-1 receptor gene polymorphisms have been shown to be correlated with longevity in studies of centenarians and octogenarians (Van Heemst et al. 2005; Suh et al. 2008), and life expectancy has been shown to be predicted by low circulating IGF-1 levels in long-lived individuals (Milman et al. 2014). Currently, the only pharmacological agents that target the IIS/GH axis are monoclonal antibodies (mABs) against IGF-1 receptor (IGF-1R), somatostatin analogues, and the GH receptor (GHR) antagonist pegvisomant used in the treatment of acromegaly (Longo et al. 2015). While multiple oncological clinical trials using IGF1R mABs have been performed (Warshamana-Greene et al. 2005; Carboni et al. 2009), these agents have not received FDA approval for cancer treatment or ageing (Chen & Sharon 2013). Somatostatin analogues are known to have a significant side effect profile due to their activity on multiple endocrine pathways (Freda 2002). Pegvisomant has been demonstrated to have a much better tolerability (Burt & Ho 2003), and was shown to decrease circulating IGF-1 levels in a dose-dependent manner (Kopchick et al. 2002). Caloric restriction, has also been shown to reduce the activity of the IIS pathway (Bonkowski et al. 2006; Katic & Kahn 2005), and transgenic mouse strains with alterations to the IIS/GH axis have been consistently

documented to modify lifespan [Table 1.8] (Junnila et al. 2013).

Table 1.8. The impact of alterations to IIS/GH axis related genes on longevity in mice. Table compiled from (Junnila et al. 2013; Bartke 2005).

Mouse model	Sample size (% of control)	Lifespan change (%)	Lifespan (days)	
			Mutant	Control
Snell dwarf	25–33	F +42 M +42	F and M 1,178 ± 235	F and M 832 ± 158
Ames dwarf	33	F +68 M +49	F 1,206±32 M 1,076±56	F 718±45 M 723±54
lit/lit	50–67	F +25 M +23	F 1,070±127 M 1,093±186	F 857±169 M 886±148
Ghr–/–	<50	F +21 M +40	F 921±41 M 917±55	F 759±41 M 656±67
Bovine GH transgenic	200	M –45	M 425±22	M 773
GHR antagonist transgenic	70	p<0.05	F 839±25 M 790±41	F 771±26 M 758±40
LI-*Igf1*–/–	75–100	F +16	F 812±33 (800 ± 33)	F 700±21 (23.0 ± 0.7 months)
Pappa–/–	40	F +38 M +38	F and M 960±28	F and M 698±23
Igf1r+/–	90	F +33 M p<0.05	F 756±46 M 679±80	F 568±49 M 585±69
Igf1r+/–	90	p<0.05	F 923±21 M 983±21	F 967±29 M 939±24
Klotho transgenic (2 strains)	100	F1 +19 F2 +19 M1 +20 M2 +31	F1 829±32 F2 830±29 M1 858±40 M2 936±47	F 697±45 M 715±44
Irs1–/–	70	F +17 M p<0.05	F 891±39 M 897±41	F 763±21 M 786±21
Irs2+/–	100	F +17 M +17	F and M 905±22	F and M 775±10
Irs2+/–	100	p<0.05	F and M 788±17	F and M 755±22
Irs2–/–	90	F –26 M –84	F 560±63 M 123±20	F 755±23 M 767±40
Brain-specific *Irs2*+/– and *Irs2*–/–	100	(+/–) F +18 M +18 (–/–) F +14 M +14	Raw data not presented	Raw data not presented
p66shc+/– and *p66shc*–/–	100	(+/–) F +7 M +7 (+/–) F +28 M +28	(+/–) F and M 815±37 (–/–) F and M 973±37	F and M 761±19

AMP-activated protein kinase pathway

Given that the IIS/GH axis acts upstream of mTOR, an integrated starvation response to CR may be the mechanism linking these pathways with ageing (de Cabo et al. 2014). Another nutrient-responsive sensor is the AMP-activated protein kinase (AMPK) pathway, which has been shown to inhibit mTOR through phosphorylation of the RAPTOR subunit of mTORC1 (Potter et al. 2010; Hardie 2014). AMPK is a serine/threonine kinase that is activated by increases in cellular AMP:ATP ratio (representing low energy states), and acts to increase ATP production to maintain energy homeostasis by activating alternate catabolic pathways, while downregulating biosynthetic pathways such as mTOR dependent protein translation (Gowans et al. 2013). This results in increased glucose uptake into skeletal muscle via GLUT4 (Kurth-Kraczek et al. 1999; Holmes et al. 1999), increased fatty acid oxidation (Merrill et al. 1997), and decreased hepatic gluconeogenesis (Ruderman et al. 2013). In addition, AMPK is also known to be involved in metabolic regulation on a larger scale by acting both on the hypothalamus to control appetite (Kola 2008; Minokoshi et al. 2004), as well through its role in mitochondrial biogenesis and mitophagy via PGC1-α phosphorylation and SIRT1 activation (Cantó et al. 2010; Jager et al. 2007). Therefore, given its fundamental role as a sensor of cellular energy and nutrient status, AMPK has been implicated in mediating the beneficial effects of CR on ageing (Cantó & Auwerx 2011; Stenesen et al. 2013). A decline in AMPK signaling has also been demonstrated to occur during the ageing process (Reznick et al. 2007; Hardman et al. 2014; Salminen et al. 2016). Pharmacological activators of AMPK such as biguanides, have been shown to delay disease onset and ageing in nematodes and mice, but had mixed results in flies and rats [Table 1.9] (De Haes et al. 2014; Cabreiro et al. 2013). Metformin is a FDA-approved anti-diabetic biguanide currently being prescribed to more than 100 million people worldwide for type II diabetes mellitus (Hardie et al. 2012), but has been shown to be possibly effective for other human age-related disease processes such as cardiovascular and metabolic disease (Group 1998), cancer (Wu et al. 2014; Coperchini et al. 2015), cognitive decline (Ng et al. 2014; Foretz et al. 2014) and frailty (Wang et al. 2017; Valencia et al. 2017).

Due its widespread use, and favorable safety profile, a multi-center placebo controlled clinical trial has been proposed to study the effects of metformin on ageing (Barzilai et al. 2016; Barzilai et al. 2018). Given that microarray studies have shown strong similarities between gene expression patterns seen in metformin and CR interventions in mice (Dhahbi et al. 2005), it will be interesting whether similar findings occur in humans.

Table 1.9. The impact of biguanides on longevity in model organisms. Table compiled from (Vaiserman et al. 2016; Burkewitz et al. 2014).

Model	Biguanide	Impact on longevity	AMPK involved	Physiological effects
C. *elegans*	Metformin	36%-40%	Yes	Slowed lipofuscin accumulation; increased autophagy, respiration and metabolic heat production
	Metformin/ Phenformin	Up to 36%; dose dependent	Yes	Altered microbial folate and methionine metabolism
D. melanogaster	Metformin	No effect	Yes	Reduced lipid stores, increased autophagy
M. musculus	Metformin	4%-38%; strain and dose dependent	Yes	Decreased appetite, blood glucose, body temperature; increased mitochondrial biogenesis, fatty acid oxidation, glycolysis and autophagy
	Phenformin	21%	Yes	No data
R norvegicus	Metformin	No effect in Fischer-344 rats	No data	Reduced body weight
	Buformin	9%	No data	1.6 fold reduction in incidence of spontaneous tumours

Mechanisms of ageing

Given that multiple dietary, pharmacological and genetic interventions have been shown to delay the ageing process in model organisms, the next important step in geroscience is to translate these discoveries into humans. Due to the difficulties involved with conducting life-long, or long-term studies measuring lifespan and ageing in humans, an approach to this problem is to interrogate the mechanistic physiological components involved with ageing as representative outcomes to parallel findings in animal models, i.e. surrogate outcomes for ageing. While improvement of lifespan and healthspan are the longer-term goals of biogerontology research, these processes can be independently studied in shorter time frames to find endpoints that can be therapeutically targeted to promote healthy ageing. In 2013, Lopez-Otin and colleagues suggested categorisation of the molecular processes underlying ageing into nine hallmarks based on three criteria [Figure 1.9, Table 1.10] (López-Otín et al. 2013):

1. Manifestation of the process during chronological ageing,
2. Aggravation of the process should cause accelerated ageing,
3. Interventions targeting the process should be seen to impede ageing.

These hallmarks form potential endpoints for clinical trials, and have been found to be useful in assessing the impact of interventions on the ageing process (López-Otín et al. 2016). Similarly, the trans-NIH Geroscience Interest Group also classified physiological processes into seven 'pillars of ageing' based on conservation of mechanistic pathways through various species and interconnectedness in their impact on the ageing process (Kennedy et al. 2014). Notably, the common themes of metabolic and proteostatic imbalance, genetic and epigenetic changes, senescence, and intracellular communication have all been demonstrated to be altered through dietary interventions such as caloric restrictions in both animal models and humans (Civitarese et al. 2007; Astrup et al.

1999; Heilbronn et al. 2006; Lecoultre et al. 2011; Ravussin et al. 2015; Most et al. 2017; Fontana & Partridge 2015), thereby advancing the case to interrogate these mechanisms in the context of diet and nutrient sensing pathways. Together, Figures 1.8 and 1.10 outline the proposed mechanistic picture of how ageing occurs, and the physiological processes underlying it.

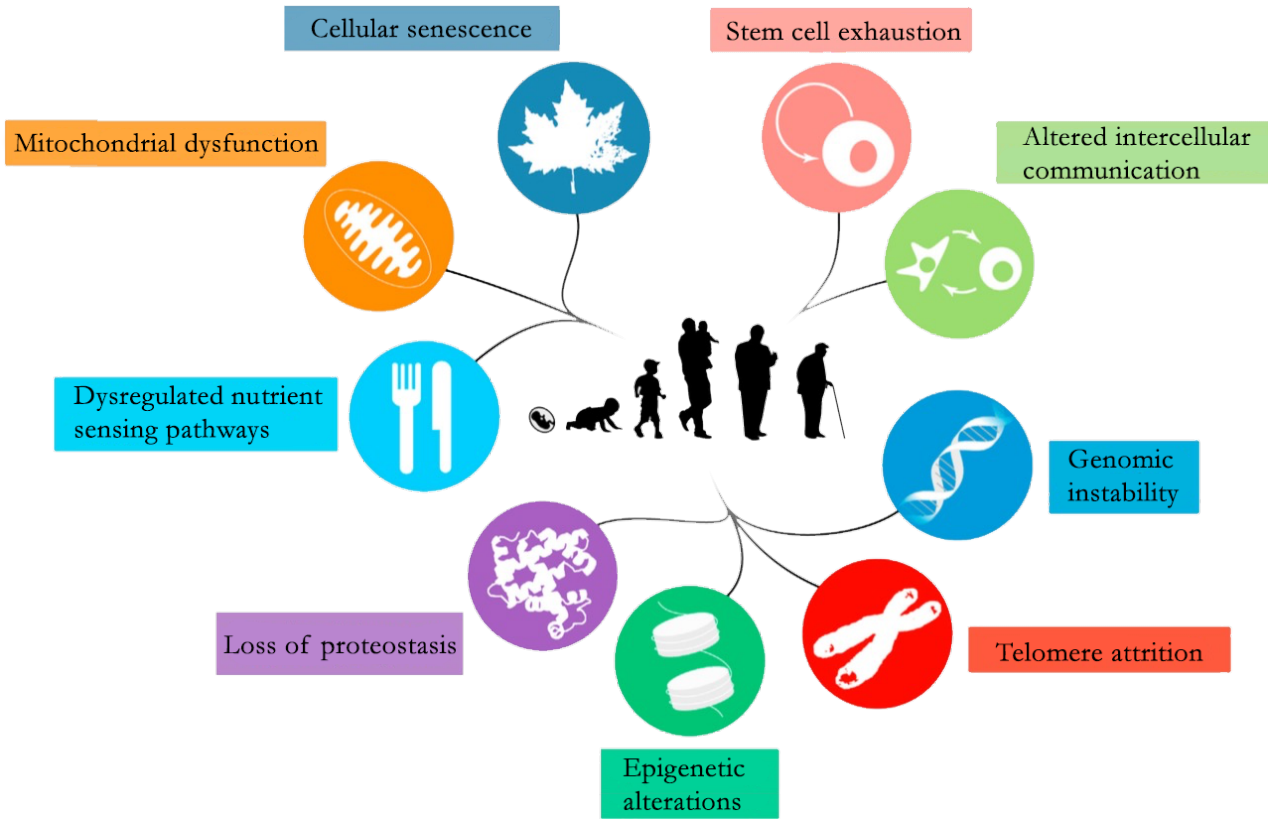


Figure 1.9. The hallmarks of ageing. Primary hallmarks (negative correlation with ageing; source of damage) are shown grouped in the bottom right hand corner, antagonistic hallmarks (hormetic; responses to damage) are shown grouped in the top left hand corner, and integrative hallmarks (aggregated; resulting from changes in the other hallmarks) shown grouped in the top right hand corner. Figure adapted from (López-Otín et al. 2016; López-Otín et al. 2013).

Table 1.10. Evidence for hallmarks of ageing in *S. cerevisiae*. Compiled from (Hu et al. 2014; Austriaco & Guarente 1997; Kim et al. 1996; Dang et al. 2009; Feser et al. 2010; Reverter-Branchat et al. 2004; Erjavec et al. 2007; Unal et al. 2011; Lord et al. 2015; Aguilaniu 2003; Lin et al. 2001; Janssens et al. 2015; Janssens & Veenhoff 2016; Hughes & Gottschling 2012; McFaline-Figueroa et al. 2011; Xie et al. 2012; Laun et al. 2005; Laun et al. 2001; Fehrmann et al. 2013; Kennedy et al. 1994; Smeal et al. 1996; Mei & Brenner 2015; Dillin et al. 2014)

Hallmark	Observations
Genomic instability	Accumulation of extra-chromosomal rDNA circles (Sinclair & Guarente 1997) Amplification of chromosomal segments (Hu et al. 2014) Increase in DNA breaks and genomic translocations (Hu et al. 2014) Increase of retrotransposon DNA content (Hu et al. 2014)
Telomere attrition	Changes in telomere length alters lifespan (Austriaco & Guarente 1997) Sub-telomeric genes are subject to transcriptional silencing with age (Kim et al. 1996)
Epigenetic alterations	Modification to histone acetylation (Dang et al. 2009) Loss of silencing at chromosome ends (Kim et al. 1996) Histone mRNA and protein level changes (Feser et al. 2010) Histone occupancy reduction (Hu et al. 2014) Changes to nucleosome positioning (Hu et al. 2014)
Loss of proteostasis	Increased oxidative stress response proteins (Reverter-Branchat et al. 2004) Aggregation of carbonyl-damaged proteins (Erjavec et al. 2007; Unal et al. 2011) Altered nuclear pore complexes (Lord et al. 2015) Oxidative protein damage (Aguilaniu 2003)
Deregulated nutrient sensing	Increased gluconeogenesis, decreased glycolysis (Lin et al. 2001) Changes in energy metabolism pathways (Reverter-Branchat et al. 2004) Altered expression of genes related to metabolism (Janssens & Veenhoff 2016)
Mitochondrial dysfunction	Altered expression of genes related to mitochondrial function (Janssens et al. 2015) Decrease in vacuolar pH and mitochondrial membrane potential, and increased mitochondrial fragmentation (Hughes & Gottschling 2012) Mitochondrial redox potential decline (McFaline-Figueroa et al. 2011) Increased ROS (Z. Xie et al. 2012; Laun et al. 2001) Genomic translocations in mtDNA, increase in mtDNA content (Hu et al. 2014)
Cellular senescence	Induction of apoptotic phenotype and terminal senescence due to oxidative stress and ageing (Laun et al. 2005; Laun et al. 2001) Increased senescence with age (Fehrmann et al. 2013)
Stem cell exhaustion	Increased chance of symmetric divisions (Kennedy et al. 1994) Reduction in replicative lifespan of daughters (Kennedy et al. 1994)
Altered intercellular communication	Decreased pheromone response (Smeal et al. 1996) Altered metabolite levels seen in CR-mediated life extension (Mei & Brenner 2015) Benefits of CR seen on neighbouring cells (Mei & Brenner 2015) Altered cellular communication with age (Dillin et al. 2014)

These hallmarks are classified into primary hallmarks (those with a negative correlation with ageing), antagonistic hallmarks (those which are responses to damage, and show a hormetic response; i.e. beneficial in low doses, but detrimental in high doses), and integrative hallmarks (aggregated responses which resulting from changes in the other processes) (López-Otín et al. 2013). Given the focus of this thesis was to investigate the role of nutrient sensing pathways, and its relationship with ageing in the context of a large scale dietary study, four main end points; a combination of primary and antagonistic hallmarks; are investigated in an attempt to cover as many of the age-related physiological changes as possible within the scope of the study [Figure 1.10]. Due to the central role of the liver in metabolism and its effects in caloric restriction (Schmucker 1998; Le Couteur et al. 2010), these four pathways were chosen as major research outcomes for this thesis, as they are known to demonstrate well-established age-related phenotypes in the liver (Cogger et al. 2014; McLean & Le Couteur 2004). These are discussed in detail in the following research chapters of this thesis:

1. Gene expression – Chapter 4
2. Telomere length – Chapter 5.1
3. Mitochondrial function – Chapter 5.2
4. Inflammation – Chapter 5.3

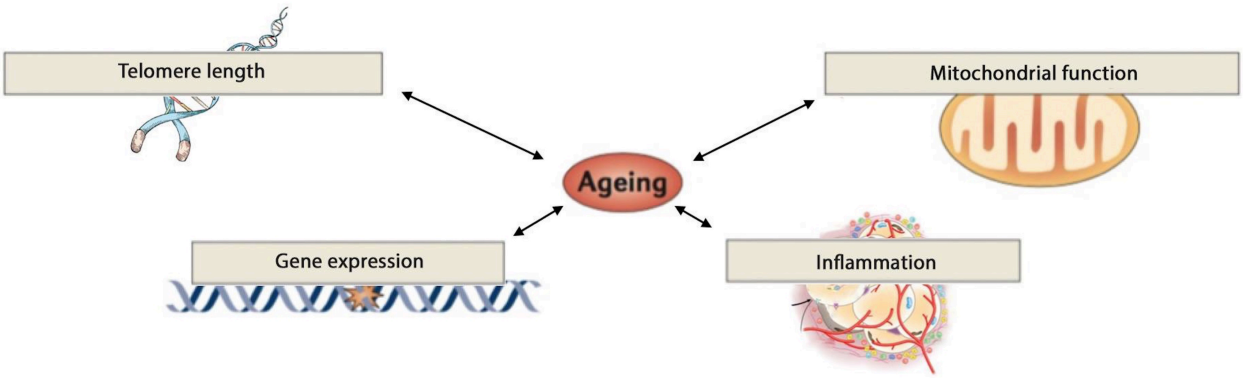


Figure 1.10. Proposed end points to be studied to elucidate the mechanisms of for the effects of nutrition on ageing within this Thesis.

Genomic, epigenetic and proteostatic changes

A plethora of evidence has shown changes in gene and protein expression during ageing, with over 10,000 publications on PubMed, and over 2,000 reviews discussing these changes (Moskalev et al. 2013). These include changes in both nuclear DNA (Gorbunova et al. 2007; Atamna et al. 2000; Trzeciak et al. 2012) and mitochondrial DNA (Gredilla et al. 2010; Yang et al. 2008), proteostasis (Sands et al. 2017; Rizzi et al. 2016; Labbadia & Morimoto 2015), and epigenetics (Fischer & Riddle 2018; Schwörer et al. 2016). Many of these effects involve the cellular growth pathways, and are in line with changes seen in caloric restriction studies (Carrano et al. 2009; Bishop & Guarente 2007; Vilchez et al. 2014).

With nuclear DNA, ageing is associated with accumulation of damage and mutations (Ou & Schumacher 2017; Fischer & Riddle 2018), loss of DNA repair mechanisms (Vermeij et al. 2016; Lord & Ashworth 2012), increased clonal mosaicism (Jones 2012; Laurie et al. 2012), and changes in gene expression patterns (Bahar et al. 2006; Melouane et al. 2018; Kyng & Bohr 2005). Epigenetic changes to post-translational histone modifications (Ashapkin et al. 2017; C. Jin et al. 2011), DNA methylation pattern shifts (Maegawa et al. 2010; Gopalan et al. 2017), chromatin remodeling (Oberdoerffer & Sinclair 2007; Schotta 2004), and transcriptional alterations (Bahar et al. 2006; Smith-Vikos & Slack 2012), enzymatic modulation of sirtuins (Someya et al. 2010; Zhong et al. 2010) have all been shown to be related to ageing, and at the same time, are thought to link with the beneficial effects of CR on lifespan (Le Couteur et al. 2011; López-Otín et al. 2013).

Homeostatic mechanisms to maintain the quality of the intracellular proteome including pathways related to protein folding, chaperoning, and degradation have also been shown to be altered by the aging process and can influence lifespan in animal models (Labbadia & Morimoto 2015; Chondrogianni et al. 2015; Yao et al. 2015). Activating enzyme mutations and supplementation of the

hexosamine pathway, which produces metabolites used in glycosylation in the endoplasmic reticulum and cytosol have demonstrated life extension benefits in *C. elegans*, (Denzel et al. 2014); supplementation of the metabolite glucosamine has been shown to extend lifespan in mice (Weimer et al. 2014), and glycoconjugates have even been hypothesised to act as predictive biomarkers for human ageing (Dall'Olio et al. 2013; Longo et al. 2015). In addition, genetic manipulation of autophagy; which is known to be altered in CR (Mariño et al. 2014; de Cabo et al. 2014); for instance through the *Atg5* or *Lamp2a* genes, have been shown to increase the lifespan of mice (Pyo et al. 2013; Zhang & Cuervo 2008). Given the role of AMPK and mTOR on autophagy, and sirtuins on epigenetic changes, the interplay between diet, the nutrient sensing pathways and the genome and proteasome, are key areas of investigation to improve the understanding of the molecular mechanisms underlying ageing (de Cabo et al. 2014; López-Otín et al. 2016).

Telomere length

Culturing most somatic cell lines have shown a limit to the number of mitotic replications possible before entering senescence due to the shortening of telomeres – a concept known as the Hayflick limit (Hayflick & Moorhead 1961). Loss of telomere length, is a major cause of replicative senescence in non-immortalised cell lines (Nehlin 2016), and telomere related pathologies are associated with early onset of diseases such as idiopathic pulmonary fibrosis, aplastic anemia, malignancies and liver fibrosis (Blasco 2005). Genetically modified mice with increased or decreased telomere lengths have also been shown to have extended or reduced lifespans respectively (Tomás-Loba et al. 2008; Armanios et al. 2009). Telomere shortening was therefore proposed as a possible mechanism contributing to ageing (Johnson et al. 1999; Aubert & Lansdorp 2008), although the use of telomere length as a biomarker for ageing has shown mixed results in humans (Vidaček et al. 2017; Mons et al. 2017). Telomere length is also known to vary amongst tissue types proportional to replicative activity, with longer telomere lengths in less proliferative somatic tissues such as liver and muscle, and shorter

lengths in haematopoeitic cells such as leukocytes (Yui et al. 1998; Gardner et al. 2007). However, while absolute telomere length is known to vary, growing evidence suggests that the rate at which telomere attrition occurs with age seems to be similar across tissue types (Daniali et al. 2013). The process of telomere attrition in ageing is faster in rodents than in humans, and CR and protein restriction have both been shown to be associated with longer telomere lengths in mice (Vera et al. 2013; Tanrikulu-Kucuk & Ademoglu 2012). Taken together, studies in humans and mice have consistently shown that nutrition is correlated with changes in telomere length, therefore providing a plausible mechanistic link for the effects of nutrition on ageing and health.

Mitochondria

Mitochondria are intracellular organelles that generate chemical energy in the form of ATP. During this process, a proton gradient is formed that drives mitochondrial respiration through an electron transport chain (ETC), allowing ATP synthesis, while also creating reactive oxygen species (ROS) (Harbauer et al. 2014). While it is unclear if progressive mitochondrial dysfunction is a cause of ageing or a byproduct, old age is associated with alterations in mitochondrial phenotype, function and number, including impairments in ATP production and increased ROS production (Johnson et al. 1999; Guarente 2008; Sahin & DePinho 2012). Mitochondrial respiratory thresholds are important regulators of CR mediated lifespan extension in yeast, while Complex I and IV of the ETC are required for the CR mediated lifespan extension in flies (de Cabo et al. 2014; Sahin & DePinho 2010).

Interestingly, paradoxical findings in model species have shown that phenotypes considered to correlate with mild mitochondrial dysfunction can, in many cases produce longevity benefits in a hormetic fashion (Palikaras et al. 2015; Munkácsy & Rea 2014), through the promotion of longevity promoting pathways (Yee et al. 2014). The IIS pathway, mTOR, AMPK and sirtuins are all known to have downstream effects on mitochondrial autophagy, biogenesis and activity (Haigis & Yankner

2010). Given the complexity in regulation of these processes and their importance in diet and ageing, study of the nutrient sensing pathways in the context of mitochondrial biology is also an area of interest in this Thesis.

Inflammation

Inflamm-aging, the so-called chronic low-grade inflammation that is associated with ageing tissue is a well characterised process, and is associated with upregulation of a multitude of genes, cytokines and inflammatory pathways (Cevenini et al. 2013; Franceschi & Campisi 2014). This process is thought to be associated with many age-related disorders such as cardiac dysfunction, neurodegeneration, myelodysplastic pathologies, metabolic dysfunction, and various hepatic changes (Le Couteur et al. 2010; Franceschi et al. 2007). Given its relationship to a multitude of systemic processes, inflammation is closely intertwined with mitochondrial function, cellular senescence, gene expression, and has many common pathways of action with the aforementioned nutrient-sensing pathways that are implicated in ageing (Fulop et al. 2015). Cellular senescence is associated with a pro-inflammatory senescence-associated secretory phenotype (SASP), involving the release of cytokines such as interleukin 6 (IL6) and tumour necrosis factor-alpha (TNF-α) (Coppé et al. 2010). In humans, pro-inflammatory glycosylated proteins, associated with changes in proteostasis, have been shown to be one of the most well correlated biomarkers of age (Dall'Olio et al. 2013), and inflammation has been linked with impairments in nutrient metabolism and metabolic function (Brestoff & Artis 2015; Umemura et al. 2014). Over-nutrition has also been seen to be linked with systemic inflammation (Franceschi et al. 2007), while CR seems to reduce inflammatory markers (Fontana et al. 2015). Given the strong relationship between inflammation, diet and ageing, and the availability of phenotypic changes to be studied, inflammation is another key area investigated in this Thesis.

The Geometric Framework for Nutrition

The traditional view of CR has undergone significant change over recent decades, with the role of specific macronutrient ratios and dietary regimens appearing to be as important in determining lifespan and health as total energy intake per se (Piper et al. 2011; Solon-Biet et al. 2015b). Given the complexity of the nutritional system, it has been difficult for reductionist models to analyse the effects of diet on ageing using simple control-versus-intervention dietary trials. Disentangling this complexity requires a systematic, integrated solution that goes beyond the simple control versus treatment group models used in most dietary studies, and a shift towards comparisons of the effects of a very wide range of environmental factors such as diet. The Geometric Framework (GF) is a method developed by Simpson and Raubenheimer, which was created to solve this problem (Simpson & Raubenheimer 1993). This framework models the nutritional environment in a n-dimensional Cartesian space, whose dimensions correspond to intake variables such as protein, carbohydrates and fat (Raubenheimer et al. 2016). An animal's interaction with its nutritional environment can be then be analysed, with outcome measures represented as a topographical heatmap. The colour of the heatmap shows the strength of the response, for instance median lifespan in Figure 1.11, where red correlates to the highest lifespan, and blue represents the lowest lifespan, and the axes represent macronutrients eaten. Similarly, Figure 1.12 shows the corresponding 3-dimensional representation across the three macronutrients: protein, carbohydrates and fat. The peaks and valleys in Figure 1.12 are indicative of the diets studied; since not all diets are possible due to certain nutritional balance causing morbidity, only a subset of the total space has data points, thereby creating a non-uniform intake space. The power of the GF lies not only in visualising the response surfaces, but also in its ability to apply statistical tests to determine significance of outcomes relative to the nutritional environment, and analyse optimal conditions to maximise these outcomes. This is done by fitting non-parametric thin-plate regression splines using generalised additive model (GAMs). A thorough description of the methods used by the GF, its development history and progress made in modeling and visualisation tools are discussed in detail in

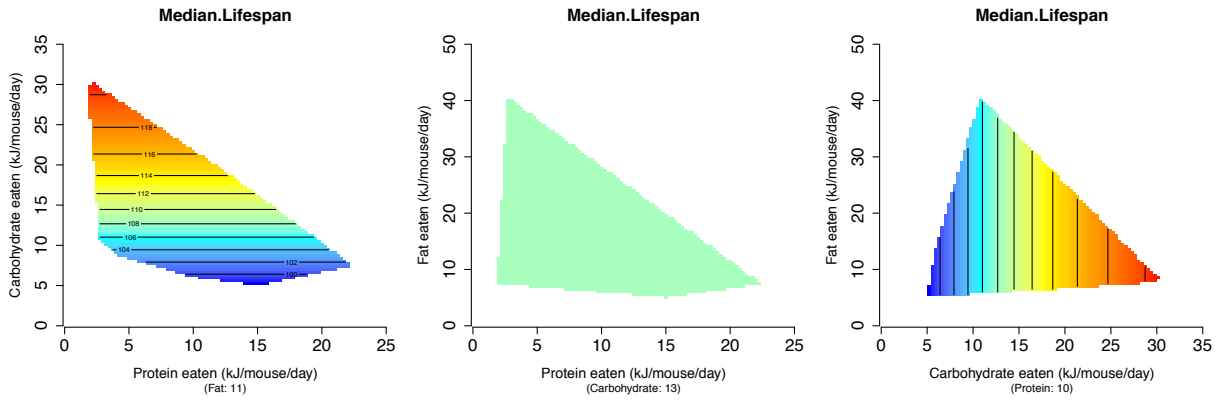


Figure 1.11. 2D GF response surfaces correlating median lifespan to macronutrient intake. Each graph shows the effects of two of macronutrients at the median point of the third macronutrient (shown in parenthesis below the X axis label). The response surfaces vary from red which is the most negative value to blue which is the most positive value.

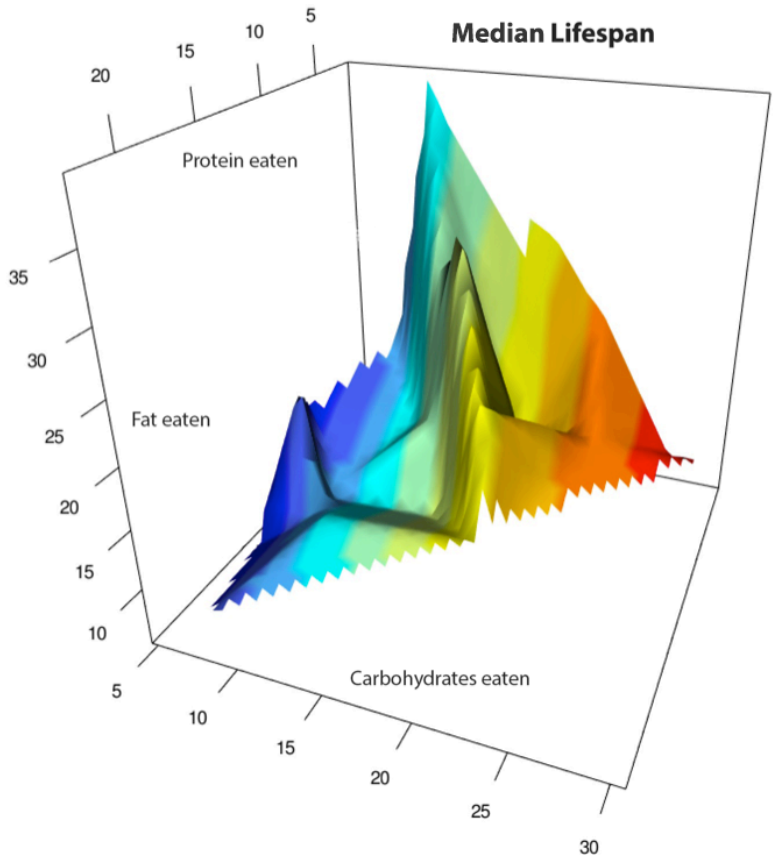


Figure 1.12. 3D GF response surfaces correlating median lifespan to macronutrient intake. Data previously published in (Solon-Biet et al. 2014).

Research Aims

Nutrition has often been the subject of conjectures and ingenious hypotheses—but our actual knowledge is so insufficient that their only use is to try to satisfy our imagination. If we could arrive at some more exact facts they could well have applications in medicine.

—François Magendie (1783–1855)

Given the ability of diet to influence lifespan, the aims of this thesis are two-fold. First, to examine and build upon the analytical tools required to disentangle the effects of nutrition on health and ageing. In order to address this aim, this thesis will discuss the history, development and progress made in methods of applying the Geometric Framework to large and diverse data sets in Chapter 3. Second, the thesis aims to investigate the mechanisms that drive ageing, by looking at the roles of nutrient sensing pathways, their regulation as a result of diet, and their impact on longevity and age-related outcomes. This aim was addressed primarily by studying tissue samples from a large dietary study of 858 mice, which has generated multiple publications previously (Solon-Biet et al. 2016; Le Couteur et al. 2014b; Solon-Biet et al. 2015c; Holmes et al. 2016; Gokarn et al. 2017; Gokarn et al. 2018). This set of work consists of transcriptome analysis of hepatic and hypothalamic tissue to investigate the impact of diet on gene regulation (Chapter 4), and its impact on telomere length, mitochondrial function, and inflammation (Chapter 5).

This thesis comprises of a novel set of work studying the regulation of nutrient sensing pathways and their impact on longevity and age-related end points through the paradigm of a dietary intervention. This study therefore represents a key step in understanding the mechanisms by which macronutrient intake influences the ageing process, and provides a basis for translational trials and guidelines to promote healthy ageing in humans.

Chapter 2: General Methods

Acknowledgements

Full credit for animal husbandry work, experimental diets, and collection of longevity and phenotypic data goes to Dr. Samantha M Solon-Biet, Dr. Aisling McMahon and Dr. Victoria Cogger. Therefore, as these methods have been previously published in detail, the animal husbandry and phenotypic data collection techniques are reproduced directly from (Solon-Biet et al. 2014), and the dietary methods are reproduced directly from Dr. Solon-Biet's PhD thesis from 2014 – 'The role of macronutrient balance on appetite, metabolic health and ageing in a mouse model'. Acknowledgements to Dr. Lindsay Wu for his assistance and guidance on western blotting and densitometry, and to Dr. Neil Youngson for his assistance and guidance on the telomere and mitochondrial PCRs.

The dietary, animal handling, phenotypic, and longevity data were mostly collected prior to the commencement of this PhD, the methods and results of which have been published (Solon-Biet et al. 2014; Solon-Biet et al. 2016; Solon-Biet et al. 2015c; Solon-Biet et al. 2015a; Le Couteur et al. 2014b; Gokarn et al. 2018). The gene and protein expression studies performed on the tissue samples described in Chapters 4 and 5 were undertaken as part of this thesis. As the purpose of this thesis was primarily to investigate nutrient sensing pathways and their endpoints, and explain how data analytical methods, particularly the Geometric Framework, can be used in the interpretation of this data, all data mining, analysis, correlations and visualisations were also performed in direct contribution to this thesis. Analytical methods related to the Geometric Framework are presented in detail in Chapter 3, as development of these methods was a major component of this thesis.

Animals and husbandry

858 three week old C57BL/6 male and female mice (Animal Resources Centre, WA, Australia) were housed three per cage in standard approved cages (Tecniplast, Varese, Italy) in the Molecular Physiology Unit of the ANZAC Medical Research Institute which is an SPF facility designed for housing transgenic mouse. A custom-designed 2-chamber Perspex insert, designed to collect food-spillage, was placed beneath the food hopper of each cage to collect food waste for quantification. Mice were maintained at 24-26°C and 44-46% humidity under a 12h:12h light-dark photoperiod, with lights on at 0600. All protocols were approved by the Sydney Local Health District Animal Welfare Committee (Protocol No. 2009/003).

30 experimental diet treatments were custom-designed and manufactured in dry, pelleted form by Specialty Feeds [Table 2.1]. The diet treatments addressed both nutritional quantity as well as quality. To manipulate diet quantity, indigestible cellulose was added to diet treatments, yielding 3 total energy (caloric) density regimes fixed at 8, 13 and 17 kJ g^{-1} (referred to as low, medium and high energy).

Food intake was measured weekly for 6 months followed by monthly thereafter and corrected for spillage and water content. Mice were checked daily and body weight measurements were recorded to correspond with food intake measurements. Animals losing more than 20% body weight were culled and the corresponding diets discontinued.

Experimental diets

30 experimental diet treatments were custom-designed and manufactured in dry, pelleted form by Gordon's Specialty Feeds, Sydney, Australia. The diet treatments addressed both nutritional quantity as well as quality and represented a wide range of macronutrient ratios that systematically sampled a protein, carbohydrate and fat simplex. To manipulate diet quantity, indigestible cellulose was added to diet treatments, yielding 3 total energy (caloric) density regimes fixed at 8, 13 and 17 kJ g^{-1} (referred to as low, medium and high energy). After approximately 13 weeks of experimental feeding, an increased incidence of rectal prolapse was observed on some of the lowest protein diets, which has previously been attributed to dietary methionine deficiency (< 0.15% of diet) (Sun et al. 2009; Miller et al. 2005). Animals with severe rectal prolapse were culled and these diets also discontinued due to ethical concerns. In total, five diets were discontinued, leaving a total of 25 experimental diets for the remainder of the study.

Table 2.1. Experimental diets. [a]Diets 2 (low energy) and 6 (medium energy) were discontinued within 23 weeks. [b]Diets 3 (low energy), 3 (medium energy) and 6 (low energy) were discontinued within 10 weeks of treatment. These diets were discontinued due to weight loss (≥ 20%), rectal prolapse or failure to thrive.

Diet		1	2[a]	3[b]	4	5	6[a]	7	8	9	10
%P		60	5	5	33	33	5	14	14	42	23
%C		20	75	20	47	20	48	29	57	29	38
%F		20	20	75	20	47	48	57	29	29	38
Low energy (8 kJ g^{-1})	**P**	5.03	0.42	0.42	2.77	2.77	0.42	1.17	1.17	3.52	1.93
	C	1.67	6.28	1.67	4.02	1.67	4.02	2.43	4.77	2.43	3.18
	F	1.67	1.67	6.28	1.67	4.02	4.02	4.77	2.43	2.43	3.18
Medium energy (13 kJ g^{-1})	**P**	7.54	0.63	0.63	4.15	4.15	0.63	1.76	1.76	5.28	2.89
	C	2.51	9.41	2.51	6.02	2.51	6.02	3.64	7.15	3.64	4.77
	F	2.51	2.51	9.41	2.51	6.02	6.02	7.15	3.64	3.64	4.77
High energy (17 kJ g^{-1})	**P**	10.06	0.84	0.84	5.53	5.53	0.84	2.35	2.35	7.04	3.86
	C	3.35	12.55	3.35	8.03	3.35	8.03	4.85	9.54	4.85	6.36
	F	3.35	3.35	12.55	3.35	8.03	8.03	9.54	4.85	4.85	6.36

Over their lifetime, mice were ad libitum-fed one of 25 diets varying in content of protein, carbohydrate and fat. Food intake was measured once per week for 6 months followed by once per month measurements thereafter and corrected for spillage and water content. Mice were checked daily and body weight measurements were recorded to correspond with food intake measurements.

The % of protein (P), carbohydrate (C) and fat (F) (as a % of total energy) is shown in Table 2.1. Each diet was replicated at 8 kJ g^{-1} (low energy), 13 kJ g^{-1} (medium energy) and 17kJ g^{-1} (high energy). Diets varied in content of P (casein and methionine), C (sucrose, wheat-starch and dextrinised cornstarch) and F (soya bean oil). All other ingredients were kept similar. Other ingredients include cellulose, a mineral mix (Ca, P, Mg, Na, C, K, S, Fe, Cu, I, Mn, Co, Zn, Mo, Se, Cd, Cr, Li, B, Ni and V) and a vitamin mix (vitamin A, D3, E, K, C, B1, B2, Niacin, B6, pantothenic acid, biotin, folic acid, inositol, B12 and choline).

Body Composition

Body composition was assessed in 180 mice across all diets by dual-energy x-ray absorptiometry (DEXA) using the GE PIXImus2 Series Densitometer (GE Medical Systems Ultrasound and BMD, Bedford, United Kingdom) under general anaesthesia (intraperitoneal ketamine:xylazine) immediately prior to culling.

Plasma insulin and leptin

Plasma insulin levels were measured using the Ultrasensitive Mouse Insulin ELISA Kit (Alpco Diagnostics, Salem, NH). Plasma leptin levels were quantified using the Mouse Leptin ELISA Kit (Millipore, St. Charles, MO).

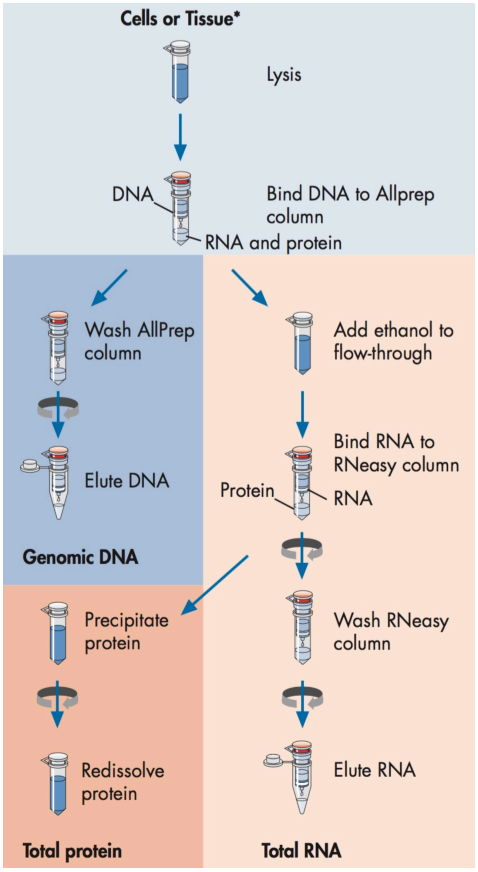
Plasma amino acids and fatty acids

Amino acids were analysed at the Australian Proteome Analysis Facility, Macquarie University, using the Waters AccQTag Ultra chemistry (Waters Corp, Milford, MA). Plasma fatty acids were measured by gas chromatography-mass spectrometry (GCMS) at the Bio21 Institute, University of Melbourne.

Blood lipids and biochemistry

Blood cholesterol, triglycerides, HDLc, LDLc, liver function tests (alanine transaminase, aspartate aminotransferase, gamma-glutamyl transpeptidase) and creatinine were performed at the Concord Hospital Pathology Department.

Extraction of Liver DNA, RNA, and protein



Frozen liver tissue samples were sectioned into 10mg blocks and homogenised using a TissueLyser LT (Qiagen, Hilden, Germany). DNA, RNA, and protein were extracted as per the protocol listed in the Qiagen AllPrep DNA/RNA/Protein Mini Handbook. RNA was eluted into RNase-free water, quantified by spectrophotometry using a NanoDrop (Thermo Scientific, Waltham MA) at 230/260/280nm and stored at -70°C. DNA concentration was also measured spectrophotometrically and stored at -20°C. Protein was pelleted and stored at -20°C.

Figure 2.1. Procedure flowchart. Figure from Qiagen AllPrep DNA/RNA/Protein Mini Handbook.

Western blots for mTOR and phosphorylated-mTOR

Protein pellets were resolubilised in SDS-PAGE buffer with protease inhibitor tablets (cOmplete, EDTA-free Protease Inhibitor Cocktail; Roche, Basel, Switzerland). Concentration of protein was quantified using bicinchoninic acid (BCA) assay (Thermo Scientific BCA Protein Assay Kit) and standard curves were developed with bovine serum albumin (BSA) controls. Samples were then diluted to a 2mg/mL concentration in 100uL volume of Laemmli buffer and 50 mM TCEP BondBreaker (Thermo Scientific). 20µg of protein was separated on 4-15% gradient mini-protean TGX gels (Bio-Rad). Gels were transferred onto nitrocellulose membranes at 25 V, 2.5 A for 10 min on a Trans-Blot Turbo transfer system (BioRad, Hercules, CA). Membranes were blocked for 1 h in a 5% skim milk solution of Tris-buffered saline (TBS). Membranes were washed three times for ten min each, in wash buffer (TBS containing 0.1% Tween-20), and incubated overnight in primary antibody solution (5% BSA, TBS 0.1% Tween 20). Membranes were washed three times as before, and incubated for one hour in secondary antibody solution (5% skim milk solution of TBS 0.1% Tween, 0.01% SDS). Membranes were washed three times as before, rinsed in water and analysed on a licor odyssey system. Densitometry was performed using licor software and results were exported using Prism (GraphPad) to excel sheets for further analysis. Antibodies used were total mTOR (Cell Signaling 4517 L27D4), phospho-Ser2448 mTOR (Cell Signaling 5536 D9C2) and α-tubulin (Sigma T6199, clone DM1A).

Gene expression and microarray

Frozen hypothalamus blocks from 24 mice (one male per group) and liver samples from 48 mice (one male and female per group, with low energy groups having less representation due to their proportional discontinuation as experimental diets as outlined above) were sectioned into 10mg segments. Total RNA was extracted using the Trizol method (Sigma) and quantified

spectrophotometrically using a NanoDrop (Thermo Scientific) at 230/260/280nm. RNA integrity from both hypothalamic and liver samples was verified using a Bioanalyzer (Agilent). 48 male liver samples (two per diet group) and 24 male hypothalamic samples (one per diet group) with an RIN>7 were analyzed by Affymetrix Mouse Gene ST array. Liver microarray was performed at the Ramaciotti Centre for Genomics, University of New South Wales; GEO: GSE85998 – for publication in (Solon-Biet et al. 2016). Gene expression data was corrected by Bonferroni adjustment and normalised to actin. Liver samples that had undergone microarray assays were run on qPCR plates using the RT2 profiler array using the Mouse Insulin Signaling Pathway kit (Qiagen – catalogue no. PAMM-030Z). A pooled sample was serially diluted, and as a standard curve in the Fluidigm Biomark software for PCR analysis.

Telomere length

Average telomere length was measured from total genomic mouse DNA by using a real-time quantitative PCR method as described (Cawthon 2002). The results were normalised using the acidic ribosomal phosphoprotein PO (36B4) gene, which is well conserved and has been used for gene-dosage studies. Primer sequences for 36B4 and telomeric repeats were as previously outlined (Callicott & Womack 2006). Forward and reverse telomeric primers were 5′ CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT 3′ and 5′ GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT 3′ respectively. Forward and reverse primers for the 36B4 gene were 5′ ACT GGT CTA GGA CCC GAG AAG 3′ and 5′ TCA ATG GTG CCT CTG GAG ATT 3′, respectively. Each reaction for the telomere portion of the assay included 12.5 µl Sybr Green PCR Master Mix (Sigma), 300 nM each of the forward and reverse primers and 20 ng genomic DNA. All PCRs were performed in duplicate. Master mix concentrations for the 36B4 portion contained 12.5 µl Sybr Green PCR Master Mix (Applied Bio- systems), 300 nM forward and reverse primers. A real time thermocycler (Roche LightCycler 480) was used with conditions as: 95 °C for 5 minutes followed by

45 cycles of 95°C for 10 s, with 60°C annealing for 15 seconds, followed by extension at 72 °C for 20 seconds for the 36B4 gene, and 95°C for 5 minutes followed by 45 cycles of 95°C for 10 seconds, with 68°C annealing for 15 seconds, followed by extension at 72°C for 15 seconds for the telomere PCR. To serve as a reference for standard curve calculation, a pooled sample of mouse DNA from all 183 samples was serially diluted over a 10-fold range, from 1 to 100 ng per well. Standard curves for 36B4 and telomeric repeats were used for absolute quantification of samples using LightCycler software.

Mitochondrial function

Mitochondrial functions were assessed in liver tissue (n=170) using the Seahorse XF Extracellular Flux Analyzer which generates the key parameters of mitochondrial function using fresh isolated mitochondria from homogenised liver tissue: basal respiration, ATP production, proton leak, maximal respiration, glycolysis and spare respiratory capacity. Different conditions were used to measure the mitochondrial oxygen consumption e.g. by providing different combinations of substrates (pyruvate-malate, glutamate-malate, succinate-rotenone, palmitoyl carnitine-malate) to the electron transport system. State III was monitored after injection of ADP and State IVo after injection of oligomycin. Respiratory Control Ratios (RCR) were calculated as State III/State IVo. Mitochondrial amount as well as substrate, ADP, and inhibitor concentrations were optimised prior to experiments. Hydrogen peroxide production was measured with an Amplex Red kit (Eugene, OR) using the same substrates as for mitochondrial respiration. Hydrogen peroxide production and enzymatic activities (3-Hydroxyacyl Coenzyme A dehydrogenase, aspartate aminotransferase and citrate synthase) were measured spectrophotometrically on the same mitochondrial isolations. Citrate synthase activity was used to normalise the results, which are therefore expressed as mitochondrial function per mitochondrion.

Mitochondrial PCR

Mitochondrial copy number was estimated from genomic mouse mtDNA by using a real-time quantitative PCR method previously described (Uddin et al. 2016). The results were normalised using the acidic ribosomal phosphoprotein PO (36B4) gene, which is well conserved and has been used for gene-dosage studies. Forward and reverse cytochrome b primers were 5′ CCCACCCCATATTAAACCCG 3′ and 5′ GAGGTATGAA GGAAAGGTATTAGGG 3' respectively. Forward and reverse primers for the 36B4 gene were 5′ ACT GGT CTA GGA CCC GAG AAG 3′ and 5′ TCA ATG GTG CCT CTG GAG ATT 3′, respectively. All PCRs were done in triplicate. To serve as a reference for standard curve calculation, a pooled sample of mouse mtDNA from all 183 samples was serially diluted over a 10-fold range, from 1 to 100 ng per well. Standard curves for 36B4 and cytochrome b were used for absolute quantitation of samples using LightCycler software.

Data analysis, visualisation and statistics

All data analysis was based on macronutrient intake, primarily kilojoules per mouse per day (kJ/mouse/day), unless otherwise stated. Data was processed by fitting of GAMs with thin-plate splines to model responses relative to macronutrient intake in protein, carbohydrates, and fat. Full data analytic methods for evaluation of statistical significance and visualisation are described in Chapter 3.

Chapter 3: Methods – Developing analytical tools for the Geometric Framework

For the better part of the last three decades, the Geometric Framework (GF) has been used to untangle the complex interactions between an animal and its nutritional environment (Raubenheimer & Simpson, 1993; Simpson & Raubenheimer, 1993). Since its conception, the application of the GF has evolved considerably, from a theoretical paradigm, to become an invaluable functional tool for the analysis and visualisation of data. This has allowed the GF to be used in a variety of experimental models, from being applied to behavioural dynamics in animal-environment interactions, to mechanistic studies in biological research. Given the complexity of the nutritional system, it has been difficult for reductionist models to analyse the effects of diet on ageing. The GF models a nutritional environment in a n-dimensional Cartesian space, whose dimensions correspond to variables such as macronutrient uptake (Raubenheimer et al. 2016). In this space, an animal's interaction with its nutritional environment can be analysed, with outcome measures represented as a topographical response surface overlaying the n-dimensional nutrient space.

Here, the evolution of the GF is reviewed, and the expansion of this model through this thesis is discussed, with specific reference to data handling techniques and integration of algorithms to improve visualisation and analysis of experimental data. Overall, a new iteration of the GF script is created in R, which can automate analytical methods for large data sets through an easily accessible interface that can be used for a wide array of scientific purposes, and lead to potential future applications such as its use in personalised nutrition in humans. While this application is not explored here, the methodological approach can be conceptualised through the use of personalised goal-seeking optimisation tools that find local maxima and minima specific to the individual response surfaces of a subject.

This chapter will review concepts from nutritional ecology theory and R script coding functions, to explain the logic and workflow used by the Geometric Framework in its application to nutritional and ageing research. The algorithms and data handling methods discussed here were developed through multiple iterations. Novel analytical tools developed for the GF as part of this thesis include:

1. Automation of the GF for large data sets
2. 3-dimensional visualisation of response surfaces
3. Correlation of data analysed through the GF, particularly for gene expression analysis

Background

In complex systems, the interplay between multiple elements makes reductionist or segmented studies difficult to interpret. For instance, manipulation of diet may lead to changes in the lifespan of an animal, but there may be a number of mechanisms underlying this change, each of which, if manipulated individually, may not produce the desired outcome [Figure 3.1]. While the GF was initially intended to study the role of nutrition, or other variable factors on sets of outcomes, major developments have taken place since its inception, which have bridged systemic and mechanistic approaches to analyse data.

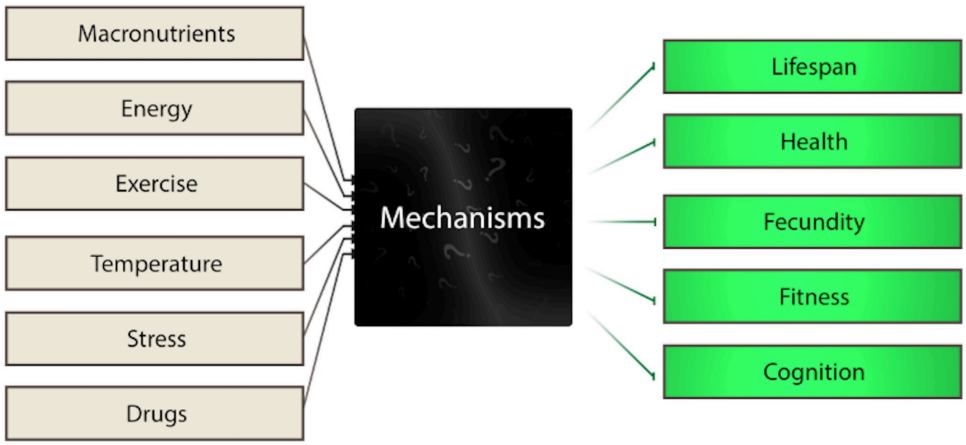


Figure 3.1. Mechanisms are often considered a black-box in ageing research due to the complexity in the interactions between the experimental inputs and response outcomes.

The most important of these was the fundamental paradigm shift away from the control versus treatment experimental model traditionally used in biological research, to analysis of systems as a whole. This is done by creating an n-dimensional Cartesian space, whose axes are represented by multiple variables of interest, where rather than comparing individual groups, a response surface is fitted by non-parametric regression modeling, effectively allowing comparison of all groups at once, thus substantially improving the predictive and explanatory power (Raubenheimer et al. 2016). This allows the effect of each variable axis on an outcome to be studied, both in isolation, as well as in combination with other axes. An example is illustrated in Figures 3.2 & 3.3, and Table 3.1 [data presented and discussed in Chapters 4 & 5]. Here, the 3-spatial axes represent macronutrient intake (Protein – P, Carbohydrates – C, Fat – F), and the topographical heatmap represents outcome.

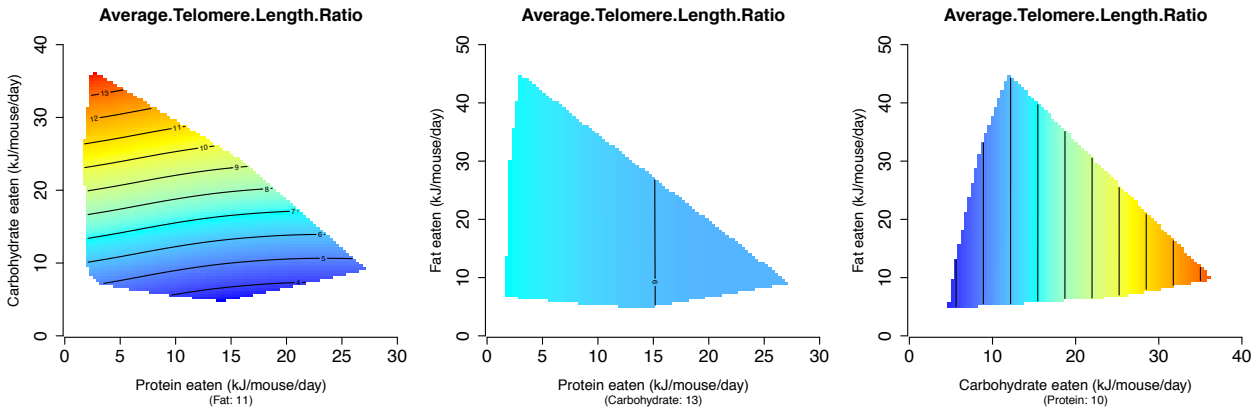


Figure 3.2. 2D GF plots for average liver telomere length ratio (ATLR).

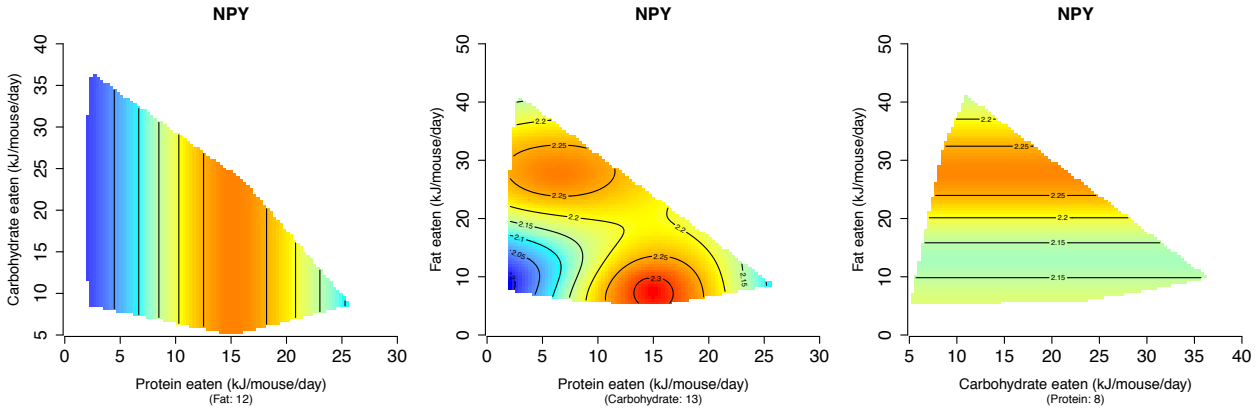


Figure 3.3. 2D GF plot for hepatic gene expression of Neuropeptide Y (NPY).

The GF plots above show two examples of how response surface fitting is beneficial to interpretation of the data. In Figure 3.2, there is a clear relationship to carbohydrates, as seen by the gradient lines being perpendicular to the carbohydrate axis, with response signal maximal at high C intake, and minimal with low C intake. Analysis of the Generalised Additive Models (GAMs) shows this to be a statistically significant result [Table 3.1].

On the other hand, with Figure 3.3, there is a mixed response to the macronutrient axes, and it is difficult to know which dimensions are relevant to the response signal. While it may seem like protein and fat are the drivers of NPY gene expression here, given the perpendicular gradient lines to each, neither of these are statistically significant, and in fact it is the ratio of P:C eaten that is the significant factor for NPY expression [Table 3.1]. This example is used only to demonstrate the power of the GF, and further analysis of gene expression data is discussed in Chapter 4.

Table 3.1. Statistical significance table, with p-values calculated from GAM surface fits for ATLR and NPY relative to the macronutrient axes, and macronutrient ratios.

p-value	**P**	**C**	**F**	**P:C**	**P:F**	**C:F**
ATLR	0.20	**0.04**	0.45	0.24	0.59	0.58
NPY	0.36	0.59	0.39	0.41	**0.01**	0.44

Taken together, this shows two things:

1. The GF is able to be used on experiments with multiple continuous or disjointed data variables such as diet, exercise, temperature in the same analysis, through the creation of an n-dimensional variable environment that corresponds to the response being studied
2. The GF is therefore able to examine the effect of input variables both in isolation, as well as in combinations with other variables, greatly increasing its power to analyse results

Evolution of the Geometric Framework

The GF was initially designed to study local optima within a nutritional space, to examine appetite interactions on feeding behaviors and their correlation to physiology and health in various species. This was used to derive evidence for aspects such as the functional, ontogenic and evolutionary basis of innate homeostatic mechanisms that existed within species (Simpson & Raubenheimer 1993). An early example of this concept is Figure 3.4, where an early GF model was used to predict the phagostimulatory surface of locusts, which were fed diets varying in P:C content (Raubenheimer & Simpson 1997). Early models such as these were primarily based on behaviour, as the predicted response surfaces were based on intake targets and protein leverage, concepts which were relatively well established at the time (Simpson & Raubenheimer 1995).

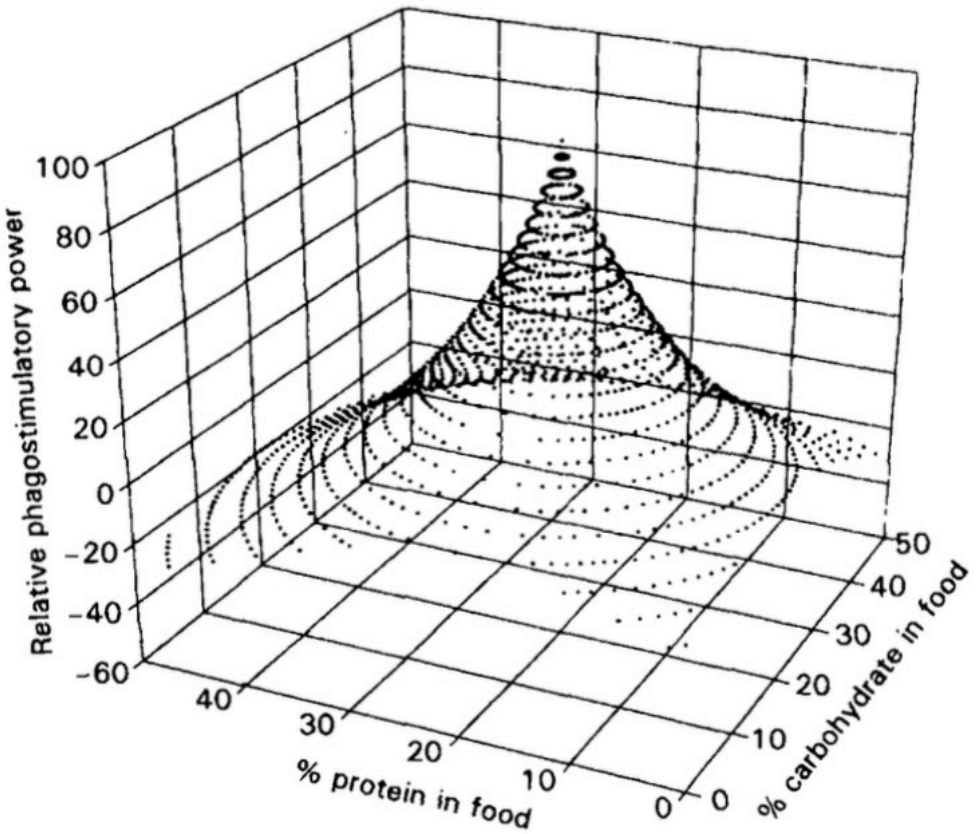


Figure 3.4. Predicted phagostimulatory surface for locusts fed diets varying in P:C ratio. Figure from (Raubenheimer & Simpson 1997).

As the model evolved, target points in the nutrient space encompassed both static points integrated over a period of time for cohort analysis, or dynamic paths over time for individual analysis (Simpson & Raubenheimer 1999). This helped expand the GF from solely analysing nutritional requirements, to also being able to compare amongst organisms, multivariate metrics such as metabolism, growth and lifespan, and their relationships to nutrition (Simpson et al. 2003).

At this point, the GF was powerful enough to be able to revisit the question of which components of dietary restriction were most important, and answer the fundamental question 'caloric restriction relative to what?'. Until this stage, the major barrier impeding the unraveling of the effects of CR was the lack of a conceptual framework from which to analyse experimental results. Since restriction implied a relative decrease, traditional approaches of control versus treatment models were inherently flawed, as they had to assume a control diet, and an arbitrary restriction relative to this. An example of this limitation is represented in Figure 3.5. Here, a study had seemingly showed that caloric restriction rather than protein restriction prolonged lifespan in rats (Davis et al. 1983). However, further analysis reveals another interpretation, that the relationship is not linear, but part of a more complex surface with a peak of 75kJ protein and 150kJ non-protein energy intake (Simpson & Raubenheimer 2007).

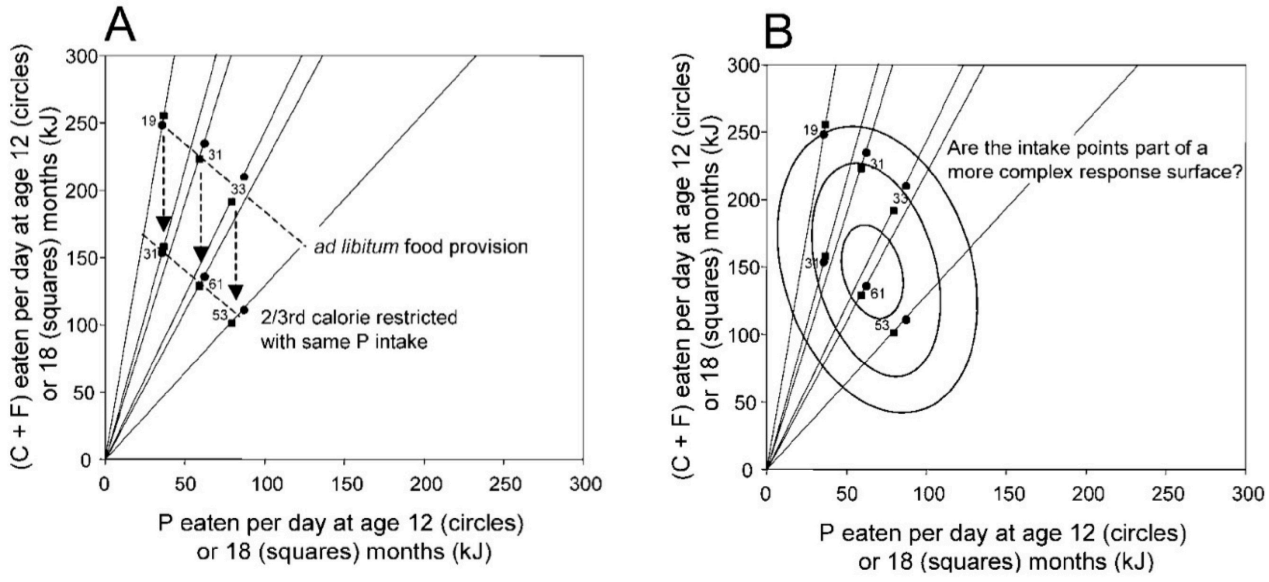


Figure 3.5. Analysing the findings of Davis et al 1983. Figure from (Simpson & Raubenheimer 2007).

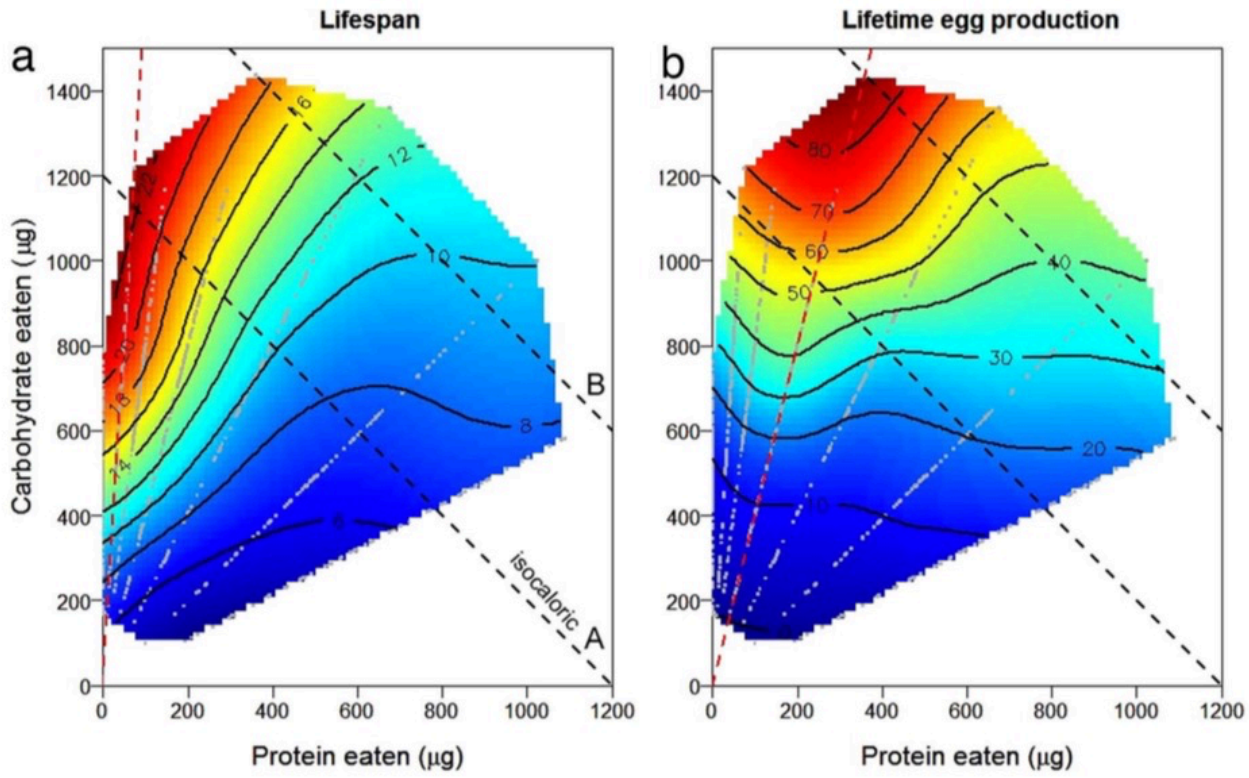


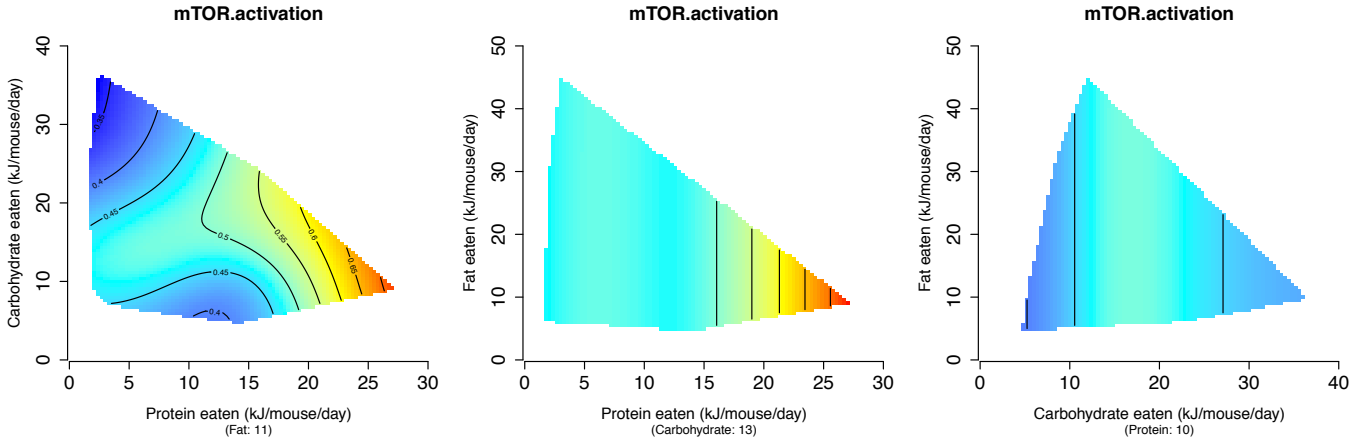
Figure 3.6. Parametric non-linear surface fitting of lifespan and lifetime egg product responses to diets varying in P:C ratio. Figure from (Lee et al. 2008).

Convincingly separating CR versus nutrient specific effects was demonstrated for one of the first times in 2008, from data collected in Drosophila, through Lande-Arnold regression estimation of a parametric non-linear response surface (Lee et al. 2008). Responses were compared by using partial F tests based on pair fitness components. In the same paper however, non-parametric thin-plate splines were fitted for data visualization, as seen in Figure 3.6.

Non-parametric regression allowed the shape and form of the predictor used to create the surface to not be confined, but rather derived from the data itself. At this stage however, the non-parametric R script techniques ('fields' package in R) used were not appropriate to run statistical analysis with, and so they were simply used for visualisation. Both methods used multiple regression analysis, which

allows computation of selection coefficients that have clear biological interpretations about forces of selection (Palacio et al. 2014). Generalised additive models (GAMs) with thin-plate splines using the 'mgcv' package in R was the next major step in the evolution of the GF, because this was able to perform log-likelihood testing to compare nested models, allowing the response surface to be analysed for direct effects, two-way interactions and three-way interactions, as needed (Wood 2003; Wood 2006; Wood 2011). GAMs are statistical models where the relationship between predictors and response variables are not constrained by the assumption of a normal distribution, and are instead generalised to encompass linear, non-linear, and even additive models. The only drawback of this approach was that it required larger sample sizes compared to standard parametric regression models, because the data not only was used for analysis, but to identify the appropriate parameter, or predictor model itself. This development in statistical protocol, while not necessarily a pre-requisite for working on herbivorous insects; for which protein and carbohydrate are generally considered the two main energy-yielding axes; allowed much greater analytic power when using the third nutritional dimension of fat in the cases of omnivorous mammals. This propelled the GF from being applied primarily on insect models, to a wider range of organisms including humans (Raubenheimer et al. 2016).

Finally, we arrive at the latest iteration of the GF, which has been used to publish multiple papers in recent years, including the breakthrough mammalian results from a large scale dietary study in mice, which showed that macronutrient ratios were more important than caloric intake in mediating the lifespan and health benefits of CR (Solon-Biet et al. 2014). An example is reproduced here in Figure 3.7, with table of statistical significance attached. While it is possible to analyse this response surfaces relative to statistical analysis alone, the directionality of a correlation can be better understood through visualisation of colour (i.e. upregulation in red vs. downregulation in blue in the example below). Interpretation of these response surfaces depends largely on the relationship between the topographical gradient lines, or isoclines, and the macronutrient axes.



p-value	P	C	F	P:C	P:F	C:F
mTOR	**0.03**	0.48	0.53	0.08	0.70	0.54

Figure 3.7. 2D GF response surfaces correlating mTOR activation to macronutrient intake. Figure generated using custom GF script in R. Data previously published in (Solon-Biet et al. 2014). Full analysis of this result is presented and discussed in Chapter 4.

One of the few drawbacks of this method was that each of the three slices that are used to visually represent the information are cut through the median of the third axis. For instance, in the left-most slice of Figure 3.7, the Protein vs. Carbohydrate plot, the slice is taken through the median of the Fat axis, indicated by the subtitle (Fat: 11). This means that while the statistical analysis may have been relatively sound, the visual representation may not present the full picture, since the visualisation is performed through slices at median points, which may have missed the key findings. In this chapter, the application of the GF is taken one step further, to a 3-dimensional visualisation (technically 4-dimensional, as the overlaid topographical heatmap represents the response axis). This too is imperfect as the topographical map is highly dependent on the number of data points in the 3-dimensional nutritional space, making the surface 'bumpy', and raising questions about what degree of mathematical smoothing is required to fit the data. An example of this visualisation is shown in Figure 3.8, and is the 3-dimensional correlate of the 2-dimensional mTOR surfaces presented in Figure 3.7.

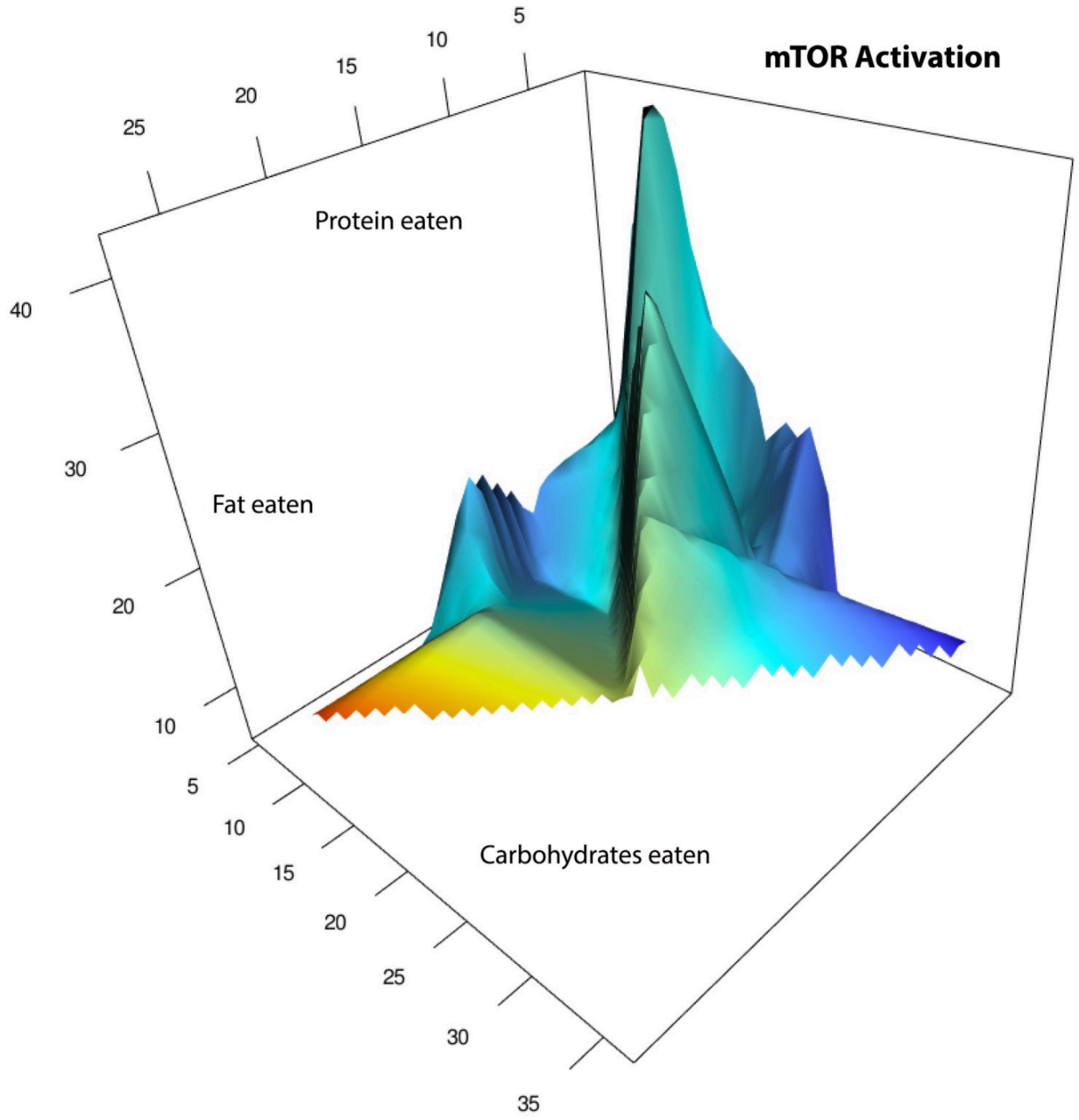


Figure 3.8. 3D GF response surfaces correlating mTOR activation to macronutrient intake. Figure generated using custom GF script in R. Here, peaks and valleys in the nutritional space represent dietary compositions, with colours at each point representing response output; in this case mTOR activation. The red areas represent highest mTOR activation, while the blue areas represent lowest mTOR activation. The 2D correlate of this plot is shown in Figure 3.7, as slices cut orthogonally to one dimension through its median point (Fat: 11, Carbohydrate: 13, Protein: 10).

New developments in statistical analysis of data through the GF

The 'mgcv' package implementation of the GAM function fits thin-plate penalised regression splines to the data, using smooth functions designed to be optimal, in this case through a Laplace approximation to the Restricted Maximum Likelihood (REML) statistic (Wood 2011). This package is unique to other GAM packages in R, as it uses:

1. Estimation of degree of smoothness is part of model fitting
2. A Bayesian approach to variance estimation is used, improving confidence interval calculation
3. Penalisation on the calculated parameters to prevent over fitting, and improve efficiency
4. A highly customisable framework to allow different methods and models of fitting

Here, an example set of data is used to demonstrate the full use of the current iteration of the GF, with detailed explanations of data handling methods, and use of R packages. All code, data visualization, and statistical modeling is handled through the R language, using R Studio as the graphical user interface (GUI).

Numerous packages were used in this application of the GF, whose names, versions and associated packages are listed here using the 'sessionInfo()' function:

```
> sessionInfo()
R version 3.3.1 (2016-06-21)
Platform: x86_64-apple-darwin13.4.0 (64-bit)
Running under: OS X 10.11.6 (El Capitan)
locale:
[1] en_AU.UTF-8/en_AU.UTF-8/en_AU.UTF-8/C/en_AU.UTF-8/en_AU.UTF-8
attached base packages:
[1] grid      stats     graphics  grDevices utils     datasets  methods   base
```

```
other attached packages:

[1] Hmisc_4.0-2     ggplot2_2.2.1   Formula_1.2-1   corrplot_0.77  fields_8.4-1
maps_3.1.1      spam_1.4-0      akima_0.5-12    rgl_0.96.0

[10] plot3D_1.1      survival_2.40-1 ellipse_0.3-8   lattice_0.20-34 sp_1.2-3
mgcv_1.8-15     nlme_3.1-128    xtable_1.8-2

loaded via a namespace (and not attached):

[1] Rcpp_0.12.7         RColorBrewer_1.1-2  plyr_1.8.4          base64enc_0.1-3
tools_3.3.1         rpart_4.1-10        digest_0.6.10

[8] checkmate_1.8.2     htmlTable_1.9       jsonlite_1.1        tibble_1.2
gtable_0.2.0        Matrix_1.2-7.1      shiny_0.14.1

[15] gridExtra_2.2.1     stringr_1.1.0       cluster_2.0.5       knitr_1.14
htmlwidgets_0.7     nnet_7.3-12         data.table_1.10.4

[22] R6_2.2.0            foreign_0.8-67      latticeExtra_0.6-28 magrittr_1.5
backports_1.0.5     scales_0.4.1        htmltools_0.3.5

[29] splines_3.3.1       assertthat_0.1      misc3d_0.8-4        mime_0.5
colorspace_1.2-7    httpuv_1.3.3        stringi_1.1.2
```

In this R environment, after the appropriate packages have been loaded, data sets such as those collected during experimentation are loaded into data frames in R. In this example, the data collected is from the large-scale dietary study, and in particular contains values of circulating amino acids (AA's), as well as the sum of the total branch chain amino acids (BCAA Sum) (Solon-Biet et al. 2014). Results of analysis are not discussed, as this chapter is focused on the coding methods used in the development of this thesis. This data set is set out as follows, with examples used in each line:

eaten.P	**eaten.C**	**eaten.F**	**Sample ID**	**Sex**	**X_1**	**X_2**	**…**	**X_n**
17.98	5.99	5.99	101	M	1	0.42	…	205.12
14.75	4.91	4.91	142	F	2	0.81	…	586.34
4.72	9.77	19.20	402	F	3	3.12	…	1094.91
…	…	…	…	…	…	…	…	…

Table 3.2. Example of a generic data set with outcome measures $X_{1\text{-}n}$ and input macronutrient intakes.

Data is first stored in comma separated value (.csv) format, and loaded into data frames as follows:

```
> aveintake <-read.csv(paste(directory,filename,".csv",sep=""),1)
```

Here, the directory and filename variables are assigned prior to loading of the data, in order to improve ease of access to files, and making bulk script uses (for multiple files/folders) much easier:

```
> directory <- "/Users/Master/Dropbox/PhD Work/GF R SCRIPTS/Data/"
> filename <- "aas"                                # Name of the file - In this case aas.csv
```

Automation of the script can be performed by the following function:

```
> filelist <- list.files(directory,pattern=".csv")
> for(y in 1:length(filelist))
> filename <- filelist[y]
```

At the same time, the column in which the outcomes start is defined by the 'starts' variable:

```
> starts <- 6                                          # Column at which the data starts at
```

Additionally, analysis outputs can be saved automatically to a specified location, and the choice of 2D sliced GF plots, 3D GF plots and heatmap correlations between data columns can be saved as pdfs. The save location directory is also created at this point, and choice is carried out by an 'if' logical:

```
> saveloc <- paste("/Users/Master/Dropbox/PhD Work/GF SCRIPTS/GF/",filename,"/",sep="")
> dir.create(saveloc)
> pdfs <- "on"                                          # pdfs of GF plots ("on" or "off")
> pdf3d <- "on"                                        # pdf of 3D GF plot ("on" or "off")
> heatmap <- "on"                              # pdf of heatmap correlations ("on" or "off")
> if(pdfs=="on"){
> if(pdfs=="on"){
> if(heatmap=="on"){
```

Once the data is loaded, it will appear as follows: only columns 1-7 displayed here):

```
> head(aveintake[,1:7])
    eaten.P   eaten.C   eaten.F Sample.ID Sex Body.weight..g. Alanine..A.
1 17.983838  5.985079  5.985079       101   M           24.22      12.394
2 14.745112  4.907220  4.907220       102   F           22.02      20.813
3  4.724988  9.767927 19.197779       103   M           23.67      53.983
4  7.776437 12.826281 12.826281       104   M           23.97      35.197
5  9.526251 13.834762  5.765345       105   F           23.15      34.806
6 11.030679  6.675834 16.019608       106   F           24.06      41.357
```

Next, the topographical colour map used in the response surface is formatted with variables that will be passed onto the plotting algorithms later on in the code:

```
> rgb.palette<-colorRampPalette(c("blue","cyan","yellow","red"),
+ space="Lab",interpolate="linear")
> no.cols<-256
> no.cols3d<-15889
> gr<-101
> rg<-15889
```

Two custom functions are now created, one to handle the response surface data frame to match the required topographical colour map, and one to correlate the correlation matrix:

```
#++++++++++++++++++++++++++
# Computing the topographical response surface
#++++++++++++++++++++++++++
# x : input variable of x-axis as data array
# y: input variable of y-axis data array
# rgnames: regulated names as data array

> findConvex<-function(x,y,rgnames){
```

```
+   hull<-cbind(x,y)[chull(cbind(x,y)),]
+   px<-pretty(x)
+   py<-pretty(y)
+   x.new<-seq(min(px),max(px),len=gr)
+   y.new<-seq(min(py),max(py),len=gr)
+   ingrid<-as.data.frame(expand.grid(x.new,y.new))
+   Fgrid<-ingrid
+   Fgrid[(point.in.polygon(ingrid[,1], ingrid[,2], hull[,1],hull[,2])==0),]<-NA
+   names(Fgrid)<-rgnames
+   return(Fgrid)
+ }
```

This first function takes the x-axis and y-axis input variables and sequences the topographical map in a segmented fashion, creating a data matrix that can be passed to the graphical algorithms used later. Next, the correlation matrix function is called. The code is a customised adaptation of:

```
> source("http://www.sthda.com/upload/rquery_cormat.r")
```

After these pre-processes are complete, the data are able to start being processed. The first step to do this, is to call the custom 'findConvex' function, to compute the required data frames (df2) from the three macronutrient axes in the data set; "eaten.P", "eaten.C", and "eaten.F":

```
> df2<-list()
> df2[[1]]<-findConvex(aveintake$eaten.P, aveintake$eaten.C,c("eaten.P","eaten.C"))
> df2[[1]]$eaten.F<-median(aveintake$eaten.F)
> df2[[2]]<-findConvex(aveintake$eaten.P, aveintake$eaten.F,c("eaten.P","eaten.F"))
> df2[[2]]$eaten.C<-median(aveintake$eaten.C)
> df2[[3]]<-findConvex(aveintake$eaten.C, aveintake$eaten.F,c("eaten.C","eaten.F"))
> df2[[3]]$eaten.P<-median(aveintake$eaten.P)
```

Computation of the GAMs can now begin, by creation of a 4-dimensional matrix, where the first three columns as the macronutrient inputs, and the fourth dimension is the response variable, which in this data frame is selected as a variable column 'h':

```
> for(h in starts:ncol(aveintake)){                # Run the process for all data columns
> aveintake2 <- aveintake[,c(1,2,3,h)]      # Macronutrients + response variable columns
> aveintake2 <- na.omit(aveintake2)             #Omit missing values from the data matrix
```

Here, the variable column h will travel between the values of 'starts', which is the first column we assigned earlier, and the total number of columns in the data set, calculated by the 'ncol' function. This was a significant development in the automation of the data processing capability of the GF script, as iterative loops allow processing of extremely large sets of data such as microarrays, as done in Chapter 4.

Next, the actual GAM is calculated using the 'gam' function in the mgcv package:

```
# rowN : the name of the response variable to be passed to the gam function
# bs: basis of smooth, in this case we use "tp", or thin-plate spline regression
# rgnames: regulated names as data array
# method: REML used here
> rowN <- colnames(aveintake2)[4]
> colnames(aveintake2)[4] <- "col1"
> gam1<-gam(col1~s(eaten.P,bs="tp")+s(eaten.C,bs="tp")+s(eaten.F,bs="tp")
+s(eaten.P,eaten.C,bs="tp") +s(eaten.P,eaten.F,bs="tp")
+s(eaten.C,eaten.F,bs="tp"),method="REML",data=aveintake2,select=TRUE)
```

The GAM data are now stored in the variable 'gam1', whose summary looks like:

```
> summary(gam1)                                          # Function used to give GAM output
Family: gaussian
Link function: identity
```

```
Formula:

col1 ~ s(eaten.P, bs = "tp") + s(eaten.C, bs = "tp") + s(eaten.F, bs = "tp") +
s(eaten.P, eaten.C, bs = "tp") + s(eaten.P, eaten.F, bs = "tp") + s(eaten.C, eaten.F,
bs = "tp")


Parametric coefficients:

            Estimate Std. Error t value Pr(>|t|)

(Intercept)  42.5719     0.9561   44.52   <2e-16 ***

---

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


Approximate significance of smooth terms:

                          edf Ref.df     F  p-value

s(eaten.P)          2.101e+00      8 1.762 0.000282 ***

s(eaten.C)          1.255e-03      8 0.000 0.382803

s(eaten.F)          6.702e-01      8 0.254 0.079892 .

s(eaten.P,eaten.C) 1.418e-01      3 0.051 0.288857

s(eaten.P,eaten.F) 6.501e-05      3 0.000 0.850069

s(eaten.C,eaten.F) 1.438e-04      3 0.000 0.781072

---

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


R-sq.(adj) =  0.136   Deviance explained = 15.4%

-REML = 548.62  Scale est. = 129.81    n = 142
```

In this example, the GAM was calculated on one the last column of the data, which was the BCAA sum data. The output of the GAM is used to determine the statistical significance of the response surface to each of the variable axes, in isolation, and in combination, as represented by the significance of the smooth terms above. Next, a function is used to capture these outputs in a stored data frame, which will be collated across all the response variables and saved as a text file. Here, the p-values from the current response are stored in ‘pvalH’, and the entire table is stored in ‘pval’:

```
> pvalH <- as.numeric(summary(gam1)$s.pv)                # Store the p-values as numerics
> pvalH[7] <- rowN                  # The column name is the name of the response variable
> pval <- rbind(pval,pvalH)              # Add the new p-values to the existing data frame
```

Smooth terms are created through the use of penalised regression splines whose parameters are selected by a Laplace approximation to REML, and an important aspect to discuss here is the way in which the p-values for smooth terms are derived. This is done based on an extension of Nychka's analysis of the frequentist properties of Bayesian confidence intervals for smoothing parameters (Marra & Wood 2012). What is therefore computed is a variant of the Bayesian, where the components of the test statistic are weighted by the iterative fitting weights, based on a likelihood ratio statistic (Wood 2011; Wood 2013). These p-values identify statistical significance to each macronutrient in isolation, and in combination.

The resulting table can be extracted from the gam data frame using the summary function, and can be tabulated as follows:

```
> summary(gam1)$s.pv
[1] 0.0002823811 0.3828032777 0.0798922369 0.2888569293 0.8500685076 0.7810717985
```

Table 3.3. GAM p-value outputs to macronutrient axes and ratios.

p-value	**P**	**C**	**F**	**P:C**	**P:F**	**C:F**
BCAA.Sum	**0.000282381**	0.382803278	0.079892237	0.288856929	0.850068508	0.781071798

2D visualisation of data through the Geometric Framework

Next, the data is passed from 'gam1' to the topographical map, by linking it to the 'df2' data frames that had been computed earlier, and now that the 2D graphs/slices are ready to be plotted, variables such as font sizes are allocated:

```
> fit1<-list()                                          # Set up the data frame as a list
> for(j in 1:3){
+ fit1[[j]]<-predict(gam1,newdata=df2[[j]])}                  # Link the data to the GAM
> fit2 <- predict(gam1,newdata=aveintake2)
> contourFontSize <- 0.4                                  # Contour label font size
> par(cex=1.0, cex.axis=1.5,cex.lab=1.2, cex.main=1.5, cex.sub=1)     # Plot label sizes
> mains1<-c(rowN,rowN,rowN)        # Labels for each slice are the response column label
> opr<-par(mfrow=c(1,3))                     # All 3 slices are plotted on the same frame
> map<-rgb.palette(no.cols)                                 # The colour gradient is set
```

Next, the medians of each axis are calculated and rounded to the nearest integer for display purposes. The max and min values are also calculated to create evenly distributed notches in the axes:

```
> sub1<-c(paste("(Fat: ",round(unique(df2[[1]]$eaten.F),0),")",sep=""),
+         paste("(Carbohydrate: ",round(unique(df2[[2]]$eaten.C),0),")",sep=""),
+         paste("(Protein: ",round(unique(df2[[3]]$eaten.P),0),")",sep=""))
> mn<-min(c(unlist(fit1[[1]]),unlist(fit1[[2]]),unlist(fit1[[3]])),na.rm=TRUE)
> mx<-max(c(unlist(fit1[[1]]),unlist(fit1[[2]]),unlist(fit1[[3]])),na.rm=TRUE)
```

The plots are finally created. This section of code is slightly inelegant, and could be improved through recursive loops, however the code runs relatively efficiently, so loops would only make the code look more presentable rather than necessarily functionally improving it:

```
> # 1st slice
> locs<-(range(unlist(fit1[[1]]),na.rm=TRUE)-mn)/(mx-mn)*no.cols
> surf<-matrix(fit1[[1]],nrow=sqrt(dim(df2[[1]])[1]))
```

```
> px<-pretty(aveintake$eaten.P)
> py<-pretty(aveintake$eaten.C)
> x.new<-seq(min(px),max(px),len=gr)
> y.new<-seq(min(py),max(py),len=gr)
> image(x.new,y.new,surf,col=map[locs[1]:locs[2]],xlab=labs1[1],ylab=labs1[2],
+ main=mains1[1],sub=sub1[1],axes=FALSE)
> axis(1)
> axis(2)
> contour(x.new,y.new,surf,add=TRUE,levels=pretty(range(mn,mx),nlev),
+ labcex=contourFontSize)
```

This is repeated three times, with changes to the appropriate variables where required for each slice. Thus the topographical response map is overlaid onto the nutritional surface, to create a 4-dimensional output, which is captured by slicing through the medians to produce 2-dimensional plots with the response variable represented by colour.

The resulting output is the response surface plot:

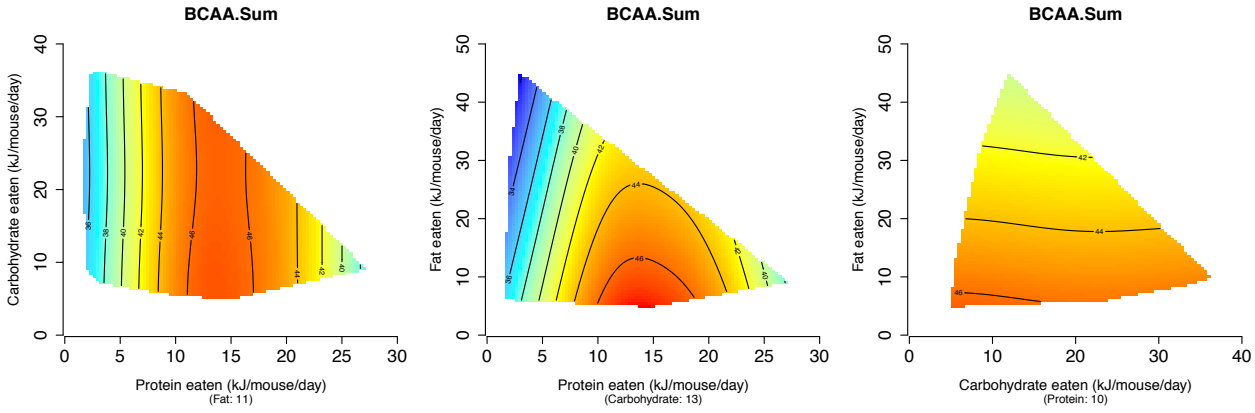


Figure 3.9. 2D GF response surfaces correlating total circulating BCAA's to macronutrient intake.

The GAM output can also be automatically captured in full as in a text file:

```
> txt1 <- file(paste(saveloc,q,s1[q],"/",rowN,".txt", sep=""))
> writeLines(capture.output(summary(gam1)),txt1)
> close(txt1)
```

Once the script has run through each response column, the total output of the p-value table is saved in two methods. The first is the table in full, with all p-values; and the second is the table with only statistically significant values (p-value < 0.05) displayed:

```
> write.table(pval, paste(saveloc,filename,"-pval.csv", sep=""), row.names = FALSE,
+ col.names = FALSE, sep=",")                                    # Full p-value table
> pvalsig <- pval                                  # Passes the table to a new data frame
> pvalsig[pvalsig>0.05] <- ""                        # Removes all non-significant values
> pvalsig[1,] <- head(pval,1)                       # Ensures the row titles are the same
> pvalsig[,1] <- pval[,1]                         # Ensures the column titles are the same
> write.table(pvalsig, paste(saveloc,filename,"-pvalsig.csv", sep=""), row.names = F,
+ col.names = F, sep=",")          # Writes the statistically significant p-value table
```

3D visualisation of data through the Geometric Framework

Next, the 4-dimensional output data is used to create a 3-dimensional plot with the response variable represented by colour. While 2D slices through medians of the 3rd axis, by being incomplete, have the potential to be visually misleading, the 3D plot produced in this section was developed to show the complete picture, which could be manipulated and moved around as an interactive plot. This section primarily uses the 'rgl' package, which is a 3D real-time rendering system for R, and the 'akima' package, which contains methods for interpolation of gridded splines to form the topographical map that is overlaid onto the 3D nutrient surface.

First, the data is passed to individual data matrices, which correspond to the macronutrient axes:

```
> aveintake2<- data.matrix(aveintake2)                # Data has to be in matrix format
> x1<- data.matrix(aveintake2[,1])
> y1<- data.matrix(aveintake2[,2])
> z1<- data.matrix(aveintake2[,3])
> s<- interp(x1,y1,z1)                 # Implements bivariate interpolation onto a grid
```

The 'interp' algorithm from the 'akima' package, implements bivariate interpolation of the irregularly spaced response surface data onto a grid, which can then be used as a topographical map for the nutritional surface (Akima 1978; Renka 1996). The data matrix 's' therefore contains the information needed for correlation with the pre-specified colour matrix. The colour matrix is now calculated, as before, however it now uses the 'interp' function rather than the 'findConvex' function:

```
> px<-pretty(x1)
> py<-pretty(y1)
> x.new<-data.matrix(seq(min(px),max(px),len=gr))
> y.new<-data.matrix(seq(min(py),max(py),len=gr))
> z.new<-data.matrix(seq(min(fit2),max(fit2),len=gr))
> surf<-data.matrix(fit2)                              # Data has to be in matrix format
> c1 <- interp(x1,y1,surf)          # interpolation data to match to the colour palette

> map<-rgb.palette(no.cols3d)                              # The 3D colour palette used
> cols <- heat.colors(no.cols3d)
> cuts <- with(c1, cut(z, breaks=no.cols3d))             # Creates the 3D contour matrix
```

The 'persp3d' function in the 'rgl' package is now used to plot the surface in a 3-dimensional space. This line of code integrates all aspects of the data collected so far, including the:

- Nutrient space
- Topographical 3-dimensional response surface
- Colour map with gradient matched to the responses

```
> with(s,persp3d(x,y,z,color=map[cuts],xlab="",ylab="",zlab="", main=NULL, box=F,
axes=T))
```

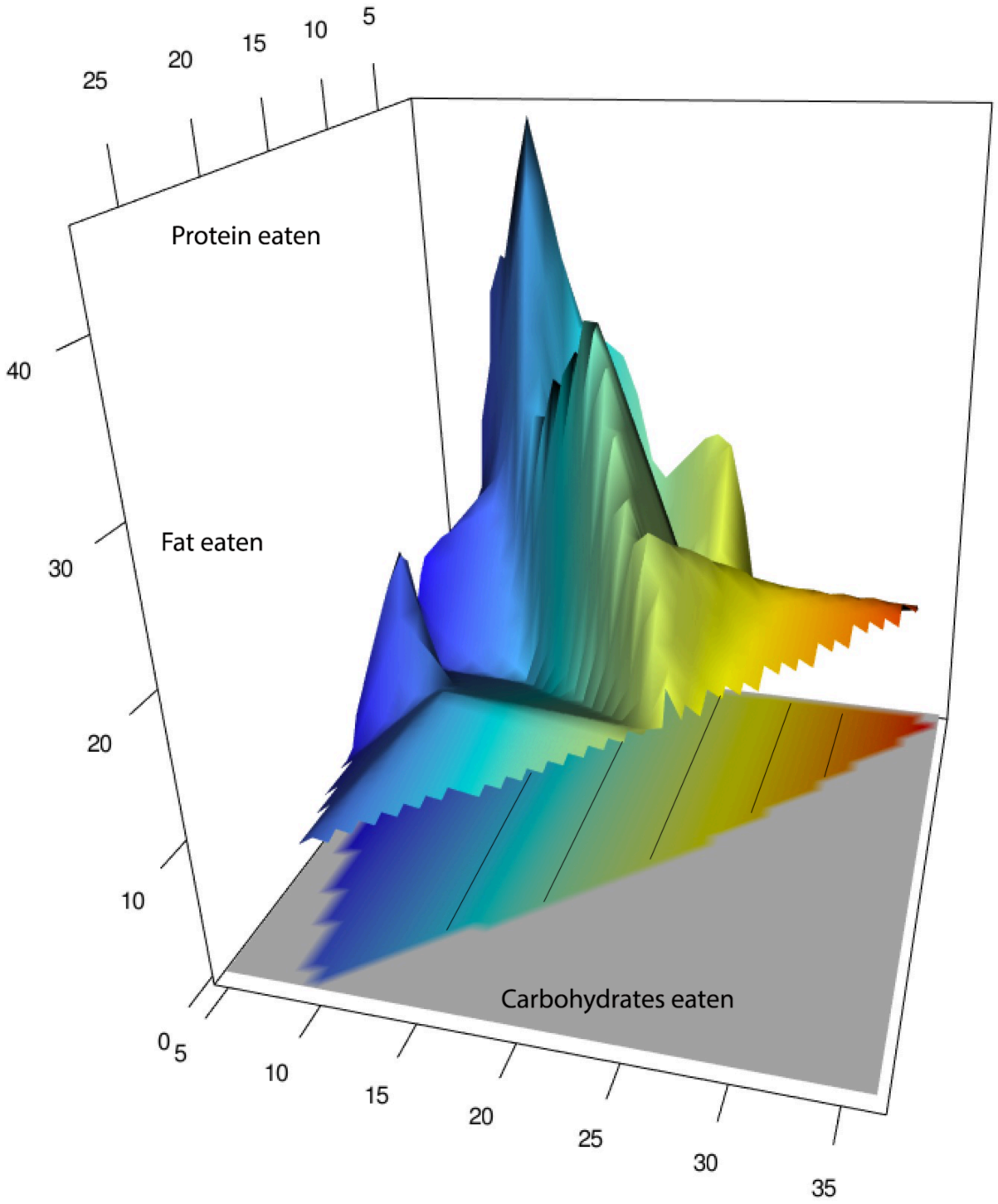


Figure 3.10. 3D response surface produced using the GF. Here, peaks and valleys in the nutritional space represent dietary compositions, with colours at each point representing response output. The red areas represent high response output, while the blue areas represent low response output. This has overlaid with the corresponding 2D slice through the median fat axis for illustration of how to read the 3D plot. In this case, the 2D plot shows that the isoclines are perpendicular to the carbohydrate axis, indicating that this response is driven primarily by carbohydrate intake.

Since the plot is angled arbitrarily, the viewpoint must be shifted by rotation. This can either be done manually, or by a preset value. Here, the optimal viewpoint was found by manual rotation, and the output was saved for future reference using the 'par3d' function:

```
> par3d()$userMatrix
           [,1]       [,2]        [,3] [,4]
[1,] -0.7052403  0.7044325 -0.08006757    0
[2,] -0.4247192 -0.3293558  0.84328991    0
[3,]  0.5676702  0.6287286  0.53146100    0
[4,]  0.0000000  0.0000000  0.00000000    1
```

This viewpoint however is only optimal for the current nutritional surface, and almost certainly require adjustment for optimization in other data sets. This is recalled using the 'view3d' function whose inputs 'pp' are the viewpoint matrix listed above:

```
> view3d(userMatrix=pp,zoom=zoom)
```

This raises two important considerations:

1. Determining the optimal viewpoint for any given surface:

The inherent draw back of a 3-dimensional visualisation is that it is not possible to view the entirety of the surface at one time. This is because optimizing one view is not possible without impacting the view of another part of the surface. Additionally, this method does not show the slice, or volume of the nutrient space, as it only provides a topographical view. For this reason, there isn't necessarily an optimal viewpoint from which to view the plot, thus reducing its publication value as a static image. On the other hand, through the method listed above, the plot generated by the rgl package in R is an interactive tool, allowing rotation and manipulation of the plot as required. Therefore, while the 3D plot may not be ideal for publication for all experimental frameworks, it is an extremely useful tool for interrogation of complex nutritional spaces in conjunction with statistics and 2D plots.

2. The degree of smoothing of the 3D surface required to appropriately interpret results:

Given that the plot is a topographical response surface overlaid onto the nutrient surface, the more complex the nutrient space, the more difficult it is to interpret the 3D plot. While the benefit of this visualisation is that it is able to show local maxima and minima to a much greater extent than 2D plots, the drawback is, as mentioned in consideration 1, that it does not display the interior volume of the nutritional space. This is akin to being able to visualise the surface of a pyramid, but not its interior. Therefore, as the number of non-surface data points in the experimental framework increase, the more difficult this visualisation becomes to interpret. While this problem is partially solved by analysing data in conjunction with statistics and 2D plots, it is certainly a critical limiting factor to 3D visualisation.

In addition, since the mgcv package in R predicts optimal degree of smoothing as part of the fitting, choosing appropriate smoothing parameters of the surface can also become an issue in some cases, and can lead to problems in calculation of p-values. Smoothing optimisation here is based on a trade-off between over- and under-fitting of the data set, which is computed through the estimation of smoothing parameters. Given that the p-values are calculated without considering uncertainty in the smoothing parameter estimates, high uncertainty in these estimates can cause artificially low p-values (Wood 2013). This especially occurs with nested smooths or with high concurvity in the model, where model parameter estimates and smoothing parameter estimates do not converge (Wood 2011). For this reason, the p-value calculations that are produced through the 3D interpolation are considered less accurate than those calculated through the 2D GAM, and the surfaces therefore must be considered in conjunction, making the 3D model unable to act as a stand alone analytical tool in most cases.

Specific add-ons to the GF script for analysis of data during this thesis

Gene expression analysis

Microarray data was processed using the 'affy', 'oligo', and 'limma' R packages from Bioconductor, and annotation, matching, clustering, and correction was performed using the 'pd.mogene.2.0.st', 'annotate', and 'mogene21sttranscriptcluster.db' database R packages from Bioconductor. Data was parsed into csv files, and processed through the GF script as described earlier. Once significance of smooth terms was calculated for each gene to macronutrient intakes and ratios, the data were analysed using a custom volcano plot script developed using the 'calibrate' package in R to find unbiased genes of interest. Unbiased genes of interest, and selected genes known to be involved in nutrient sensing pathways are identified and analysed in detail in Chapter 4. Since there are multiple variable-responses present for each macronutrient axis, a unidirectional volcano plot is formed for p-value versus fold change plots. For Pearson's correlations, a bivariate volcano plot is formed. Full volcano plot script details are provided below.

The 'calibrate' package was used for this script, as shown by the 'sessionInfo()' function:

```
> sessionInfo()
R version 3.3.1 (2016-06-21)
Platform: x86_64-apple-darwin13.4.0 (64-bit)
Running under: OS X 10.11.6 (El Capitan)
locale:
[1] en_AU.UTF-8/en_AU.UTF-8/en_AU.UTF-8/C/en_AU.UTF-8/en_AU.UTF-8

attached base packages:
[1] stats     graphics  grDevices utils     datasets  methods   base

other attached packages:
[1] calibrate_1.7.2 MASS_7.3-45
```

```
loaded via a namespace (and not attached):
[1] tools_3.3.1
```

Data is called from the comma separated value file, and loaded into data frames as follows:

```
> aveintake <-read.csv(paste(directory,filename,".csv",sep=""),1)
```

In this R environment, the data set used was the microarray data parsed into a comma separated value file as described above. Table 3.4 shows this with the first 4 genes. FC – fold change; log2fc – log base 2 of fold change; pval(x) – p-value calculated using the GF for each macronutrient, and ratio.

Table 3.4. Gene expression and statistical significance data set from microarray analysis.

Gene	FC	log2fc	pvalp	pvalc	pvalf	pvalpc	pvalpf	pvalcf
Serpinb3c	391.92	8.61	0.97	0.27	0.27	0.69	0.39	0.04
Gm8693	55.01	5.78	0.01	0.09	1.00	0.33	0.82	0.39
Zfp874b	51.25	5.68	0.68	0.56	0.06	0.66	0.38	0.68
Scgb2b12	37.67	5.24	0.01	0.36	0.39	0.75	0.42	0.21

The percentile cut off for fold change is set to find unbiased genes of interest (GOI). This value can be varied depending on the list of genes that are captured by the volcano plot.

```
> fcstat <- 0.99                    # Fold change cut-off value for volcano plot
```

Data can now be plotted using the standard 'plot' function in R, and labeling of genes is performed using the 'textxy' function in the 'calibrate' package. The statistical threshold for p-values was chosen at $p<0.05$. Log_2(fold change) was chosen for the x-axis as per volcano plot convention, while log_{10}(p-value) was chosen for the y-axis to limit axis size.

The resulting output is a unidirectional volcano plot, with genes of interest labeled by colour, as shown in Figure 3.10 below.

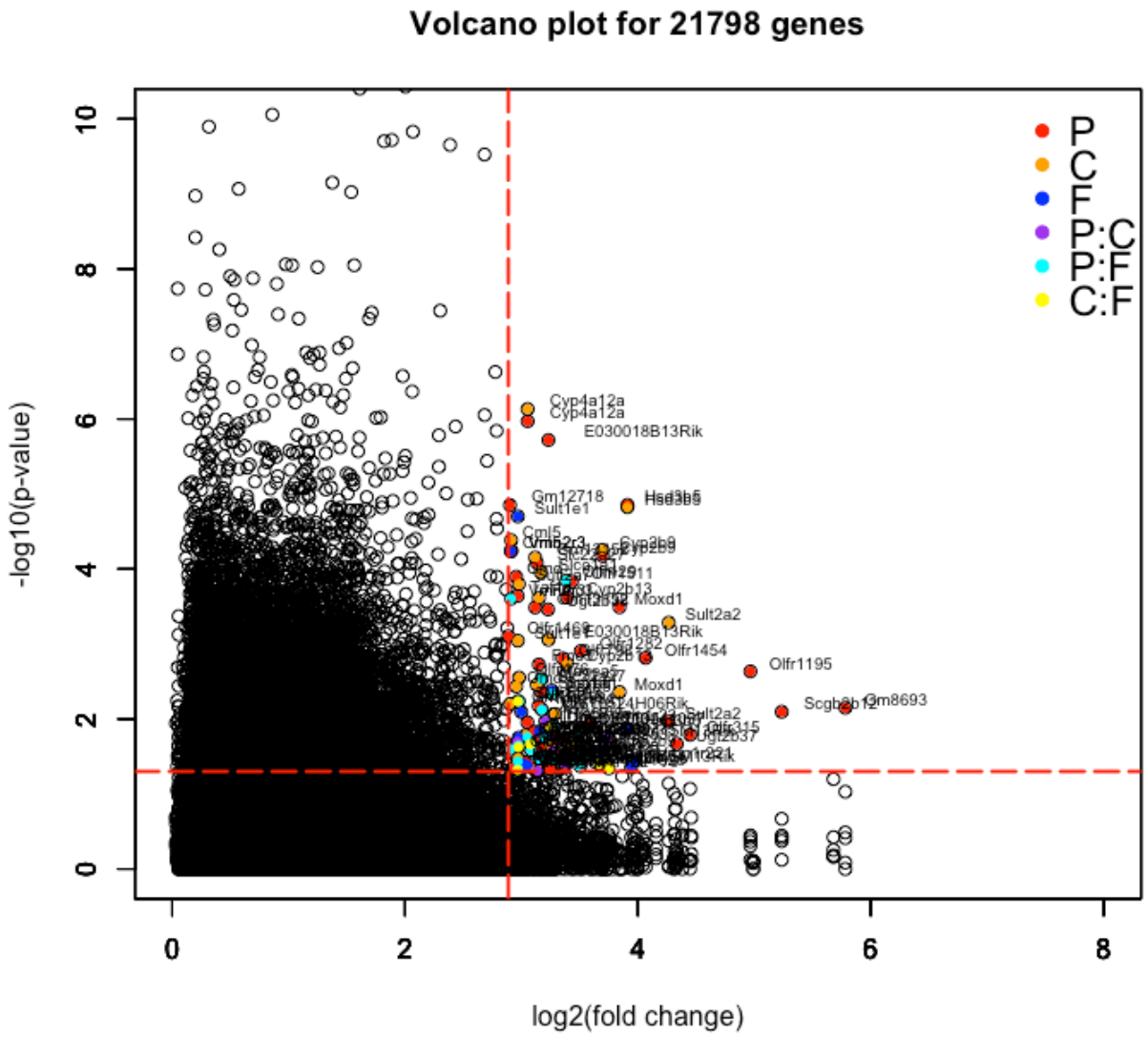


Figure 3.10. Example unidirectional volcano plot for unbiased genes of interest from a microarray. Outputs can be seen in Table 3.5

The output of this volcano plot can be tabulated to contain the fold change and p-values for genes of interest sorted by macronutrient to which they are significantly correlated [Table 3.5].

Table 3.5. Example output table for unbiased genes of interest, sorted by macronutrient correlation.

Gene	Fold Change	log2fc	pval	BY
Gm8693	55.00524782	5.781497362	0.007088124	P
Scgb2b12	37.66513381	5.23515775	0.008008181	P
Olfr1195	31.26318164	4.966392703	0.002319759	P
Olfr315	21.82934031	4.448196627	0.016244258	P
Ugt2b37	20.17806289	4.334715776	0.021455283	P
Slc22a27	8.764625436	3.131692438	0.003507577	C
Bcl2a1a	9.134831758	3.191378158	0.030501294	P:F
Apol7c	12.6910257	3.665736768	0.039717287	C:F

Correlation of data

Another additional tool developed to complement the GF script is a correlation matrix, used to compare various data sets. Here, the method of correlation can be varied depending on the data set used. For many data sets, the Pearson product-moment correlation coefficient can be used, when the responses are predicted to be linearly correlated with macronutrient intake. Additional correlations such as Spearman and Kendall rank correlations can be substituted if the correlations are not predicted to be linear, or there is significant skew in the data set. First, we can check the distribution of the example data sets, and their approximate correlation plots as follows:

```
> testset <- aveintake[,31:34]              # Example test set containing last 4 columns
> hist(testset)                                          # Creates histogram of the set
> plot(testset)                                               # Creates x-y plot of set
> bagplot(testset)          # Creates bagplot of set (only bagplot of BCAA shown below)
```

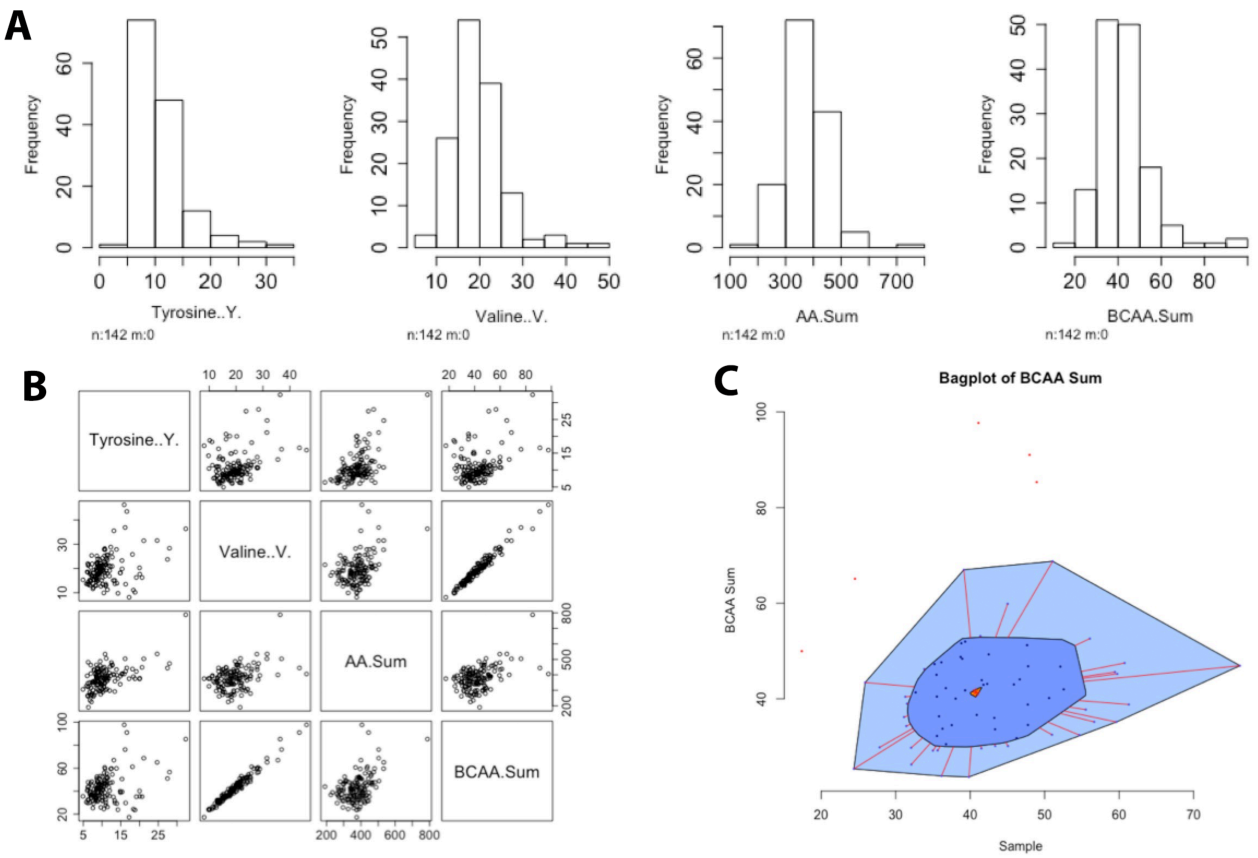


Figure 3.11. Data plots of an example data set as (a) histograms, (b) dotplots, (c) bagplots.

As we can see, the distributions are relatively normal, and the correlations can in some case be linear, as seen in Figure 3.11. The bagplot is a bivariate generalization of a boxplot, and is used to demonstrate the distribution of the data points. Here we see that Pearson's correlations would work best for the data set, as they are predicted to be linearly correlated.

The application of this method to data sets that are significantly correlated to a specific nutrient or ratio allows directionality to be quickly identified. The custom 'rquery.cormat' function (as described earlier in this chapter) can now be called to create a clustered correlation matrix – in this case, with non-significant correlations left blank. This can only be done with the subset of responses that we already know are correlated with a particular macronutrient intake or ratio, as a correlation between two unrelated response surfaces would not provide any data that can be appropriately interpreted:

```
> a2 <- aveintake[,starts:ncol(aveintake)]          # 'a2' contains all the response data
> ps <- pvalsig[c(1,ux),]       # 'ps' contains significant p-vals to a chosen axis 'ux'
> a3 <- data.frame(a2[,is.na(match(colnames(a2),ps[1,]))==0])       # Significant subset
> rquery.cormat(a2, labcex=0.5)                          # Creates the correlation matrix
```

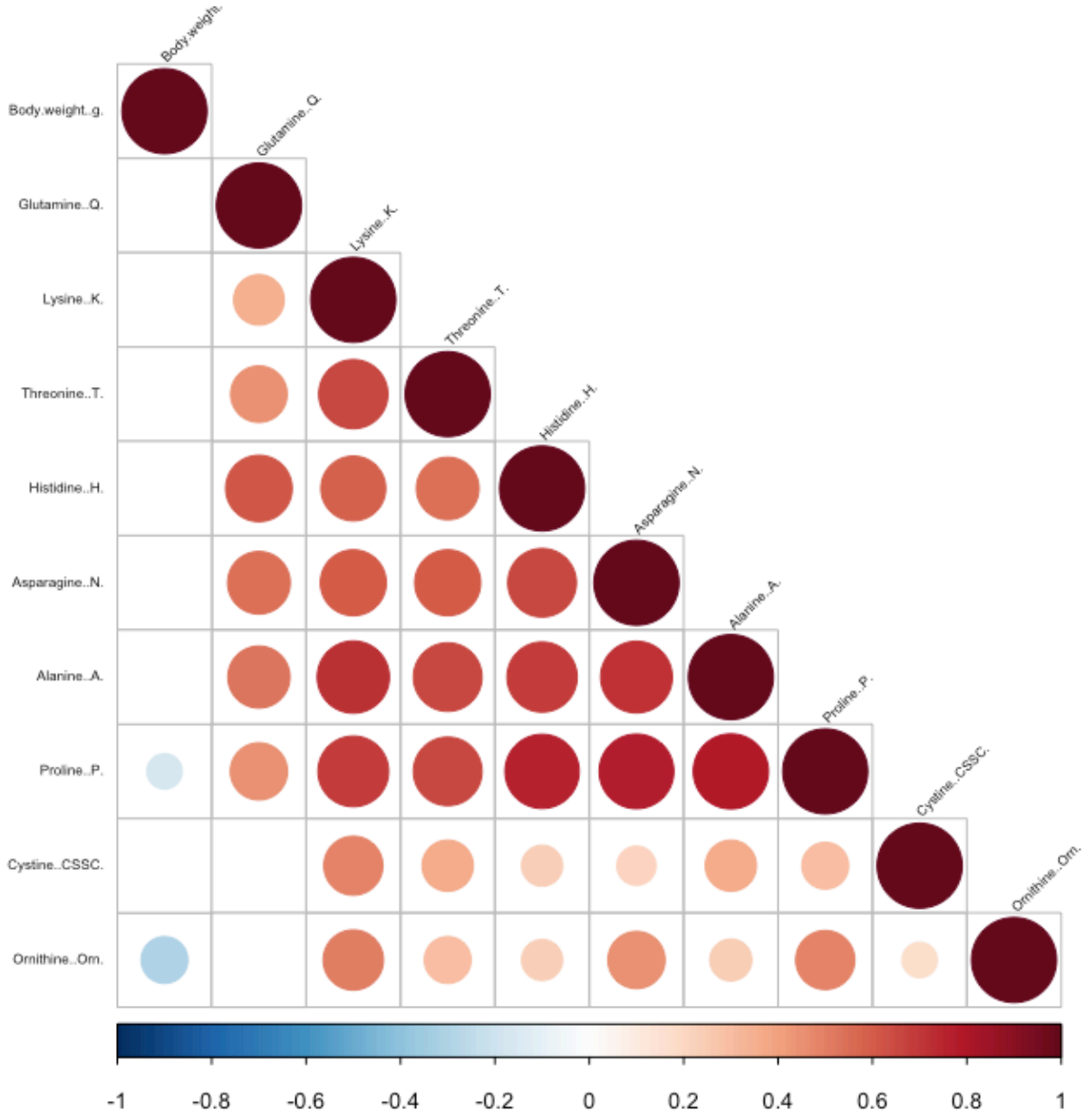


Figure 3.12. Correlation matrix of response variables that were significantly correlated to P:F ratio. Here we can see that almost all the responses are in the same direction with P:F ratio, with only body weight being correlated in the opposite direction to any amino acid result.

This matrix can now be used to check the correlations and directionality of the response variables. For instance, in this case, we can see that Ornithine is positively correlated with Lysine, but negatively correlated with body weight. Inspection of the 2D GF plots confirm this relationship, as shown in Figure 3.13, where the maxima and minima in Lysine and Ornithine localise to the same space, while the relationship is opposite between Ornithine and body weight.

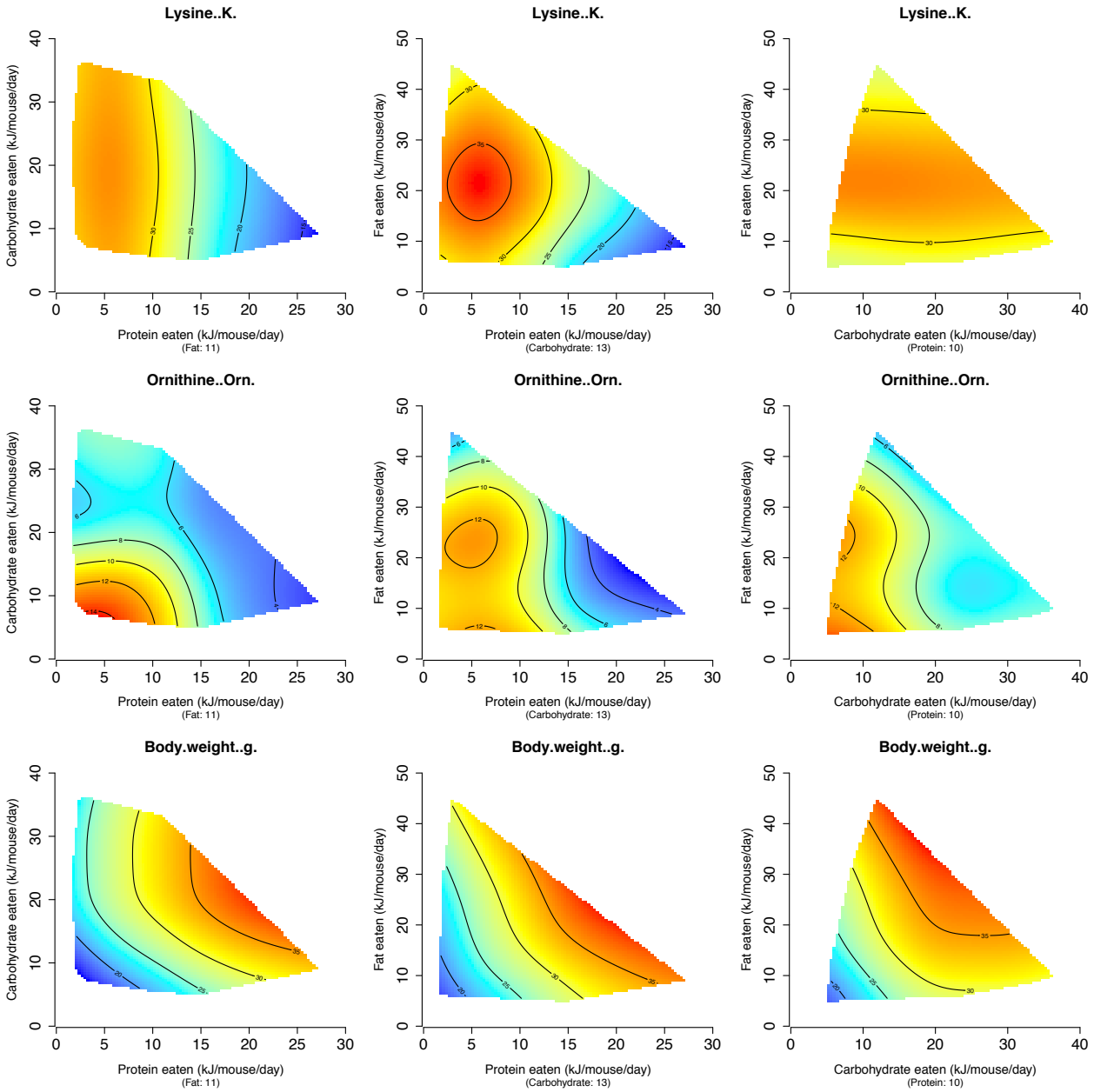


Figure 3.13. 2D GF surfaces correlating Lysine, Ornithine, and body weight to macronutrient intake.

General comments

The evolution of the geometric framework since the early 90's has allowed integration of evolutionary and mechanistic approaches to ageing research, by creating a unifying framework in which to analyse the seemingly disparate data (Raubenheimer et al. 2016). Despite this, wider application of the GF across other scientific fields has been limited, due to a lack in accessibility, particularly of a user-friendly functional interface, until the last decade with the use of R scripts. This has allowed complex response surface fitting and data visualisation methods to be applied on a large scale, allowing the GF to be propelled into the public sphere through its application in a variety of publications (Hew et al. 2016; Speakman et al. 2016; Wahl et al. 2016; Solon-Biet et al. 2016; Simpson et al. 2017a).

Here, we reviewed the progressive iterations of the GF since its inception, and have outlined developments made to the GF script as part of this thesis through example data sets. The problem in application of the GF to large data sets is addressed through automation using iterative loop scripts, and the discord between analysis and visualisation in 2D planes is aided by the creation of a 3D visualisation tool. Finally, additional tools used to analyse data such as gene regulation are outlined, and methods of data correlation are discussed.

Future work on this area should include methods to optimise the coding structures used to improve efficiency and computation. Additionally, a new algorithm could be developed which is able to analyse the local maxima and minima of multiple response surfaces to create a goal-seeking optimisation tool that could be used to answer questions such as "What is the optimal diet to maximise cardiovascular health, while minimizing liver fibrosis?" This would no doubt be an invaluable research tool, and could help broaden the horizons of the GF beyond nutritional and ageing research, to a much more widely encompassing experimental paradigm.

Chapter 4: The Impact of Diet on Nutrient Sensing Pathways

Excerpts from this chapter related to the liver have been published in (Gokarn et al. 2018); excerpts related to the hypothalamus are in review for submission; data related to mTOR activation have been published in (Solon-Biet et al. 2014).

Background

While caloric restriction (CR) has been shown to have the potential to extend lifespan, there is still uncertainty about the mechanisms for its effects. Debate also exists as to whether it is reduction of calories, or macronutrients that primarily mediates the effects of CR on ageing (Levine et al. 2014; Brown-Borg & Buffenstein 2016; Speakman et al. 2016; Le Couteur et al. 2015). Regardless of which of these is true, a common mechanism by which nutritional interventions influence ageing is related to the modulation of several nutrient sensing cellular growth pathways (Vijg & Campisi 2008; Le Couteur, McLachlan, et al. 2012b; López-Otín et al. 2016). Experimental modulation of these pathways; mTOR, AMPK, IIS and sirtuins; have been shown to influence lifespan and health in a variety of taxa, through their effects on cellular processes such as metabolism, mitochondrial biogenesis, autophagy, gene expression, growth, replication, and other pleiotropic signaling effects [Figure 4.1] (Houtkooper et al. 2012; Hofmann et al. 2015; Baur et al. 2006; Longo et al. 2015; Junnila et al. 2013; Zoncu et al. 2010; Finkel 2015; Pazoki-Toroudi et al. 2016).

In humans however, the evidence for CR is not as robust as in other model organisms, partly due to the difficulty in implementing long-standing dietary regimens and subsequently measuring their impact on lifespan. To circumvent these issues of 1) CR vs. macronutrients, and 2) the difficulty of implementing long-term nutritional strategies in humans, modulation of these nutrient sensing

pathways, which are largely evolutionarily conserved, seem to be a robust method to investigate translatable therapeutics (Longo et al. 2015; da Costa et al. 2016; Partridge 2012). This can be achieved through either ad libitum diets varying in macronutrient and caloric ratio, or with pharmaceutical agents and gene mutations directly targeting the nutrient sensing pathways.

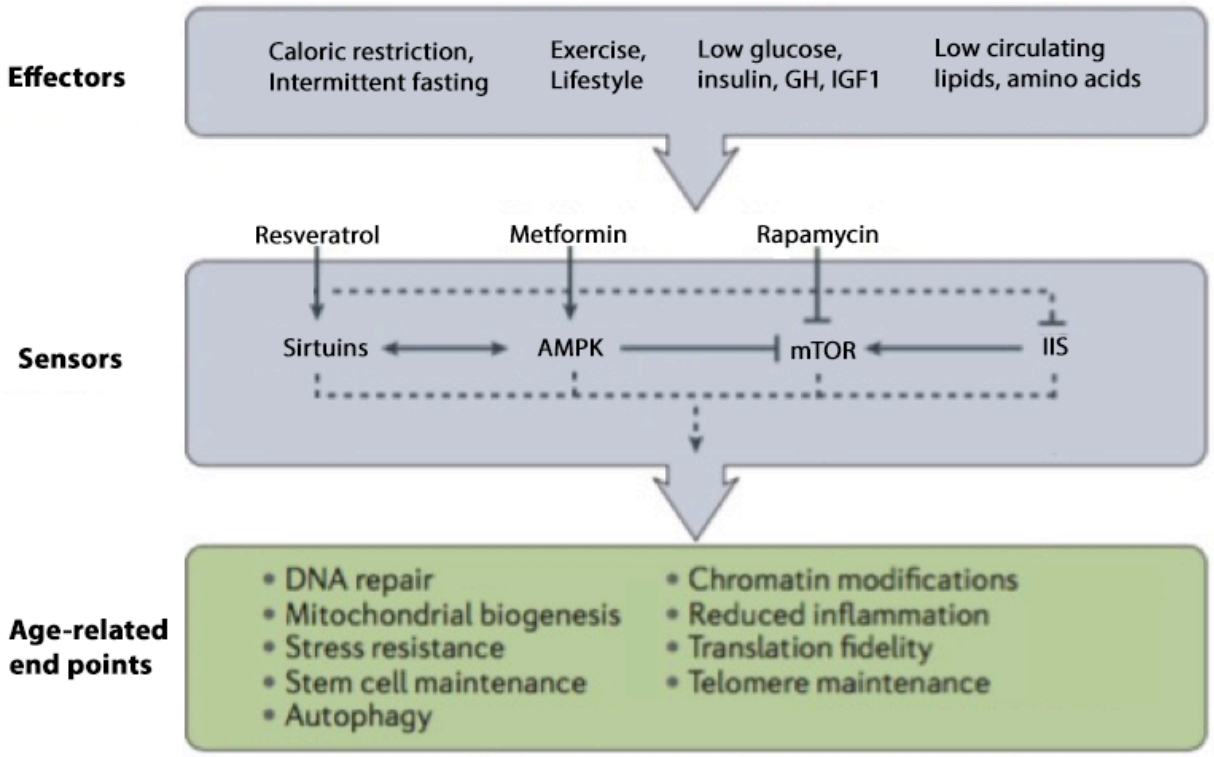


Figure 4.1. The four major nutrient sensing pathways (Sirtuins, AMPK, mTOR, and IIS) and their relationship with age-related end points. Figure adapted from (Bonkowski & Sinclair 2016).

As yet, there have not been any reports of the effects of low protein, high carbohydrate diets on gene expression, nor the long-term effects of dietary macronutrients on gene expression. The liver is the primary organ that responds to nutrition, and regulates systemic metabolic responses to diet. Therefore hepatic gene expression is a strong candidate to be responsive to dietary perturbations, and it is hypothesised that protein will be the key driver of gene expression. There have been many, mostly short-term studies of the effects of diet on the hepatic transcriptome that have identified a wide range of genes and pathways that are influenced by nutrition and these are mostly involved with the regulation of the metabolism of nutrients and energy (Osada 2013; Schmucker 1998; Dhahbi et al.

2005; Schwarz et al. 2012). There has also been increasing interest in discovering which genes and pathways mediate this response, partly because this can provide a platform for the discovery of pharmacological agents for delaying aging (Benayoun et al. 2015; Le Couteur et al. 2012b). Key nutrient sensing pathways that are thought to influence aging include mTOR, SIRT1, insulin/IGF-1 and AMPK, and more recently FGF21 (Bishop & Guarente 2007; Solon-Biet et al. 2015b; Solon-Biet et al. 2016). The effects of caloric restriction on mammalian gene expression has also been undertaken to provide data-driven insights into pathways and genes that influence healthy aging (Swindell 2009; Plank et al. 2012). In one meta-analysis of over 50 animal studies, it was found that caloric restriction is associated with overexpression of 101 genes and underexpression of another 73 genes in the liver. Pathways affected by caloric restriction included growth hormone signaling, lipid metabolism and immune responses (Plank et al. 2012). Another similar meta-analysis identified genes associated with oxidative stress, inflammation and tumorigenesis in 22 different tissue types in mice (Swindell 2009).

The hypothalamus also plays a key role in mediating the effects of diet on the body, since it is not only involved in food intake, but also in the neuroendocrine interactions that mediate functions such as growth, reproduction and metabolism (Waterson & Horvath 2015). By virtue of its location, the hypothalamic arcuate nucleus is able to use fenestrated capillaries that make up the blood brain barrier to sense nutrient and hormonal signals, and coordinate responses through orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) and anorexigenic pro-opiomelanocortin (POMC) neuronal feedback loops (Timper & Brüning 2017). In addition, mutations in the POMC, brain-derived neurotrophic factor (BDNF) and melanocortin-4-receptor (MC4R) genes have been shown to result in increased appetite and obesity in both humans and rodents (Krude et al. 1998; Yeo & Heisler 2012). Both CR and intermittent fasting, have also been shown to cause behavioural fluctuations in mice, with significant changes to gene expression in the central nervous system (CNS), varying across the prefrontal cortex, amygdala and hypothalamus (Yamamoto et al. 2009). It is therefore

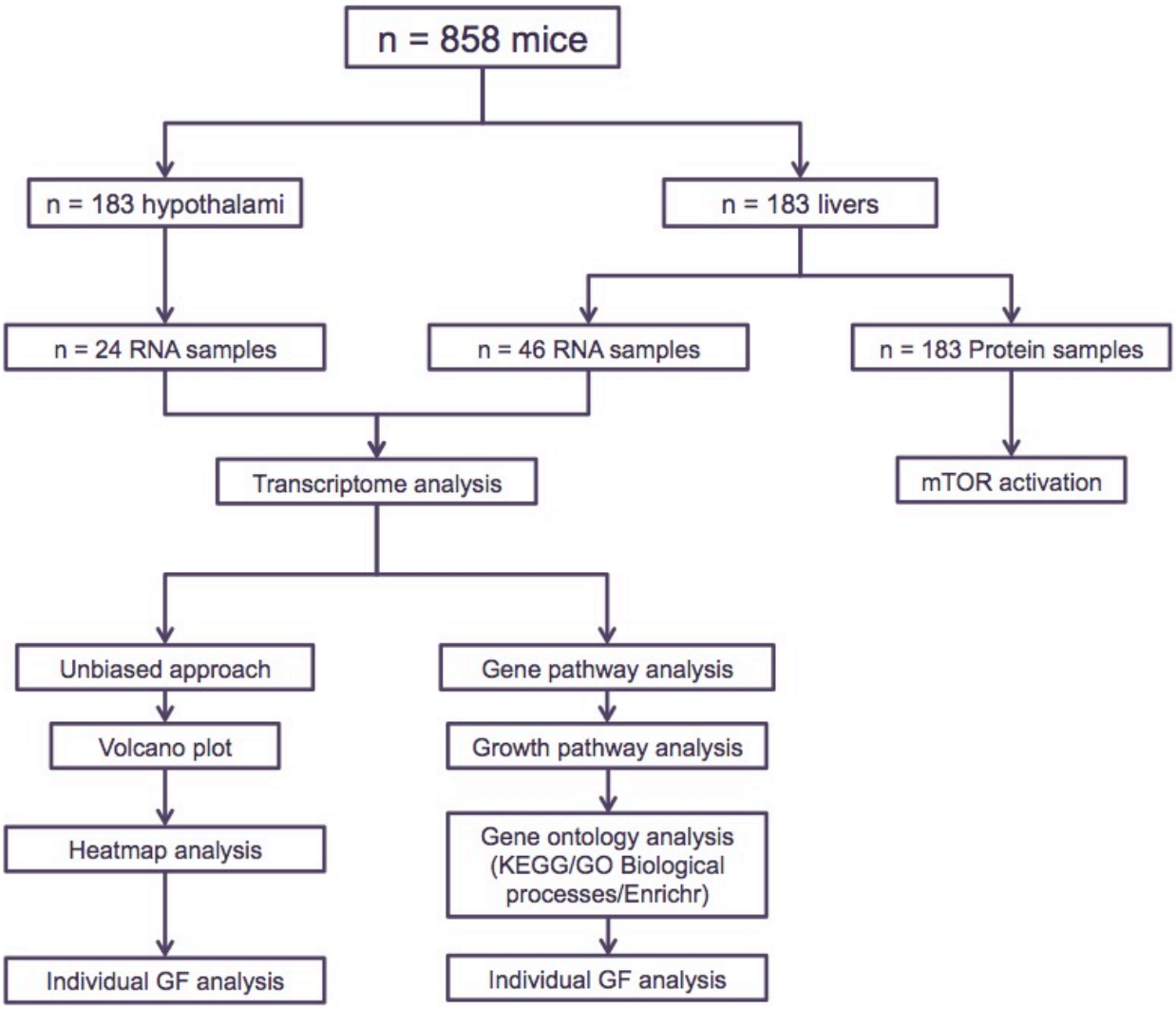
hypothesised that energy intake will be a key driver of gene expression in the hypothalamus. Several meta-analyses on genome-wide association studies (GWAS) have also shown multiple single nucleotide polymorphisms (SNPs) in hypothalamic genes which are associated with high BMI and obesity (Speliotes et al. 2010; Xu & Xie 2016). While GWAS and quantitative trail loci (QTL) mapping are the most common methods used to investigate the genetic basis for energy and macronutrient intake, so far almost no genetic associations have been found that correspond to longevity (Broer & van Duijn 2015; Yashin et al. 2015; Collaku et al. 2004). This limits the translation of findings from animal model GWAS, since even though dietary studies can be performed in a much more accurate manner in animal laboratory conditions, results do not necessarily have an immediate correlate to humans. On the other hand, more targeted molecular techniques such as microarray, polymerase chain reactions (PCR) and western blotting have been used to good effect in determining common genes and pathways that are relevant to humans (Bonkowski & Sinclair 2016; Han & Hickey 2005; Razi et al. 2017; Dhahbi et al. 2005). Given the multitude of roles of the liver and hypothalamus on ageing, food intake, metabolism and health, investigations were undertaken here to examine the effects of protein, carbohydrate and fat on hepatic and hypothalamic gene expression in ageing mice maintained on lifelong ad libitum diets varying in the ratios of these macronutrients. To analyse the impact of both individual macronutrients and ratios, the Geometric Framework (GF) was applied to this experimental design, as previously described (Simpson et al. 2015; Simpson & Raubenheimer 2007). Here, the GF was used to study the effects of varying dietary composition on these nutrient sensing pathways using tissue collected from a large-scale dietary study in mice. This was primarily performed through interrogation of genetic expression of the pathways and their downstream targets, through the use of microarray and PCR assays on hepatic and hypothalamic tissue. In addition, direct measures were performed to validate the gene expression data through measures of circulating protein and western blot analyses. These sets of data were then correlated with each other, and lifespan data collected from the same cohort, to determine their correlation with the ageing process.

Methods

Chapters 2 and 3 describe the details of the methods used in this chapter. The work done in this thesis primarily focused on extraction of DNA, RNA and protein from tissue samples, performing assays (microarray, PCR, western blots), development of data analysis tools (R scripts and frameworks) and data analysis. Briefly, three-week-old male and female mice (C57Bl6/J, n=858) were ad libitum-fed one of 25 experimental diets varying in protein, carbohydrate, fat and energy content. Energy manipulations were achieved by addition of cellulose generating low, medium and high energy density diets (8, 13, and 17 kJ/g) [Table 4.1]. At 15 months of age, one cohort of mice (n=183) spanning the diets was euthanised and tissues collected, while the remaining mice were maintained for lifespan analysis.

Frozen liver (n=183) and hypothalamic (n=24) tissue samples from mice at 15 months of age were sectioned into 10mg blocks, and DNA, RNA, and protein were extracted using the Qiagen AllPrep Mini Kit. Isolated RNA (n=24 male/n=22 female from the liver, n=24 male from the hypothalamus [See Table 4.1]) was analysed using Affymetrix Mouse Gene ST arrays at the Ramaciotti Centre for Genomics, University of New South Wales; GEO: GSE85998 (Solon-Biet et al. 2016). Gene expression data were normalised to actin expression. Liver samples that had undergone microarray assays were run on qPCR plates using the RT2 profiler array using the Mouse Insulin Signaling Pathway kit (Qiagen). A pooled sample was serially diluted, and as a standard curve in the Fluidigm Biomark software for PCR analysis. Protein pellets were resolubilised and used to run western blots for mTOR and phosphorylated-mTOR.

A flowchart of the methods performed in this chapter are outlined below:



Data analyses were based on macronutrient intake (kJ/mouse/day). The relationship between macronutrients and gene expression was determined using two methods. First, the correlation between the intake of each macronutrient and the expression of each gene was calculated using a Pearson's correlation coefficient. Second, the Geometric Framework approach was used where the statistical significance of the relationships between each gene and macronutrients and the interactions between macronutrients was calculated with Generalised Additive Models (GAMs). In addition to the GAM statistics (GAMS), graphical representation of the relationship between macronutrients and gene expression was assessed for some genes of interest using the GF as described in Chapter 3.

Genes of interest were determined from the P values returned by each of these methods and application of Benjamini Hochberg correction with a FDR of 0.05. Heatmaps were performed in R using gplots package. Gene enrichment analysis was performed in Enrichr to determine gene pathways based on the KEGG database and biological pathways based on the GO Biological Processes 2017 (Kuleshov et al. 2016). Only pathways and processes with more than two genes represented and an adjusted P value <0.05 were considered to be potentially significant. Once significance of smooth terms was calculated for each gene to macronutrient intakes and ratios, the data was analysed using a custom volcano plot script developed using the 'calibrate' package in R to find unbiased genes of interest (GOI). Unbiased genes of interest, and selected genes known to be involved in nutrient sensing pathways were identified and analysed in more detail using the GF script.

Table 4.1. Sex, diets and dietary intakes of mice included in transcriptome analysis of liver.

Sex	Protein eaten	Carbohydrates eaten	Fat eaten	Energy density	Protein/Carbohydrate /Fat ratio
M	25.82	8.59	8.59	High	60/20/20
F	22.04	7.33	7.33	High	60/20/20
M	2.44	36.49	9.73	High	5/75/20
F	2.02	30.19	8.05	High	5/75/20
M	2.13	8.5	31.89	High	5/20/75
F	2.75	10.98	41.18	High	5/20/75
M	16.19	23.51	9.79	High	33/48/20
F	2.45	23.49	23.49	High	5/48/48
F	6.72	13.9	27.32	High	14/29/57
F	5.51	22.39	11.39	High	14/57/29
M	12.48	20.59	20.59	High	23/38/38
M	2.21	33.06	8.82	High	5/75/20
F	13.01	7.87	18.89	High	33/20/48
F	6.11	12.64	24.84	High	14/29/57
M	25.77	8.58	8.58	High	60/20/20

M	15.67	22.76	9.48	High	33/48/20
F	11.8	7.14	17.14	High	33/20/48
M	2.05	19.61	19.61	High	5/48/48
M	8.19	16.94	33.3	High	14/29/57
M	6.53	13.51	26.56	High	14/29/57
M	9.58	15.81	15.81	High	23/38/38
F	7.46	12.31	12.31	High	23/38/38
M	1.92	28.8	7.68	High	5/75/20
M	2.3	22.03	22.03	High	5/48/48
F	2.34	22.44	22.44	High	5/48/48
M	5.95	24.17	12.3	High	14/57/29
M	14.85	10.23	10.23	High	42/29/29
M	7.78	12.83	12.83	Low	23/38/38
M	16.61	5.53	5.53	Low	60/20/20
F	15.15	5.04	5.04	Low	60/20/20
F	7.24	11.95	11.95	Low	23/38/38
F	13.8	9.51	9.51	Low	42/29/29
M	6.48	13.41	26.35	Medium	14/29/57
F	4.7	19.13	9.73	Medium	14/57/29
F	14.14	9.74	9.74	Medium	42/29/29
F	7.11	11.73	11.73	Medium	23/38/38
M	13.81	8.35	20.05	Medium	33/20/48
F	13.5	9.3	9.3	Medium	42/29/29
F	8.04	13.26	13.26	Medium	23/38/38
F	13.48	9.29	9.29	Medium	42/29/29
M	11.29	16.4	6.83	Medium	33/48/20
M	5.39	21.93	11.16	Medium	14/57/29
F	10.33	15	6.25	Medium	33/48/20
M	12.01	7.27	17.45	Medium	33/20/48
F	11.35	6.87	16.48	Medium	33/20/48
M	8.13	13.42	13.42	Medium	23/38/38

Results

The effects of macronutrients on overall liver gene expression

Global transcriptome analysis of the liver was performed using Affymetrix Mouse Gene ST arrays representing 21,798 hepatic genes. To form an unbiased assessment of these genes, fold change and p-values, calculated through the Geometric Framework, were passed to a volcano plot script in R, to identify the genes with maximum fold change that were significantly correlated with diet [Figure 4.2]. The most highly expressed gene was *Alb* (albumin) and there was about a 100-fold difference in the expression of the highest versus the lowest expressed genes within each liver. Of the three macronutrients, dietary protein was associated with changes in the expression of the highest number of genes using either correlation analysis (n=1279 genes, 648 positive correlation, 631 negative correlation) or GAMS (n=2933 genes). This compared to only 8 genes by correlation analysis and 72 genes by GAMS for dietary carbohydrates; and only 3 genes by correlation analysis and 19 genes by GAMS for dietary fat (Figure 4.3 A-B). For dietary protein, there were 980 genes (77% of genes from correlation analysis) in common using either GAMS or correlation analysis, while for dietary carbohydrates there were 4 genes (50% of genes from correlation analysis) in common using both types of analysis, indicating that both types of analysis are identifying similar overall trends.

Heatmap analysis [See Figure 4.4] revealed a very strong inverse relationship between gene expression that correlated with protein intake compared with intake of fat or carbohydrates, such that genes that were positively correlated with the intake of dietary protein were negatively correlated with the intake of either fat or carbohydrates, and vice versa. Although the inverse relationships between the macronutrients on the heatmaps are very distinct, it should be noted that only a few genes were statistically significantly associated with the intakes of carbohydrates or fats.

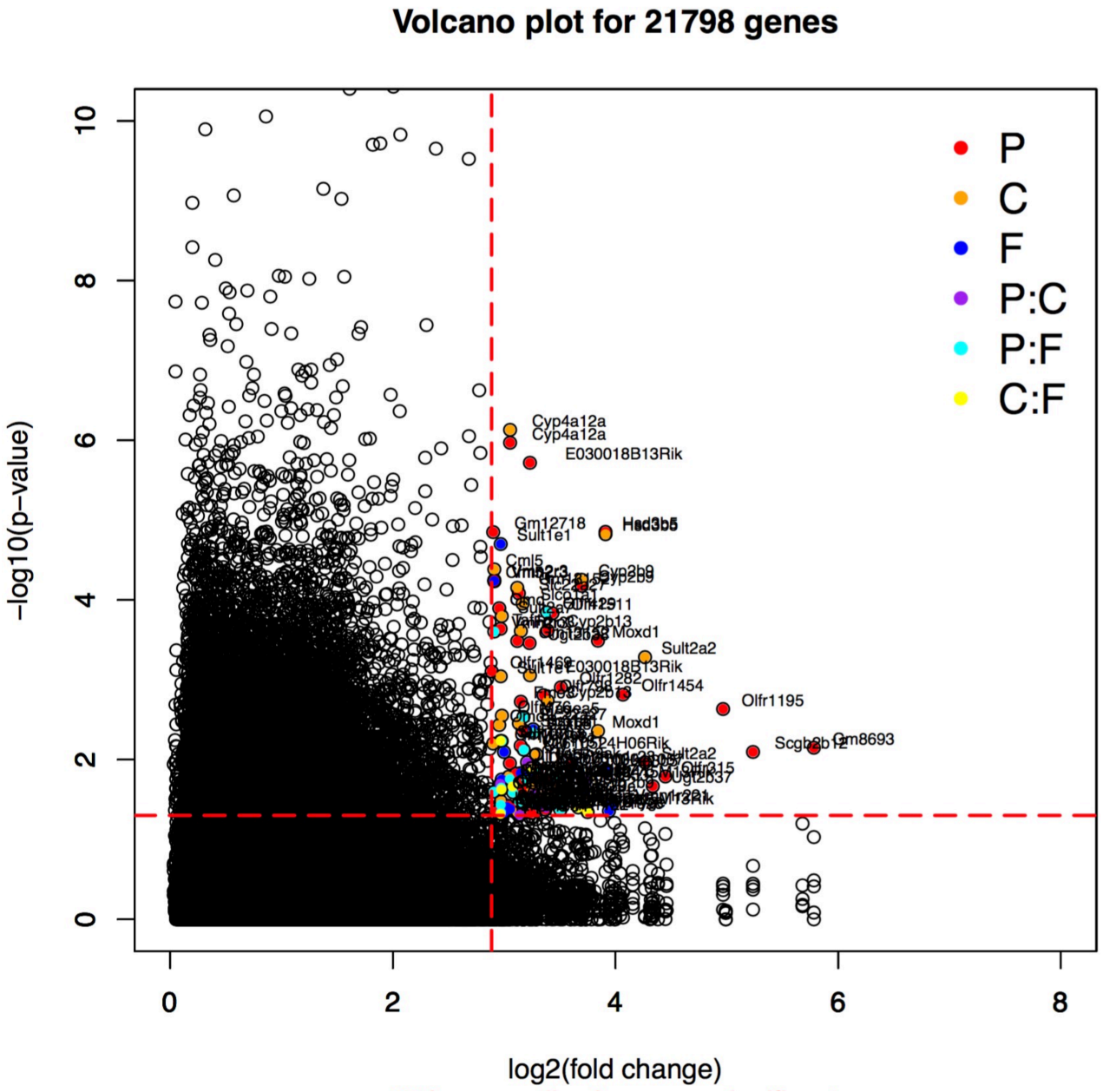


Figure 4.2. Volcano plot showing genes of interest identified through an unbiased analysis of the hepatic microarray. Genes are colour coded based on correlation to macronutrient axis or ratio. Genes of interest (GOI's) were selected based on both fold change, as well as having a p-value greater than p=0.05.

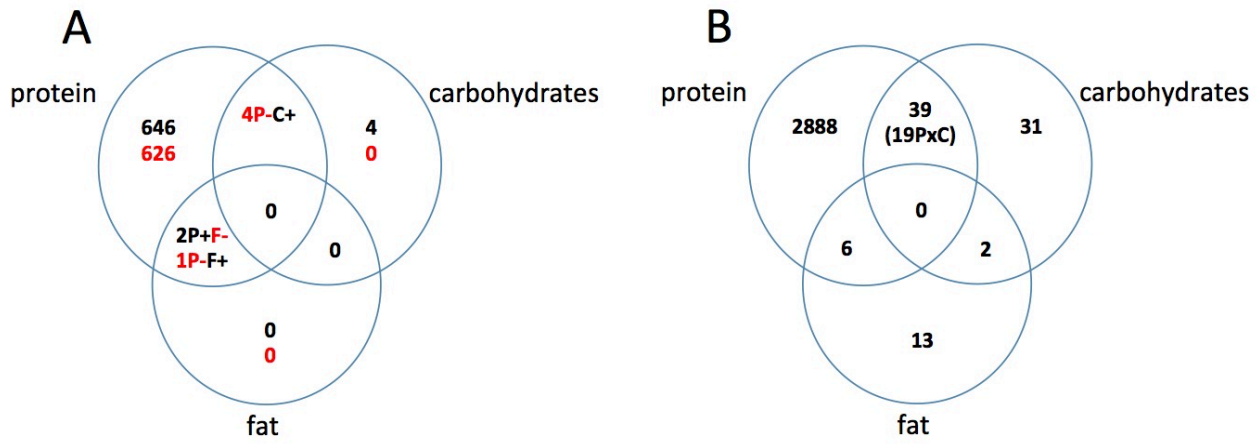


Figure 4.3. Venn diagrams showing the effects of macronutrient intake on gene expression in the liver. 4.3A shows the results determined using Pearson's correlation coefficient (black positive correlation, red negative correlation). 4.3B shows the results determined using GAMS (PxC interactive term between carbohydrates and protein).

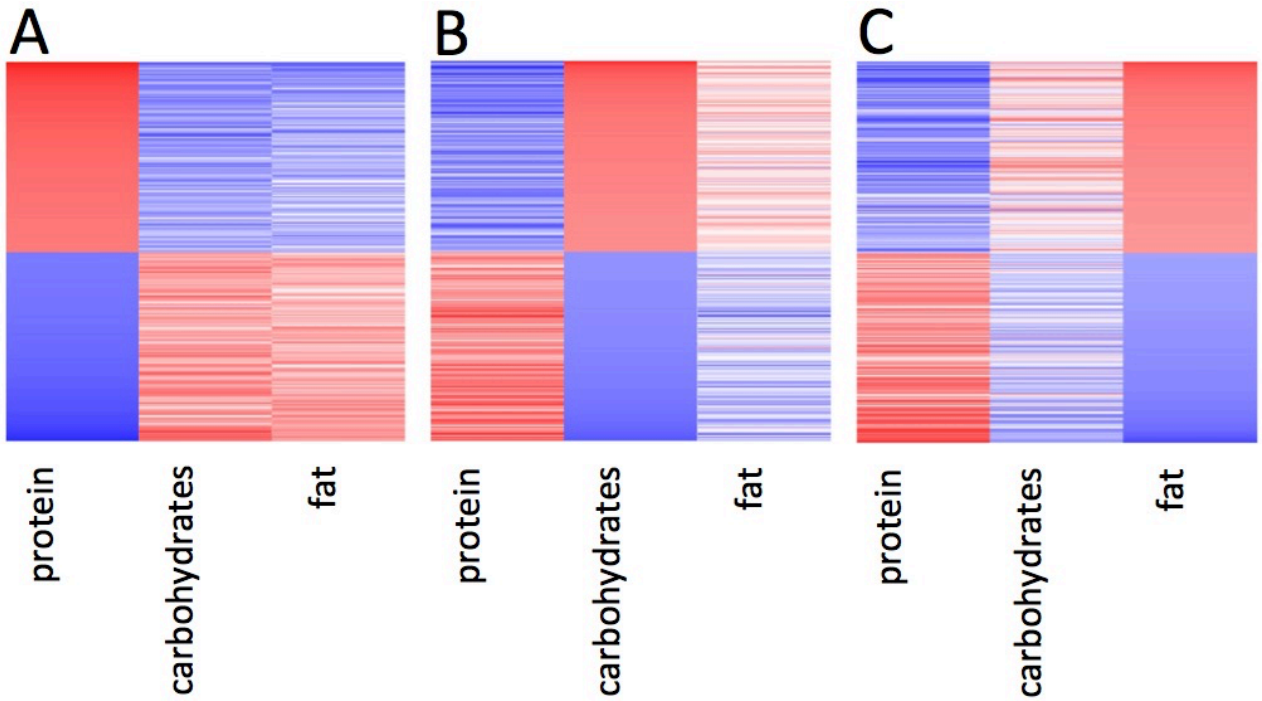


Figure 4.4 Heatmaps comparing the overall pattern of expression of genes in the liver. The values are Pearson's correlation coefficients ranked from most positive correlation in red to the most negative correlation in blue. The most positive 1000 genes and the most negative 1000 genes are included. The genes have been ranked according to significance of the correlation with protein (A), carbohydrates (B) and fat (C).

The genes with the most statistically significant association with macronutrient intake are shown in Table 4.2. Four representative surfaces using the Geometric Framework approach are shown in Figure 4.5. These genes are shown because they were highly significantly associated with macronutrient intake and demonstrate characteristic responses to macronutrients that can be easily visualised by the Geometric Framework method [Table 4.3]. Nicotinamide N-methyltransferase (*Nnmt*) was positively associated with protein intake (Figure 4.5A), while Insulin-like growth factor 2 mRNA-binding protein 2 (*Igf2bp2*) was negatively associated with protein intake (Figure 4.5B). Glucosylceramidase beta 2 (*Gba2*) was positively associated with carbohydrate intake (Figure 4.5C), while Solute carrier family 15 member 5 (*Slc15a5*) was positively associated with both protein and carbohydrate intake (Figure 4.5D).

Table 4.2. Gene expression with the highest statistical association with macronutrient intake in the liver determined using either GAMS or correlation analysis. For correlation analysis, genes are divided into those with positive and negative correlation. Benjamini Hochberg correction with a FDR of 0.05 has been performed for the P values. Genes in the top twenty for both GAMS and correlation analysis are bolded.

Protein	**p**	**Carbohydrates**	**p**	**Fat**	**p**
Top genes by GAMS analysis					
Igf2bp2	7.34E-21	Slc15a5	5.83E-07	Sag	2.9E-11
Adgrg2	6.31E-12	Reg1	3.11E-06	**Gm5424**	9.18E-09
Orm1	1.71E-10	Sox13	0.00072	Hsd3b1	2.58E-07
Nnmt	1.57E-10	Alb	0.00073	**Cps1**	1.99E-05
Igsf23	6.3E-10	Lrrc16a	0.00108	Cfap54	0.00054
Spink5	6.72E-10	**Fam131c**	0.00168	Mir192	0.00150
Gm5424	6.24E-09	Cyp4a12a	0.00220	**Gpc1**	0.00288
Corin	1.63E-08	Hsd17b6	0.00358	X1700012C14Rik	0.00759
Itga6	9.14E-08	Rnf128	0.00610	Zfp449	0.01249

Impa2	1.83E-07	**Gba2**	0.00927	Fam19a5	0.01529
Gpi1	2.41E-07	Rtn4	0.01255	Pcdh18	0.02078
Slc15a5	2.57E-07	Elovl3	0.01706	Gfod2	0.02631
Fgf21	3.06E-07	X1700012C14Rik	0.01613	Sema5b	0.02444
Vtcn1	2.95E-07	Wasf3	0.01623	Sult1e1	0.02970
Psat1	3.09E-07	Pdilt	0.01556	Rnf133	0.03283
Arhgef2	9.22E-07	Adgrg7	0.01509	Trpc3	0.03193
Hsd17b6	1.05E-06	Derl3	0.01469	Slc39a12	0.03276
Slc13a2	1.09E-06	**Pard3b**	0.01428	Sel1l3	0.03158
Cps1	1.16E-06	Cyp2u1	0.01425	Cidec	0.03688
Soat2	5.73E-06	Hsd3b5	0.01567		

Top genes by correlation analysis, positive correlation with intake

Cth	1.92E-05	Slc17a8	0.00711	**Gpc1**	0.038492
Lrtm1	2.64E-05	**Pard3b**	0.01925		
Slc13a2	2.36E-05	**Gba2**	0.02124		
Gpx6	6.05E-05	Tubg1	0.02399		
Acmsd	9.08E-05	A230050P20Rik	0.02411		
Sdhb	0.00022	**Fam131c**	0.02692		
Nnmt	0.00021	Unc13b	0.02818		
Acadsb	0.00022	Ergic1	0.03640		
Gm5424	0.00025				
Slc43a1	0.00037				

Top genes by correlation analysis, negative correlation with intake

Adgrg2	4.78E-06	**Cps1**	0.00722
Impa2	2.72E-05	**Gm5424**	0.01158
Igf2bp2	4.72E-05		
Slc9a7	4.76E-05		
Unc13b	5.69E-05		
Lhx6	6.77E-05		
Slc17a8	0.00025		
App	0.00028		
Slc7a7	0.00030		
Gpi1	0.00032		

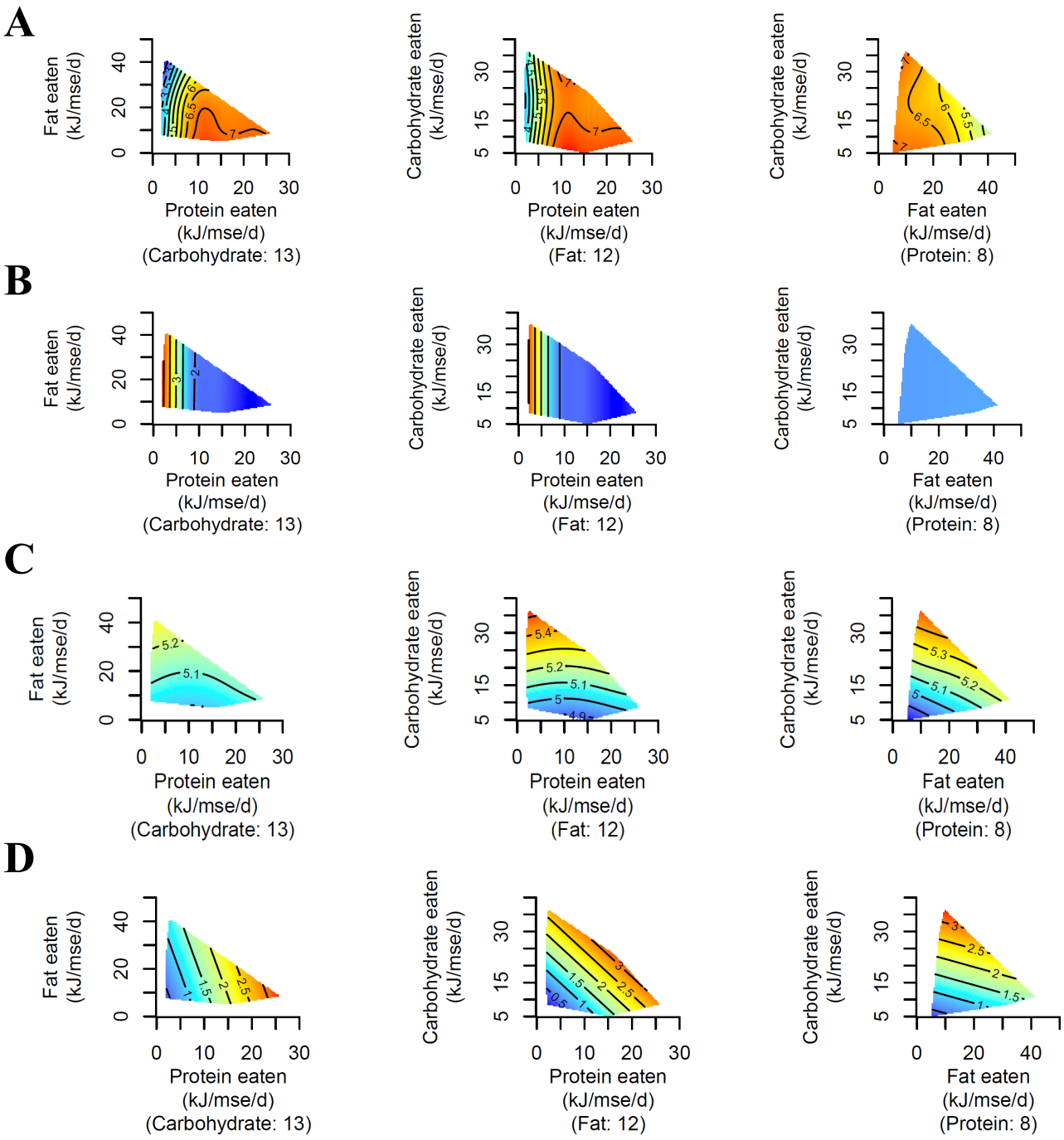


Figure 4.5. Two dimensional response surfaces created using the GF. The relationships between the macronutrients and four hepatic genes (A *Nnmt*, B *Igf2bp2*, C *Gba2*, D *Slc15a5*) are demonstrated. Each graph shows the effects of two of macronutrients at the median point of the third macronutrient (shown in parenthesis below the X axis label). The response surfaces vary from red which is the most negative value to blue which is the most positive value. The GAM statistics are shown in Table 4.3.

Table 4.3. Statistics for the Geometric Framework figures in Figure 4.5. GAMS data for hepatic expression of *Slc15a5, Nnmt, Igf2bp2* and *Gba2*.

	edf	df	F	P
Nnmt				
Protein	3.602e+00	8	11.861	3.08e-14
Fat	9.190e-01	8	1.417	0.000488
Carbohydrates	1.266e-05	8	0.000	0.603552
P x F	7.364e-06	3	0.000	1.000000
P x C	1.879e-05	3	0.000	0.334256
F x C	1.291e+00	3	1.346	0.048472
Slc15a5				
Protein	9.824e-01	8	6.962	1.48e-10
Fat	8.469e-01	8	0.691	0.0138
Carbohydrates	9.842e-01	8	7.781	2.79e-11
P x F	3.323e-06	3	0.000	0.7224
P x C	5.495e-05	3	0.000	0.4078
F x C	1.487e-06	3	0.000	0.9572
Igf2bp2				
Protein	4.066e+00	8	22.398	<2e-16
Fat	9.167e-02	8	0.013	0.299
Carbohydrates	1.573e-05	8	0.000	0.481
P x F	4.107e-06	3	0.000	0.503
P x C	4.094e-06	3	0.000	0.678
F x C	1.762e-06	3	0.000	0.985
Gba2				
Protein	8.442e-01	8	0.168	0.1930
Fat	8.244e-01	8	0.587	0.0108
Carbohydrates	9.536e-01	8	2.568	4.4e-06
P x F	2.580e-06	3	0.000	0.5303
P x C	4.501e-06	3	0.000	0.3722
F x C	4.107e-01	3	0.168	0.2799

The effects of macronutrients on hepatic gene pathways

Gene pathway analysis based on correlation coefficients was able to be analysed according to whether the genes had a positive or a negative correlation with macronutrient intake [See Table 4.4, Figure 4.6A]. Pathways enriched by genes whose expression was *positively* correlated with protein intake included oxidative phosphorylation and multiple pathways associated with amino acid metabolism, as well as a pathways associated with neurodegenerative diseases. Pathways enriched by genes whose expression was *negatively* correlated with protein intake include several key metabolic signaling pathways, PI3k-Akt, mTOR, AMPK and insulin signaling as well as pathways associated with various cancers. Gene pathway analysis based on GAMS only found five pathways associated with protein intake all of which were also present in the pathways found using correlation analysis. There were few if any significant or relevant pathways linked to carbohydrate or fat intake with either analytical approach.

The effects of macronutrients on biological processes in the liver

Biological processes associated with genes whose expression was *positively* correlated with protein intake included a very large number of processes associated with mitochondria as well as amino acid metabolism [See Table 4.5, Figure 4.6B]. Biological processes associated with genes whose expression was negatively correlated with protein intake included transcription, protein metabolism and cell differentiation and maturation. There were no pathways associated with fat intake and only two with carbohydrate intake, and none identified using GAMS analysis.

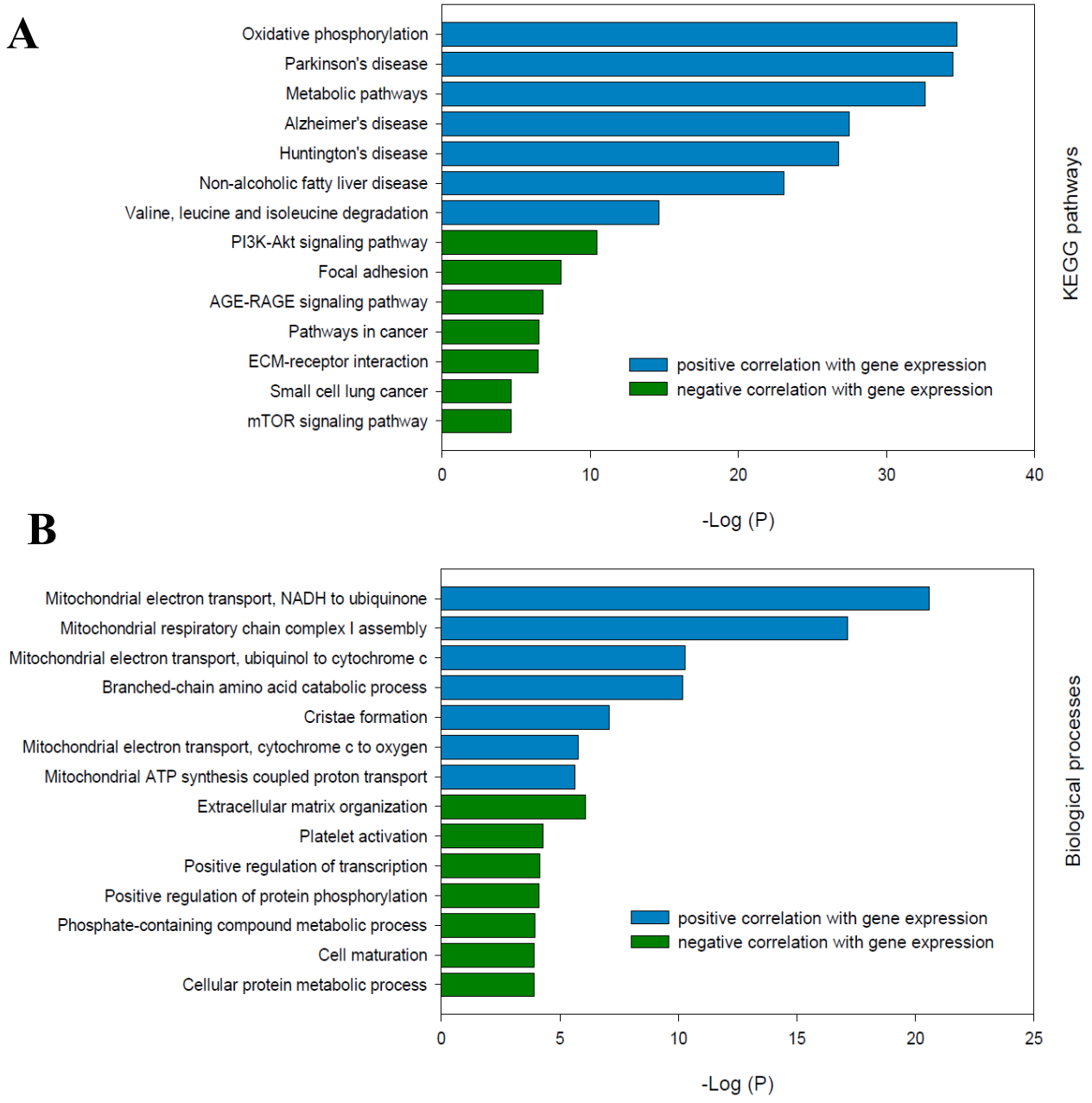


Figure 4.6. (A) Gene pathways and (B) Biological processes associated with protein intake, where the association between protein intake and hepatic gene expression has been determined using Pearson's correlation coefficient. Pathways analysis was performed using Enrichr. Full datasets are in Tables 4.4 and 4.5.

Table 4.4. Gene pathway analysis based on KEGG database in Enrichr. The genes of interest in the liver were determined using either Pearson's correlation coefficient or GAMS to evaluate the relationship between each macronutrient and gene expression, and a FDR of 0.05. There were no pathways that were significant for carbohydrates, fat or the interactions between macronutrients.

Gene pathway analysis using Enrichr and KEGG database				
	overlap	-log(p)	p	adj p
Pathways associated with genes *positively* correlated with protein intake using Pearson's coefficient				
Oxidative phosphorylation	46/133	34.7474	0.0000	0.0000
Parkinson's disease	47/142	34.4844	0.0000	0.0000
Metabolic pathways	127/1239	32.5861	0.0000	0.0000
Alzheimer's disease	44/168	27.4649	0.0000	0.0000
Huntington's disease	46/193	26.7592	0.0000	0.0000
Non-alcoholic fatty liver disease	38/151	23.0914	0.0000	0.0000
Valine, leucine and isoleucine degradation	18/48	14.6411	0.0000	0.0000
Arginine biosynthesis	8/20	7.0726	0.0000	0.0000
Cardiac muscle contraction	14/78	6.8751	0.0000	0.0000
Propanoate metabolism	9/32	6.3681	0.0000	0.0000
Biosynthesis of amino acids	13/74	6.3146	0.0000	0.0000
Alanine, aspartate and glutamate metabolism	9/35	6.0039	0.0000	0.0000
Tryptophan metabolism	9/40	5.4771	0.0000	0.0000
Glycine, serine and threonine metabolism	9/40	5.4771	0.0000	0.0000
Carbon metabolism	14/113	4.8876	0.0000	0.0002
Cysteine and methionine metabolism	8/45	4.1418	0.0001	0.0008
Glyoxylate and dicarboxylate metabolism	6/28	3.6980	0.0002	0.0022
Peroxisome	10/83	3.5550	0.0003	0.0028
beta-Alanine metabolism	6/31	3.4419	0.0004	0.0035
Histidine metabolism	5/24	3.0999	0.0008	0.0073
Arginine and proline metabolism	7/50	3.0339	0.0009	0.0081
Pentose and glucuronate interconversions	5/36	2.2871	0.0052	0.0394
Lysine degradation	6/52	2.2427	0.0057	0.0419
Pathways associated with genes *negatively* correlated with protein intake using Pearson's coefficient				
PI3K-Akt signaling pathway	38/341	10.4612	0.0000	0.0000
Focal adhesion	25/202	8.0027	0.0000	0.0000
AGE-RAGE signaling pathway	16/101	6.8180	0.0000	0.0000
Pathways in cancer	34/397	6.5509	0.0000	0.0000
ECM-receptor interaction	14/82	6.4542	0.0000	0.0000
Small cell lung cancer	12/86	4.6848	0.0000	0.0007

mTOR signaling pathway	10/60	4.6824	0.0000	0.0007
Amoebiasis	12/100	4.0266	0.0001	0.0026
Choline metabolism in cancer	12/101	3.9844	0.0001	0.0026
Proteoglycans in cancer	18/203	3.9423	0.0001	0.0026
Toxoplasmosis	13/118	3.9275	0.0001	0.0026
Colorectal cancer	9/62	3.7883	0.0002	0.0033
Pancreatic cancer	9/66	3.5770	0.0003	0.0049
Progesterone-mediated oocyte maturation	11/98	3.4813	0.0003	0.0057
Thyroid hormone signaling pathway	12/118	3.3460	0.0005	0.0071
Acute myeloid leukemia	8/57	3.3248	0.0005	0.0071
Rap1 signaling pathway	17/211	3.2686	0.0005	0.0076
Protein digestion and absorption	10/90	3.1798	0.0007	0.0088
AMPK signaling pathway	12/124	3.1512	0.0007	0.0089
Insulin resistance	11/109	3.0855	0.0008	0.0092
Phospholipase D signaling pathway	13/144	3.0799	0.0008	0.0092
Fc gamma R-mediated phagocytosis	10/93	3.0674	0.0009	0.0092
TNF signaling pathway	11/110	3.0524	0.0009	0.0092
Hepatitis B	13/146	3.0240	0.0009	0.0092
N-Glycan biosynthesis	7/49	3.0201	0.0010	0.0092
Renal cell carcinoma	8/66	2.8944	0.0013	0.0118
Central carbon metabolism in cancer	8/67	2.8514	0.0014	0.0125
Dorso-ventral axis formation	5/27	2.8041	0.0016	0.0135
MAPK signaling pathway	18/255	2.7630	0.0017	0.0143
HTLV-I infection	18/258	2.7072	0.0020	0.0157
Thyroid cancer	5/29	2.6597	0.0022	0.0169
Arrhythmogenic right ventricular cardiomyopathy	8/74	2.5733	0.0027	0.0200
Ras signaling pathway	16/227	2.5114	0.0031	0.0224
Osteoclast differentiation	11/132	2.4192	0.0038	0.0269
Jak-STAT signaling pathway	12/158	2.2649	0.0054	0.0373
Endometrial cancer	6/52	2.1877	0.0065	0.0433
Cell cycle	10/124	2.1481	0.0071	0.0450
Viral carcinogenesis	14/205	2.1471	0.0071	0.0450
Pathways associated with genes linked to protein intake by GAMS				
Non-alcoholic fatty liver disease (NAFLD)	45/151	5.8505	0.0000	0.0004
Oxidative phosphorylation	35/133	3.5035	0.0003	0.0320
Focal adhesion	48/202	3.4093	0.0004	0.0320
Huntington's disease	46/193	3.3239	0.0005	0.0320
Parkinson's disease	36/142	3.2516	0.0006	0.0320

Table 4.5. Biological pathways analysis based on Gene Ontology Biological Process 2017 database in Enrichr. The genes of interest in the liver were determined using either GAMS or Pearson's correlation coefficient to evaluate the relationship between each macronutrient and gene expression, and a FDR of 0.05. There were no processes detected using GAMS or for fat.

Biological processes using Enrichr and GO Biological processes 2017 database

	overlap	-log(p)	p	adj p
Biological processes associated with genes *positively* correlated with protein intake using Pearson's coefficient				
mitochondrial electron transport, NADH to ubiquinone	22/46	20.5891	0.0000	0.0000
mitochondrial respiratory chain complex I assembly	22/62	17.1563	0.0000	0.0000
mitochondrial electron transport, ubiquinol to cytochrome c	7/14	10.2933	0.0000	0.0000
branched-chain amino acid catabolic process	9/16	10.1865	0.0000	0.0000
cristae formation	9/27	7.0838	0.0000	0.0000
mitochondrial electron transport, cytochrome c to oxygen	7/20	5.7876	0.0000	0.0002
mitochondrial ATP synthesis coupled proton transport	7/21	5.6234	0.0000	0.0002
tryptophan catabolic process	5/9	5.4572	0.0000	0.0003
urea cycle	5/10	5.1676	0.0000	0.0005
mitochondrial ATP synthesis coupled electron transport	4/6	4.8540	0.0000	0.0010
lysine catabolic process	5/12	4.6931	0.0000	0.0013
cellular nitrogen compound metabolic process	6/23	4.2116	0.0001	0.0037
mitochondrial translational termination	11/87	4.0461	0.0001	0.0046
mitochondrial translational elongation	11/87	4.0461	0.0001	0.0046
ATP biosynthetic process	6/25	3.9908	0.0001	0.0049
oxidative phosphorylation	4/10	3.7518	0.0002	0.0079

L-phenylalanine catabolic process	4/11	3.5665	0.0003	0.0114
cellular amino acid biosynthetic process	5/25	3.0143	0.0010	0.0346
aerobic respiration	5/25	3.0143	0.0010	0.0346
mitochondrial respiratory chain complex III assembly	3/8	2.8084	0.0016	0.0484
Biological processes associated with genes *negatively* correlated with protein intake using Pearson's coefficient				
extracellular matrix organisation	18/142	6.0919	0.0000	0.0011
platelet activation	12/94	4.2924	0.0001	0.0244
positive regulation of transcription of nuclear large rRNA transcript from RNA polymerase I promoter	4/8	4.1619	0.0001	0.0244
positive regulation of protein phosphorylation	13/113	4.1215	0.0001	0.0244
phosphate-containing compound metabolic process	5/16	3.9428	0.0001	0.0244
cell maturation	4/9	3.9179	0.0001	0.0244
cellular protein metabolic process	17/186	3.9158	0.0001	0.0244
endodermal cell differentiation	6/26	3.8254	0.0001	0.0263
positive regulation of gene expression	18/214	3.6549	0.0002	0.0346
regulation of small GTPase mediated signal transduction	13/129	3.5387	0.0003	0.0407
receptor-mediated virion attachment to host cell	3/5	3.4916	0.0003	0.0412
cellular response to hypoxia	8/55	3.4328	0.0004	0.0432
Ras protein signal transduction	8/56	3.3782	0.0004	0.0453
cell migration	13/136	3.3153	0.0005	0.0486
Biological processes associated with genes correlated with carbohydrate intake using Pearson's coefficient				
synaptic vesicle priming	6/17	2.6202	0.0024	0.0102
bile acid metabolic process	6/17	2.6202	0.0024	0.0102

The effect of macronutrients on longevity regulating pathways in the liver

The KEGG 'longevity regulating pathways, multiple species' gene set (ko0413, http://www.genome.jp/dbget-bin/www_bget?pathway+ko04213) includes 59 genes of which 50 were present in this microarray data set, some with two variants. Using GAMS analysis there were only seven genes in common with these longevity regulating genes (*Atg5, Mtor, Pik3r2, Igf1r, Prkab2, Hsap1b, Adcy3*) and three using correlation analysis (*Atg4, Mtor, Pik3r2*). The correlation coefficients between each of these genes and macronutrients were plotted on a heatmap [Figure 4.7] to determine the overall pattern of the relationship between macronutrient intake and expression of genes associated with the regulation of aging. There were many more genes whose expression were negatively correlated with protein intake than positively correlated with protein intake. Genes associated with carbohydrate intake had the opposite pattern. Consequently, there was a clear inverse pattern between genes associated with protein intake versus those associated with carbohydrate intake (and a similar, but less marked pattern when comparing protein intake and fat intake).

The Geometric Framework was used to evaluate five nutrient sensing genes that are considered to link nutrition with aging: *Mtor, Igf1, Sirt1, Prkab2 (*a subunit of AMPK) and *Ffg21* [See Figure 4.8, Table 4.6]. Surprisingly the expression of *Mtor*, which is one of the key signaling pathways stimulated by protein, was upregulated with lower protein intake. The effects of macronutrients on mTOR protein phosphorylation in these mice has been previously published (Solon-Biet et al. 2014), and comparison of these data with *Mtor* gene expression showed that there was no correlation ($r=-0.052$, $p=0.7$). Low protein intake was associated with reduced expression of *Igf1* and increased expression of *Prkab2* and *Fgf21*. The expression of *Sirt1* was not significantly influenced by diet by dietary macronutrients. The genes influenced by protein intake were compared to those reported to be influenced by caloric restriction. A meta-analysis identified 174 genes where expression is influenced by caloric restriction (Plank et al. 2012) of which only 30 were in common with those genes

influenced by protein intake determined by GAMS, and only 11 in common with those genes influenced by protein intake determined by correlation [Table 4.7].

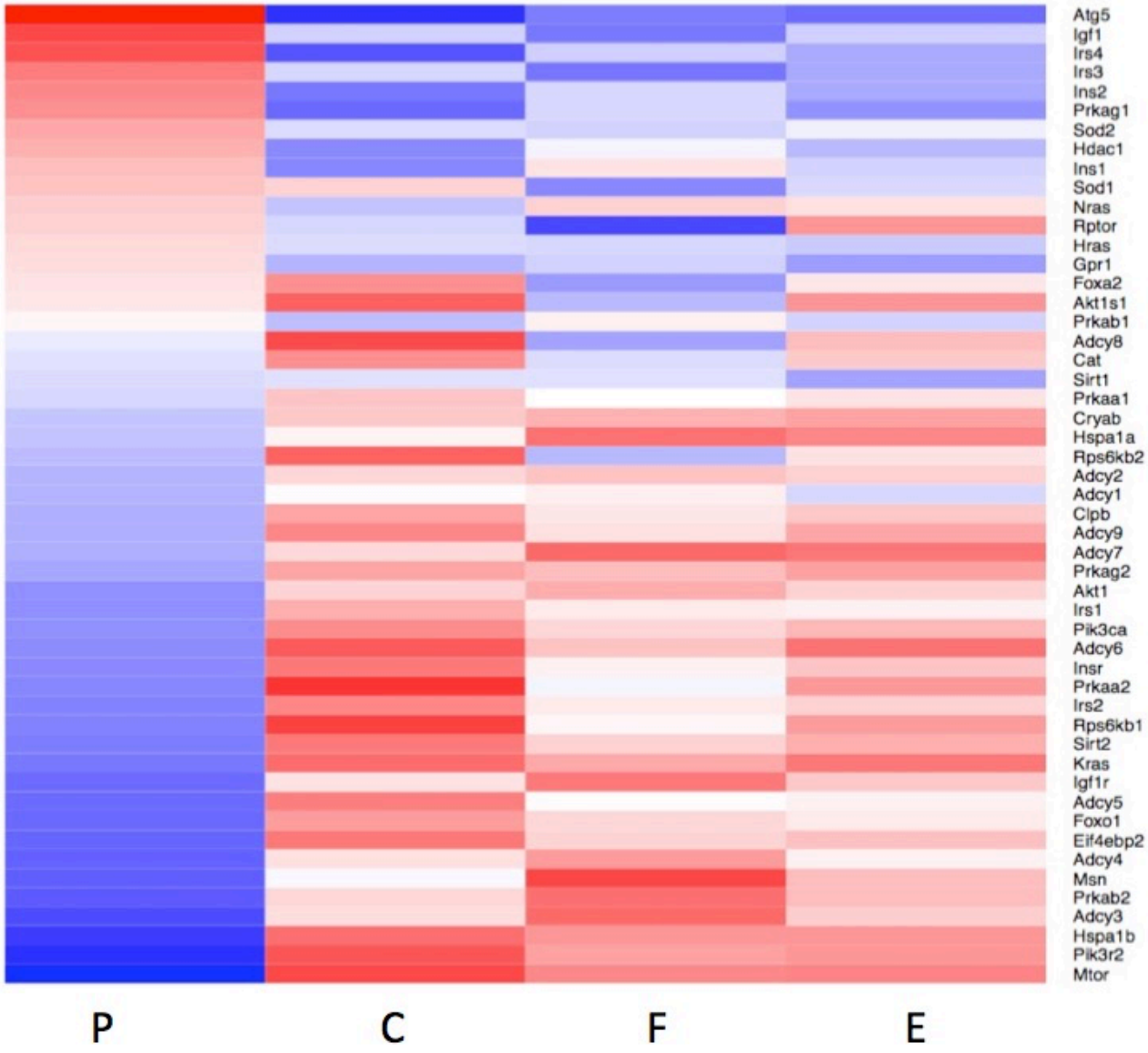


Figure 4.7. Heatmap showing the relationship between longevity regulating genes in the liver according to KEGG, and the correlation coefficients that link each of these genes with protein, carbohydrates, fat and total energy intake. Positive correlation coefficients are shown in red and negative coefficients in blue.

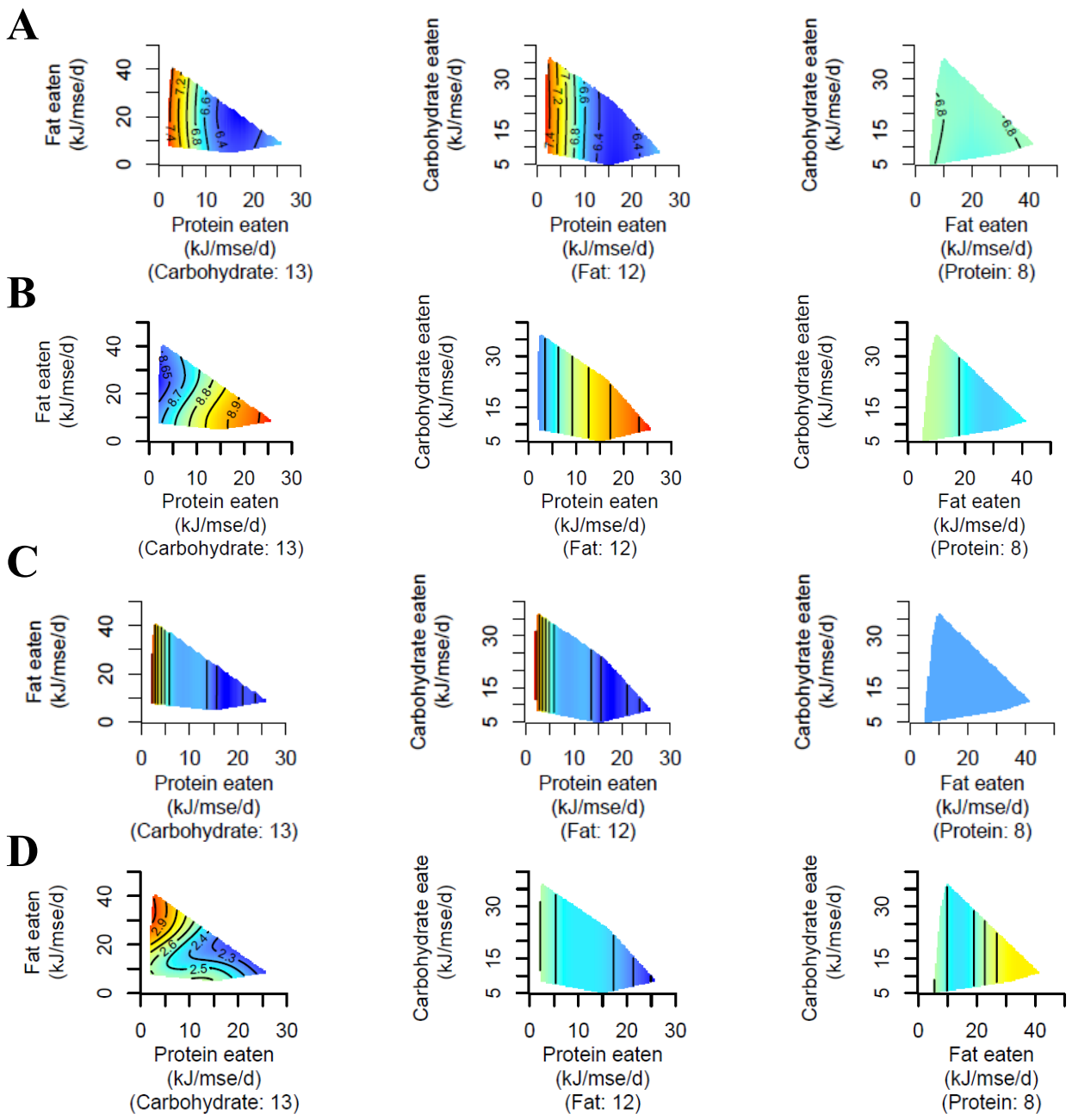


Figure 4.8. Three dimensional response surfaces created using the GF. The relationships between the macronutrients and four nutrient sensing hepatic genes (A *Mtor*, B *Igf1*, C *Pkrab2*, D *Fgf21*) are demonstrated. Each graph shows the effects of two of macronutrients at the median point of the third macronutrient (shown in parenthesis below the X axis label). The response surfaces vary from red, which is the most negative value to blue which is the most positive value. The GAM statistics are shown in Table 4.6.

Table 4.6. Statistics for the Geometric Framework figures in Figure 4.8. GAMS data for hepatic expression of *Mtor, Igf1, Prkab2 and Ffg21.*

	edf	df	F	P
Mtor				
Protein	2.170e+00	8	3.016	1.45e-05
Fat	2.873e-05	8	0.000	0.507
Carbohydrates	1.795e-05	8	0.000	0.765
P x F	2.350e-01	3	0.085	0.286
P x C	1.122e-05	3	0.000	0.364
F x C	2.588e-01	3	0.099	0.291
Igf1				
Protein	8.092e-01	8	0.53	0.0193
Fat	9.036e-06	8	0.00	0.3452
Carbohydrates	5.297e-07	8	0.00	0.5722
P x F	7.436e-01	3	0.47	0.1522
P x C	2.033e-07	3	0.00	1.0000
F x C	2.067e-07	3	0.00	0.6170
Prkab2				
Protein	1.276e+00	8	1.082	0.0031
Fat	1.790e-05	8	0.000	0.7183
Carbohydrates	1.067e-05	8	0.000	0.8009
P x F	1.653e+00	3	2.840	0.0076
P x C	8.471e-07	3	0.000	0.6440
F x C	6.838e-06	3	0.000	0.6708
Fgf21				
Protein	4.313e+00	8	8.913	1.91e-10
Fat	8.544e-05	8	0.000	0.361
Carbohydrates	6.244e-06	8	0.000	1.000
P x F	5.098e-06	3	0.000	0.648
P x C	1.639e-05	3	0.000	1.000
F x C	5.026e-06	3	0.000	0.670

Table 4.7. Genes in common between those influenced by protein intake from this study and compared with those influenced by caloric restriction as published by (Plank et al. 2012) where there were 101 genes upregulated by caloric restriction and 73 downregulated. Genes of interest were determined using either a correlation analysis or GAMS with a FDR of 0.05 for their association with protein intake.

Upregulated in caloric restriction	**Downregulated in caloric restriction**
Altered expression with protein intake determined by GAMS	
Klf10	C9
Rgs16	Col15a1
Cyp2j6	Hsd3b2
Zbtb16	Slc10a2
Cyp2b13	Extl1
BC089597	Cyp2f2
Sds	Hipk2
Mt2	R3hdm2
Lpin1	Col3a1
Per1	Stac3
Angptl4	
Plin4	
St3gal5	
Fmo3	
Rhobtb1	
Cbr1	
Nat8	
Cpt1a	
Arrdc2	
Por	
Altered expression with protein intake determined by correlation analysis	
Cyp2j6	C9
Sds	Cyp2f2
Sult1d1	Col15a1
Fam195a	
Plin4	
Zbtb16	
Klf10	
Rgs16	

PCR confirmation of hepatic microarray analysis

In order to validate the results of the transcriptome analysis, quantitative RT-PCR was performed on a set of 84 genes associated with nutrient sensing pathways. Gene expression values from the microarray and PCR were correlated using a Q-Q plot to determine distribution of gene expression (Warnat et al. 2005). The expression of the majority of genes from the microarray were closely correlated with their PCR counterparts [Figure 4.9, Table 4.8].

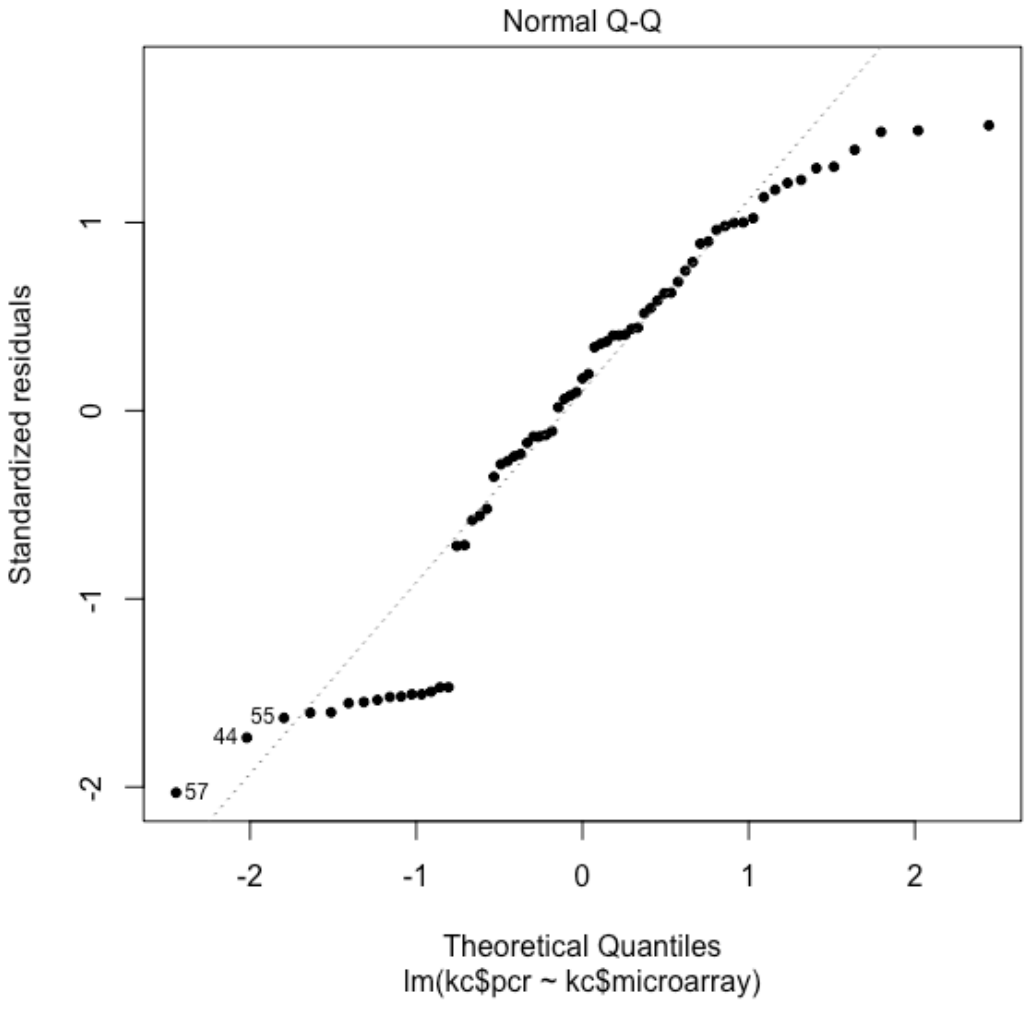


Figure 4.9. Correlation between Affymetrix microarray gene expression data and PCR data for 84 genes listed in Table 4.8.

Table 4.8. List of genes on Qiagen Insulin Signalling Pathway RT^2 Profiler PCR Array

Insulin Receptor-Associated Proteins

Insulin & Receptors: Ins1, Insl3, Irs1, Irs2, Sorbs1.

Insulin-Like Growth Factors & Receptors: Grb10, Igf1r, Igfbp1.

SH3 / SH2 Adaptor Proteins: Cbl, Grb10, Grb2.

Other Insulin Receptor-Associated Proteins: Dok1, Dok2, Dok3, Eif4ebp1, Frs2, Frs3, Gab1, Nck1, Ppp1ca, Ptpn1 (PTP1B), Ptprf, Shc1.

PI3 Kinase Signaling

Genes: Akt1, Akt2, Akt3, Eif2b1, Mtor, Pdpk1, Pik3ca (p110alpha), Pik3cb, Pik3r1 (p85alpha), Pik3r2 (p85beta), Prkcg, Prkci, Prkcz.

PI3 Kinase Signaling Target Genes: Adra1d, Bcl2l1 (Bcl-XL), Dusp14, G6pc, G6pc2, Hk2, Igfbp1, Pck2, Serpine1 (PAI-1), Srebf1, Ucp1, Vegfa.

MAP Kinase Signaling

Genes: Araf, Braf, Dok2, Dok3, Gab1, Grb2, Hras, Kras, Map2k1 (Mek1), Mapk1 (Erk2), Raf1, Rps6ka1, Rras, Rras2, Shc1, Sos1.

MAP Kinase Signaling Target Genes: Bcl2l1 (Bcl-XL), Ercc1, Fos, Nos2 (iNOS), Klf10 (Tieg1), Ucp1.

Primary Insulin Signaling Target Genes: Cebpb, Fos, Jun, Lep (Leptin), Prl.

Secondary Insulin Signaling Effector Target Genes: Npy, Pck2, Tg.

PPAR Targets

Acox1, Cfd (Adn), Cap1, Cebpb, Gpd1, Pck2, Pparg, Retn, Slc27a4.

SREBP1 Targets

Acaca, Fbp1, G6pc, Gck, Pck2, Pklr.

Carbohydrate Metabolism

Glucose Metabolism: Fbp1, G6pc, Gck, Gpd1, Hk2, Ins1, Lep (Leptin), Pck2, Pklr.

Glycogen Metabolism: G6pc, Ppp1ca.

Carbohydrate Transport: Ins1, Slc2a1, Sorbs1.

Lipid Metabolism

Cholesterol Metabolism: Ldlr, Lep (Leptin), Srebf1.

Fatty Acid Metabolism: Acox1, Slc27a4.

Lipid Transport: Ldlr, Slc27a4, Sorbs1.

Other Lipid Metabolism Genes: Araf, Prkcg, Prkci, Prkcz, Raf1, Shc1.

Protein Metabolism

Protein Phosphatases: Dusp14, Ppp1ca, Ptpn1 (PTP1B), Ptprf.

Protein Kinases: Akt1, Akt2, Akt3, Araf, Gsk3b, Igf1r, Map2k1 (Mek1), Mapk1 (Erk2), Pdpk1, Pik3ca (p110alpha), Pik3r1(p85alpha), Prkcg, Prkci, Prkcz, Raf1,Rps6ka1.

Protein Biosynthesis: Eif2b1, Eif4ebp1, Ppp1ca.

Protein Transport: Gsk3b, Hras, Prkci, Rras2.

Other Protein Metabolism Genes: Bcl2l1 (Bcl-XL), Cebpa, Cebpb, Dok3, Frs2, Gab1, Gck, Grb10, Grb2, Jun, Ldlr, Lep(Leptin), Nck1, Nos2 (iNOS), Pik3r2 (p85beta), Serpine1 (PAI-1), Shc1, Sorbs1, Sos1, Ucp1.

Transcription Factors & Regulators: Aebp1, Cebpa, Cebpb, Fos, Jun, Pparg, Srebf1, Klf10 (Tieg1).

Cell Growth & Differentiation

Cell Cycle: Gsk3b, Hras, Igf2, Jun, Kras, Mapk1 (Erk2), Vegfa.

Cell Proliferation: Gsk3b, Igf2, Irs2, Lep (Leptin), Nos2 (iNOS), Shc1, Vegfa.

Growth Factors & Receptors: Frs2, Igf1r, Igf2, Igfbp1, Lep (Leptin), Shc1, Vegfa.

Cell Differentiation: Cebpa, Cebpb, Gsk3b, Jun, Map2k1 (Mek1), Pik3r1 (p85alpha), Pparg.

The effects of macronutrients on overall hypothalamic gene expression

Across the 10,908 genes probed by the hypothalamic microarray [Figure 4.10], diet caused changes in the expression of 157 genes when analysed using correlation statistics (123 up-regulated, 34 down-regulated). The majority of these genes were influenced fat intake (52 up-regulated, 3 down-regulated) and total energy intake (44 up-regulated, 6 down-regulated) [Figure 4.11A]. 140 genes had over 5-fold differences in expression between the highest and lowest groups, while 25 genes had greater than 10-fold changes in expression.

GF analysis allowed ratios to be examined in addition to individual macronutrients, with a total of 934 genes having changes in their regulation as a result of varying macronutrient composition [See Figure 4.11B]. Interestingly, the GF analysis showed similar results to the correlation method, with fat once again playing the most significant role in regulating hypothalamic gene expression (n=205 genes), although the protein-to-carbohydrate (P:C) ratio, or the non-fat (fat intake vs non-fat intake) ratio was also picked up as a key regulator of gene expression (n=469 genes). Volcano plot analysis was used to identify genes of interest [Figure 4.10], with the top ten genes by macronutrient intake outlined in Table 4.9, both by GF and correlation analysis.

Representative surfaces for each macronutrient (protein, carbohydrates, fat) are shown in Figure 4.12 with their corresponding GAM statistics shown in Table 4.10. These genes were chosen because they are each in the top 10 most significantly altered expression levels based on GF analysis [Table 4.9], as well as demonstrate characteristic responses that are easily identified visually. Spindle assembly abnormal protein 6 (*Sass6*) is negatively correlated with protein intake, as seen in Figure 4.12A, where lower expression (blue colour) is associated with high protein, while high expression (red colour) is associated with low protein in the diet. Ubiquinol-cytochrome c reductase (*Uqcr*) is positively associated with carbohydrate intake [See Figure 4.12B], and B cell leukemia/lymphoma 2 related

protein A1b (*Bcl2a1b*) was positively associated with fat intake [See Figure 4.12C]. Of the genes significantly influenced by each macronutrient and ratio, several were involved in cellular growth and metabolism. As shown in Figure 4.11, the protein-to-carbohydrate ratio was the key driver of changes in gene expression. Key genes of interest here were: carbohydrate intake significantly influenced expression of *Slc6a3, Igf1 and Sirt2*; protein intake significantly influenced *Npy, Akt3, Sirt1*; and P:C ratio significantly influenced *Sirt5, Rps6a4/5, Igf2r, Igf1r and Sirt3*.

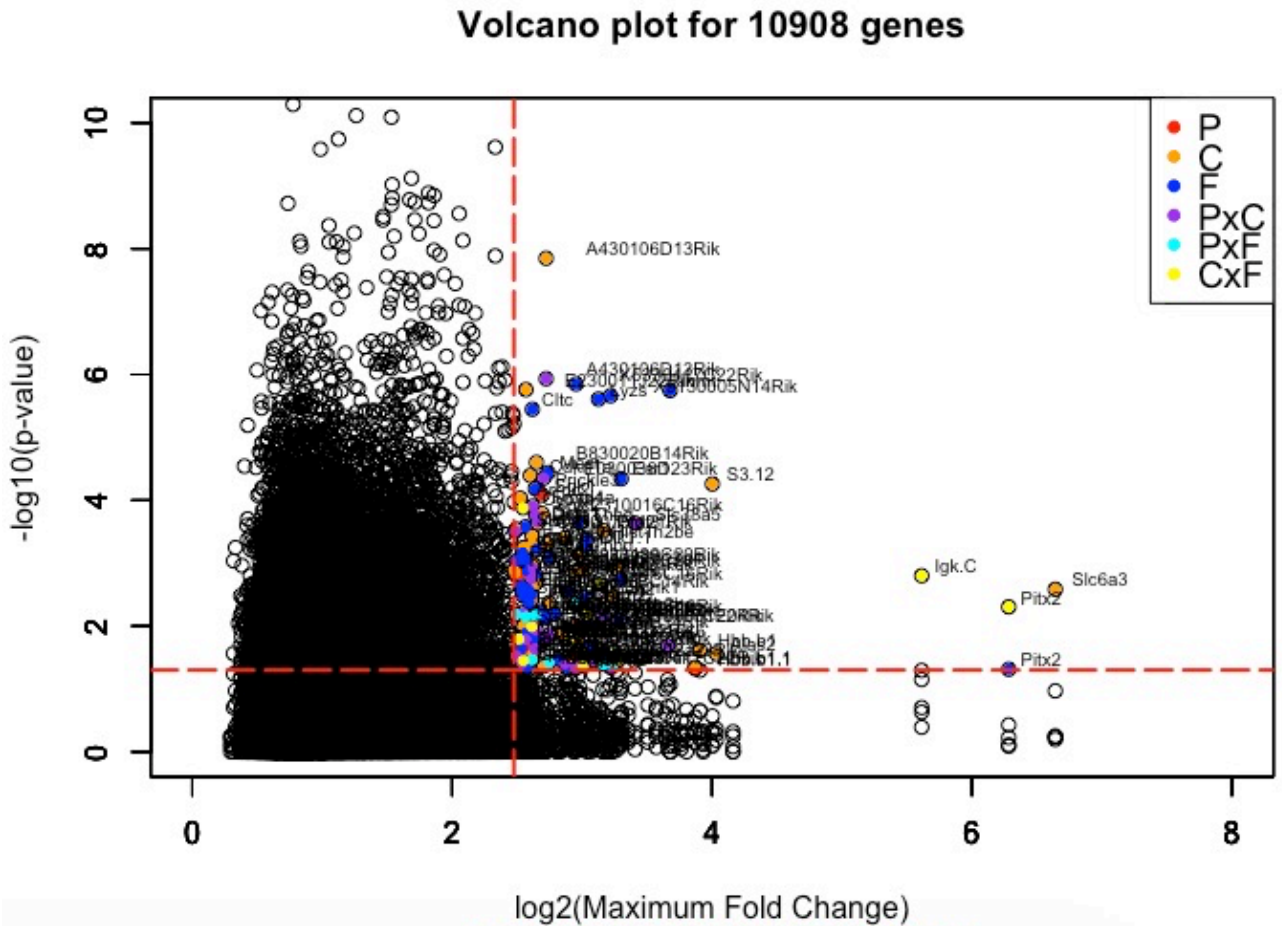


Figure 4.10. Unidirectional volcano plot of the 10908 genes in the hypothalamic microarray based on fold change versus p-value as derived from the GF. Genes are colour coded based on correlation to macronutrient intake (P, C, F) or ratio (PxC, PxF, CxF) as outlined in the legend.

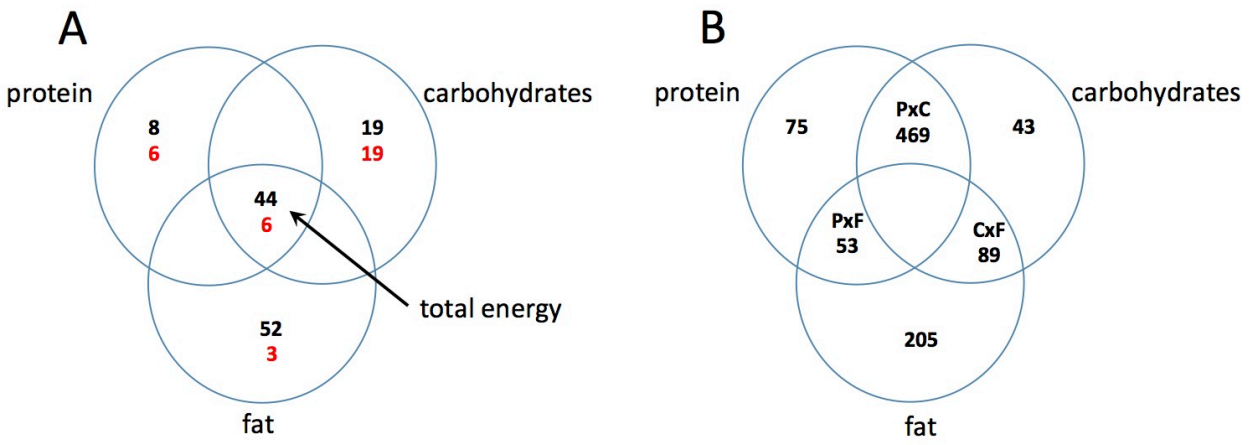


Figure 4.11. Venn diagrams showing the effects of macronutrient intake on hypothalamic gene expression. In addition to macronutrients, total energy is also shown in Figure 4.11A, with results determined using Pearson's correlation coefficient (black positive correlation, red negative correlation). Figure 4.11B uses GF analysis, with macronutrient ratio results also shown (PxC, PxF, CxF interactive terms between protein, carbohydrates and fat).

Table 4.9. Gene expression with the highest statistical association with macronutrient intake determined using either GF or correlation analysis in the hypothalamus. Benjamini Hochberg correction with a FDR of 0.05 has been performed for the p-values. Expression is broken down into up- or down-regulation.

Protein

GF		Correlation		
Gene	**p-value**	**Gene**	**Expression**	**p-value**
Hgs	9.25E-07	Spg21	Up	2.51E-04
Sass6	1.02E-06	D630014A15Rik	Down	2.95E-04
Tmem87a	8.56E-06	BC031181	Up	7.23E-04
LOC100046457	8.90E-06	Arl3	Down	9.52E-04
Psat1	9.00E-06	Asah3l	Down	1.24E-03
Ivns1abp.2	1.37E-05	Trpm6	Up	1.38E-03
Atp1b1	1.78E-05	LOC100045680	Down	1.48E-03
Psma7.1	2.66E-05	LOC381420	Down	2.01E-03
X4933426M11Rik	3.76E-05	Gtpbp6	Up	2.45E-03
LOC329416	4.12E-05	Sec22c	Down	2.50E-03

Carbohydrates

GF		Correlation		
Gene	**p-value**	**Gene**	**Expression**	**p-value**
A730094H17Rik	1.31E-11	4930538K18Rik	Down	2.79E-05
LOC386330	8.12E-11	Pik3ip1	Down	1.41E-04
X2810403A07Rik	1.81E-10	Txnrd3	Down	1.96E-04
Ptprs	2.43E-10	Odf2	Down	2.03E-04
X9530053J19Rik	7.60E-10	Aloxe3	Down	2.26E-04
X4933439J20Rik	9.58E-10	6330444G18Rik	Up	2.65E-04
Kcnq2.1	1.30E-09	Setd1a	Down	2.70E-04
Adrbk2	1.84E-09	Man1b1	Down	2.94E-04
Uqcr	1.90E-09	Eng	Up	2.95E-04
X9430091F09Rik	3.07E-09	Zfp704	Down	3.13E-04

Fat

GAMS		Correlation		
Gene	**p-value**	**Gene**	**Expression**	**p-value**
X4933421H10Rik	2.77E-09	Tmed9	Up	3.73E-05
E030007H10Rik	1.36E-08	Zfr	Up	5.16E-05
Csnk1g2	3.15E-08	BC005537	Up	1.16E-04
Loxl1	2.23E-07	LOC100039786	Up	1.59E-04
Pgcp	2.95E-07	Pbrm1	Up	1.71E-04
Vamp8	3.44E-07	Sui1-rs1	Up	1.74E-04
Bcl2a1b	4.30E-07	2400001E08Rik	Up	1.77E-04
Supt16h.1	6.00E-07	Tmem176b	Up	1.92E-04
Unk	1.14E-06	LOC268700	Up	2.18E-04
Hspg2	1.26E-06	Tmed2	Up	2.60E-04

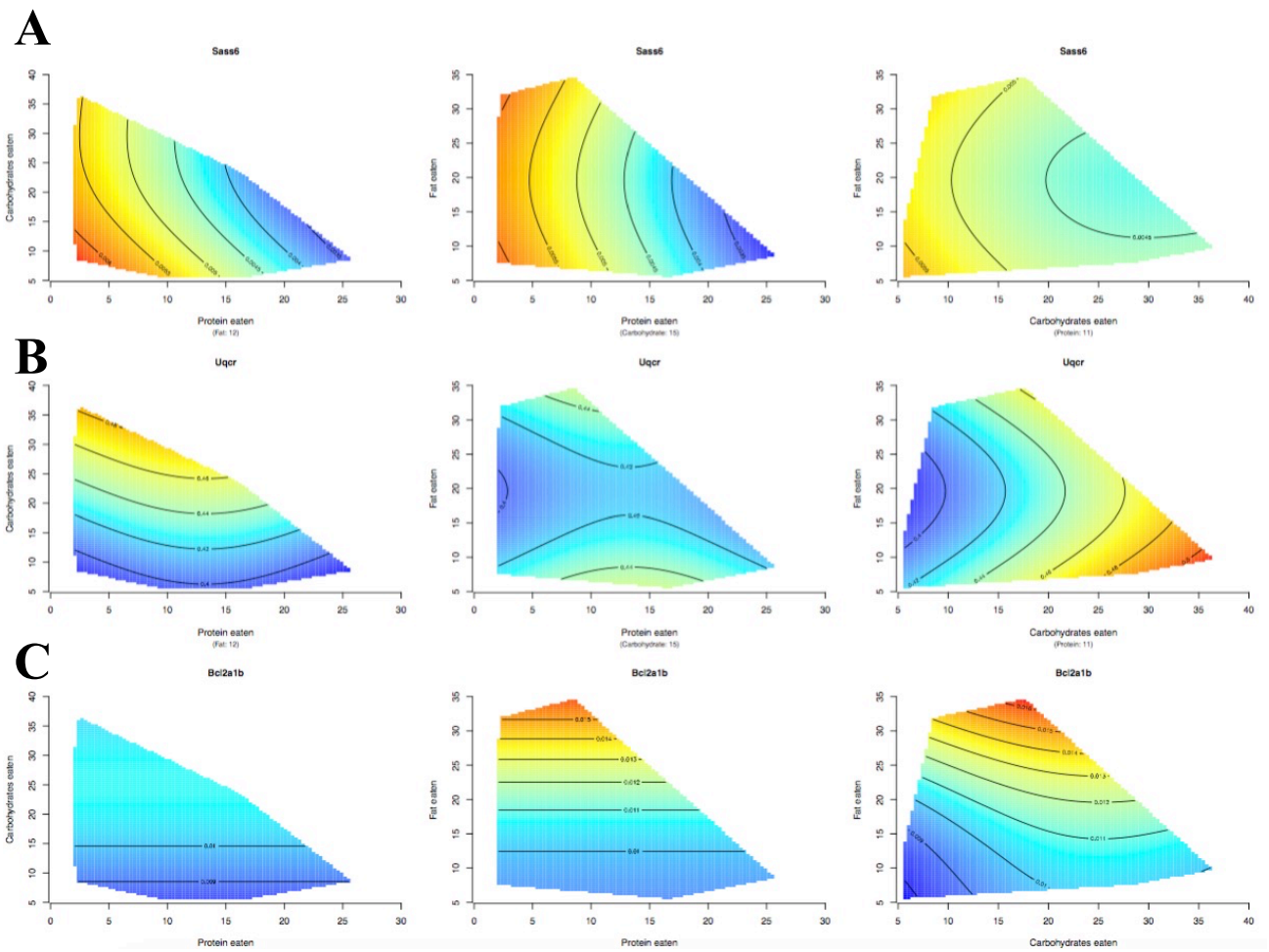


Figure 4.12. Representative three dimensional response surfaces created using the GF. The relationships between the macronutrients and the hypothalamic genes are demonstrated using the colour maps, with red being the most positive response, and blue being the most negative response. Slices are taken through the median of each of the axes (shown in parenthesis below the x-axis label). Figure 4.12A is a representative surface for correlation with protein intake on the *Sass6* gene. Figure 4.12B is a representative surface for correlation with carbohydrate intake on the *Uqcr* gene, and Figure 4.12C is a representative surface for correlation with fat intake on the *Bcl2a1b* gene. GAM statistics are provided in Table 4.10.

Table 4.10. Statistics for the Geometric Framework figures in Figure 4.12. GAMS data for hypothalamic expression of *Sass6, Uqcr,* and *Bcl2a1b*.

	edf	df	F	p-value
Sass6				
Protein	9.73E-01	8	12.497	1.02E-06
Carbohydrates	7.46E-01	8	2.781	2.89E-02
Fat	4.69E-01	8	0.641	1.17E-01
P x C	1.42E+00	3	0.000	1.22E-02
P x F	7.74E-01	3	0.000	1.18E-01
C x F	1.22E+00	3	0.000	2.66E-02
Uqcr				
Protein	9.02E-01	8	0.646	3.18E-03
Carbohydrates	1.93E+00	8	4.567	1.90E-09
Fat	1.40E-05	8	1.150	6.62E-01
P x C	1.78E+00	3	0.000	2.09E-07
P x F	1.78E+00	3	0.000	1.09E-06
C x F	1.28E+00	3	0.000	2.43E-03
Bcl2a1b				
Protein	3.32E-05	8	0.000	9.38E-01
Carbohydrates	5.89E-01	8	4.089	9.69E-02
Fat	9.75E-01	8	15.509	4.30E-07
P x C	5.75E-01	3	0.000	2.15E-01
P x F	1.63E-05	3	0.000	7.77E-01
C x F	1.60E+00	3	0.000	1.05E-02

Similar to the results from hepatic gene expression in the same cohort of animals, heatmap analysis [See Figure 4.13] showed a strong inverse relationship between gene expressions correlating to protein intake compared with fat intake.

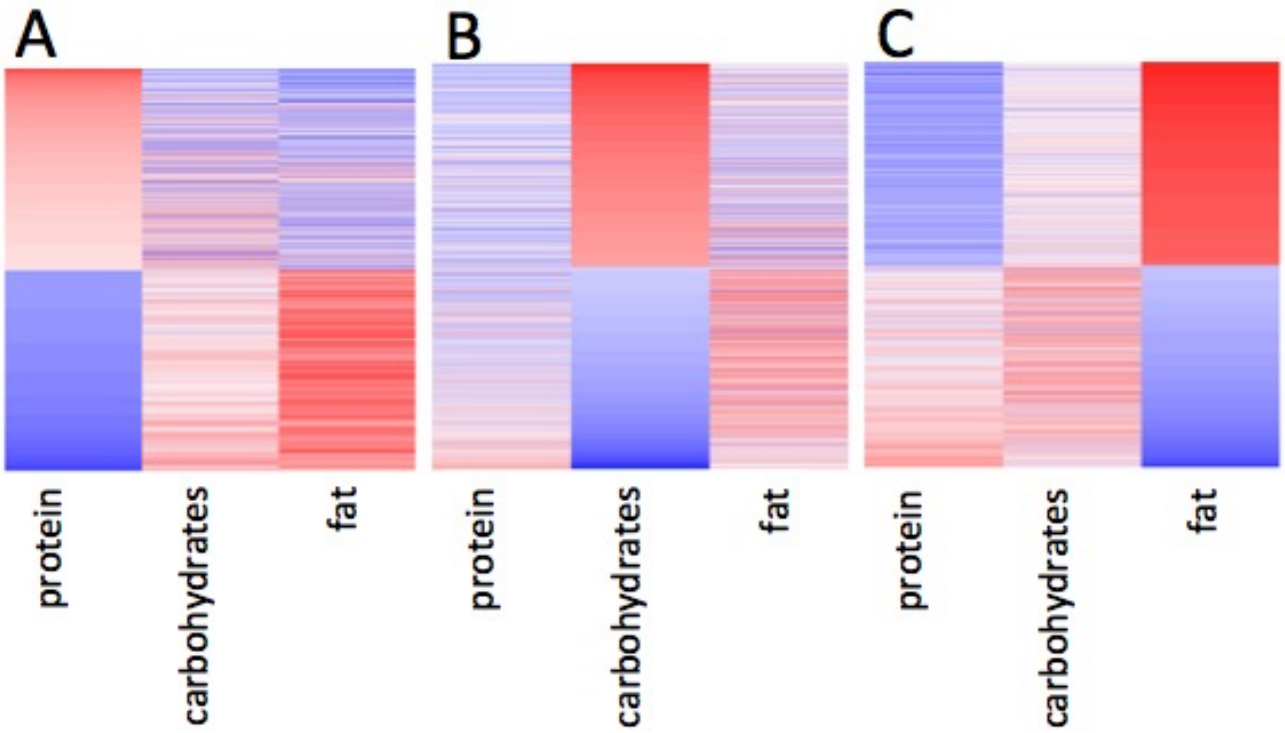


Figure 4.13. Heatmaps comparing the effects of protein, carbohydrates and fat on overall gene expression patterns in the hypothalamus. Here, the values are Pearson's correlation coefficients ranked from most positive correlation in red to the most negative correlation in blue. The most positive 1000 genes and the most negative 1000 genes are included. The genes have been ranked according to significance of the correlation with protein (Figure 4A), carbohydrates (Figure 4B) and fat (Figure 4C).

The effects of macronutrients on hypothalamic genes related to biological processes

Enrichr gene pathway analysis performed using the KEGG and GO biological process databases is outlined in Tables 4.11 and 4.12. Gene pathway analysis here is based on significant correlation alone, and does not differentiate between positive and negative correlations. Protein was found to be involved with several pathways associated with metabolism and biosynthesis of macronutrients, as well as inflammation, in particular the regulation of NF-kB. Carbohydrate intake was associated with pathways involved in mineralocorticoid and fluid homeostasis, as well as activity of ion transport channels. Fat on the other hand, which affected the most number of genes, was associated primarily with protein transport and formation pathways, including multiple processes related to the endoplasmic reticulum, and Golgi apparatus. The protein-to-carbohydrate ratio had multiple correlations to disease pathways, particularly neurodegenerative diseases, as well as protein translation, transportation and localisation through SRP-mediated protein targeting [See Table 4.13].

The effect of macronutrients on longevity regulating pathways in the hypothalamus

43 of the 59 genes that are described in the KEGG 'longevity regulating pathways, multiple species' gene set (ko0413, http://www.genome.jp/dbget-bin/www_bget?pathway+ko04213) were present in this microarray data set, some with two variants. GF analysis revealed that 28 of these genes had their expression significantly influenced by macronutrient intake [Table 4.14]. Of these, 13 were correlated with P:C ratio, and 12 to carbohydrate intake, with protein and fat influencing 6 genes each. Correlation coefficients of these genes have been plotted on a heatmap to each of the macronutrients, as well as total energy [See Figure 4.14]. Once again, protein and fat seemed to have inverse effects on gene regulation.

Table 4.11. Gene pathway analysis based on the KEGG database in Enrichr. The hypothalamic genes of interest were determined using either Pearson's correlation coefficient or GAMS to evaluate the relationship between each macronutrient and gene expression, and a FDR of 0.05.

Protein

Pathway	**p-value**
Carbon metabolism	8.83E-03
Aldosterone-regulated sodium reabsorption	9.40E-03
Carbohydrate digestion and absorption	1.24E-02
Biosynthesis of amino acids	3.15E-02
Proximal tubule bicarbonate reclamation	8.28E-02
Biosynthesis of unsaturated fatty acids	8.28E-02
Circadian rhythm	1.07E-01
Herpes simplex infection	1.53E-01
cAMP signaling pathway	1.71E-01
Viral carcinogenesis	1.79E-01

Carbohydrates

Pathway	**p-value**
Chemokine signaling pathway	7.54E-03
Proximal tubule bicarbonate reclamation	4.83E-02
Aldosterone-regulated sodium reabsorption	8.06E-02
Vasopressin-regulated water reabsorption	9.04E-02
Carbohydrate digestion and absorption	9.24E-02
Endocrine and other factor-regulated calcium reabsorption	9.63E-02
N-Glycan biosynthesis	1.00E-01
Mineral absorption	1.04E-01
Endocytosis	1.07E-01
Arachidonic acid metabolism	1.25E-01

Fat

Pathway	**p-value**
Protein export	2.30E-02
Systemic lupus erythematosus	5.04E-02
Ubiquitin mediated proteolysis	5.27E-02
Viral carcinogenesis	6.03E-02
Glycine, serine and threonine metabolism	6.33E-02
Endocrine and other factor-regulated calcium reabsorption	8.37E-02
Hedgehog signaling pathway	9.30E-02
Alzheimer's disease	9.47E-02
Protein processing in endoplasmic reticulum	9.62E-02
Alcoholism	1.12E-01

Table 4.12. Gene pathway analysis based on the GO Biological process database in Enrichr. The hypothalamic genes of interest were determined using either Pearson's correlation coefficient or GAMS to evaluate the relationship between each macronutrient and gene expression, and a FDR of 0.05.

Protein

GO Biological Process	**p-value**
Central nervous system myelination	4.92E-03
Negative regulation of co-receptor activity involved in epidermal growth factor receptor signaling pathway	6.01E-03
Extra-ocular skeletal muscle development	7.20E-03
Cardiac muscle cell contraction	8.05E-03
Negative regulation of NF-kappaB import into nucleus	1.19E-02
Positive regulation of receptor internalisation	1.24E-02
Negative regulation of epidermal growth factor-activated receptor activity	1.46E-02
Cytoplasmic sequestering of NF-kappaB	1.69E-02
Regulation of neuron migration	1.81E-02
Positive regulation of cytokine production involved in inflammatory response	2.41E-02

Carbohydrates

GO Biological Process	**p-value**
positive regulation of voltage-gated potassium channel activity	1.25E-04
positive regulation of calcium:sodium antiporter activity	1.25E-04
positive regulation of large conductance calcium-activated potassium channel activity	2.01E-04
atrial cardiac muscle cell to AV node cell communication by electrical coupling	4.04E-04
membrane repolarisation during SA node cell action potential	4.66E-04
SA node cell to atrial cardiac muscle cell communication by electrical coupling	4.66E-04
membrane repolarisation during atrial cardiac muscle cell action potential	7.54E-04
regulation of cardiac muscle contraction by regulation of the release of sequestered calcium ion	9.24E-04
positive regulation of potassium ion transmembrane transporter activity	1.11E-03
membrane repolarisation during ventricular cardiac muscle cell action potential	1.11E-03

Fat

GO Biological Process	**p-value**
Endoplasmic reticulum tubular network formation	3.59E-03
Endoplasmic reticulum tubular network maintenance	2.12E-03
Positive regulation of PERK-mediated unfolded protein response	3.59E-03
Endoplasmic reticulum tubular network membrane organisation	4.46E-03
PERK-mediated unfolded protein response	6.45E-03
Negative regulation of protein localisation to plasma membrane	6.45E-03
Negative regulation of Golgi to plasma membrane protein transport	7.57E-03
Positive regulation of actin filament bundle assembly	8.77E-03
Negative regulation of cytoplasmic translational initiation	1.29E-02
Protein autoubiquitination	1.57E-02

Table 4.13. Gene pathway analysis based on the KEGG and GO biological process databases in Enrichr for the protein-to-carbohydrate ratio. The hypothalamic genes of interest were determined using either Pearson's correlation coefficient or GAMS to evaluate the relationship between each macronutrient and gene expression, and a FDR of 0.05.

Protein-to-Carbohydrate ratio (P x C)	
KEGG Pathway	p-value
SRP-dependent co-translational protein targeting to membrane, translocation	1.02E-05
Cytoplasmic translation	8.71E-05
SRP-dependent cotranslational protein targeting to membrane, docking	4.02E-05
Maintenance of translational fidelity	2.52E-04
Plastid translation	2.52E-04
N-terminal peptidyl-proline demethylation involved in translation	2.68E-04
SRP-dependent cotranslational protein targeting to membrane, signal sequence recognition	1.42E-04
Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	1.76E-04
Translational termination	3.82E-04
Translational elongation	4.52E-04
GO Biological Process	p-value
SRP-dependent cotranslational protein targeting to membrane, translocation	1.02E-05
Cytoplasmic translation	8.71E-05
SRP-dependent cotranslational protein targeting to membrane, docking	4.02E-05
Maintenance of translational fidelity	2.52E-04
Plastid translation	2.52E-04
N-terminal peptidyl-proline dimethylation involved in translation	2.68E-04
SRP-dependent cotranslational protein targeting to membrane, signal sequence recognition	1.42E-04
Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	1.76E-04
Translational termination	3.82E-04
Translational elongation	4.52E-04

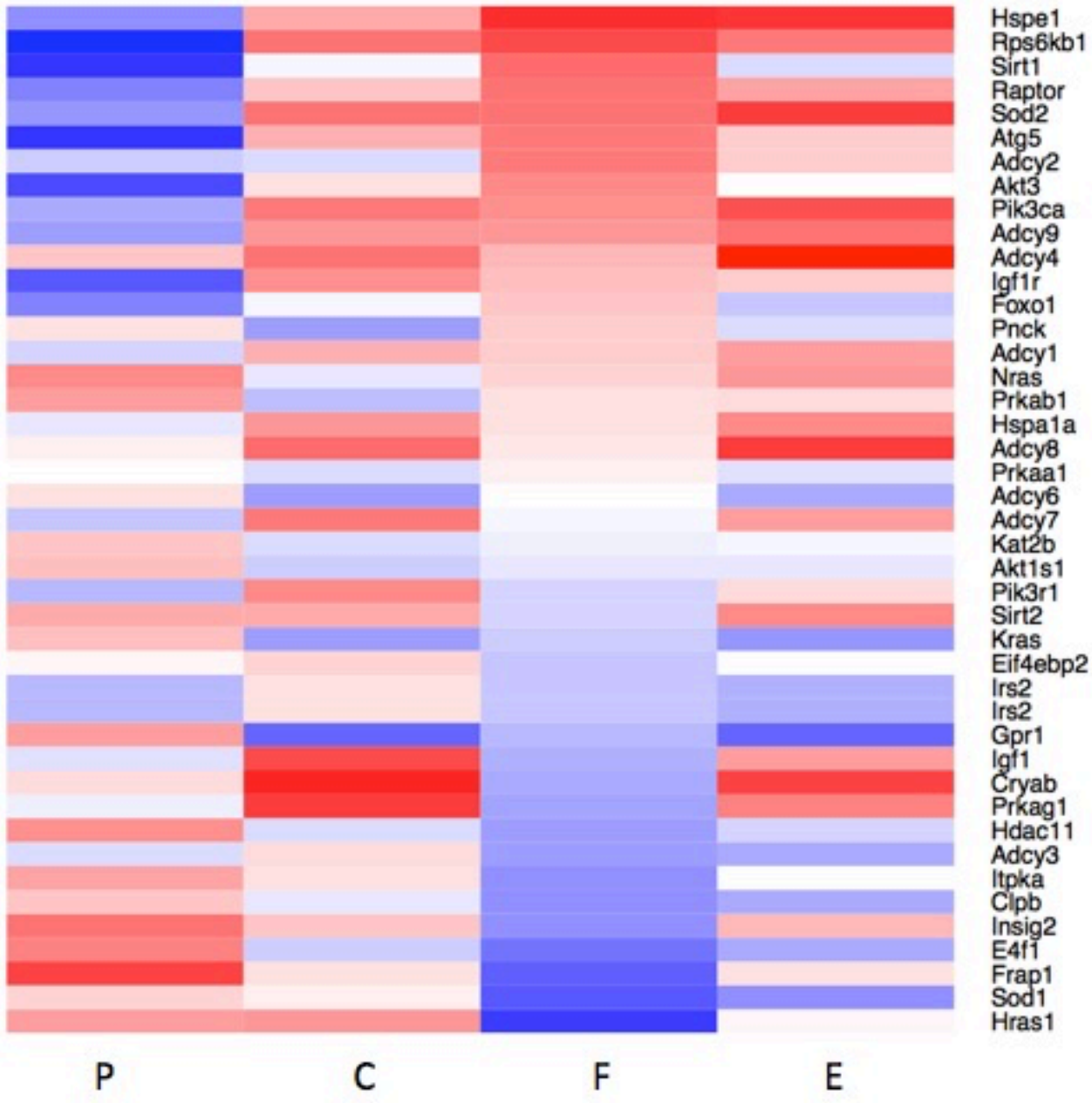


Figure 4.14. Heatmap showing the relationship between longevity regulating genes in the hypothalamus according to KEGG, and the correlation coefficients that link each of these genes with protein, carbohydrates, fat and total energy intake. Positive correlation coefficients are shown in red and negative coefficients in blue. Full results are provided in Table 4.14.

Table 4.14. Pearson correlations and GF analysis for hypothalamic genes identified in KEGG 'longevity regulating pathways, multiple species' gene set (ko0413, http://www.genome.jp/dbget-bin/www_bget?pathway+ko04213). Pearson correlations are denoted as cor(X), where X refers to either protein (P), carbohydrate (C), fat (F), or energy (E). GF p-values are denoted as pval(X), with X referring to the same nomenclature as above. Non-significant values are left blank.

Gene	cor(P)	cor(C)	cor(F)	cor(E)	pval(P)	pval(C)	pval(F)	pval(PxC)	pval(PxF)	pval(CxF)
Hspe1	-0.257	0.191	0.481	0.452			**1.02E-04**			**1.02E-02**
Rps6kb1	-0.519	0.309	0.404	0.301	**3.43E-02**			**9.57E-04**	**2.34E-03**	**1.10E-03**
Sirt1	-0.461	-0.019	0.328	-0.078	**2.42E-02**			**2.80E-02**		
Raptor	-0.292	0.131	0.322	0.212			**3.46E-02**	**3.30E-02**		
Sod2	-0.231	0.314	0.313	0.449				**6.23E-04**		
Atg5	-0.461	0.174	0.308	0.111	**4.09E-02**			**6.56E-03**		
Adcy2	-0.104	-0.072	0.297	0.117				**5.05E-03**		
Akt3	-0.407	0.058	0.270	-0.007						
Pik3ca	-0.192	0.295	0.248	0.398						
Adcy9	-0.214	0.243	0.232	0.310						
Adcy4	0.124	0.321	0.162	0.594		**1.44E-03**				
Igf1r	-0.373	0.252	0.137	0.106	**1.34E-02**					
Foxo1	-0.289	-0.018	0.125	-0.130	**5.35E-03**		**9.09E-03**			
Pnck	0.065	-0.216	0.114	-0.077						
Adcy1	-0.094	0.177	0.109	0.219						
Nras	0.267	-0.055	0.099	0.242				**5.99E-03**		
Prkab1	0.222	-0.146	0.067	0.080			**1.82E-02**			
Hspa1a	-0.050	0.232	0.066	0.273						**4.36E-02**
Adcy8	0.037	0.338	0.048	0.438		**2.04E-03**				

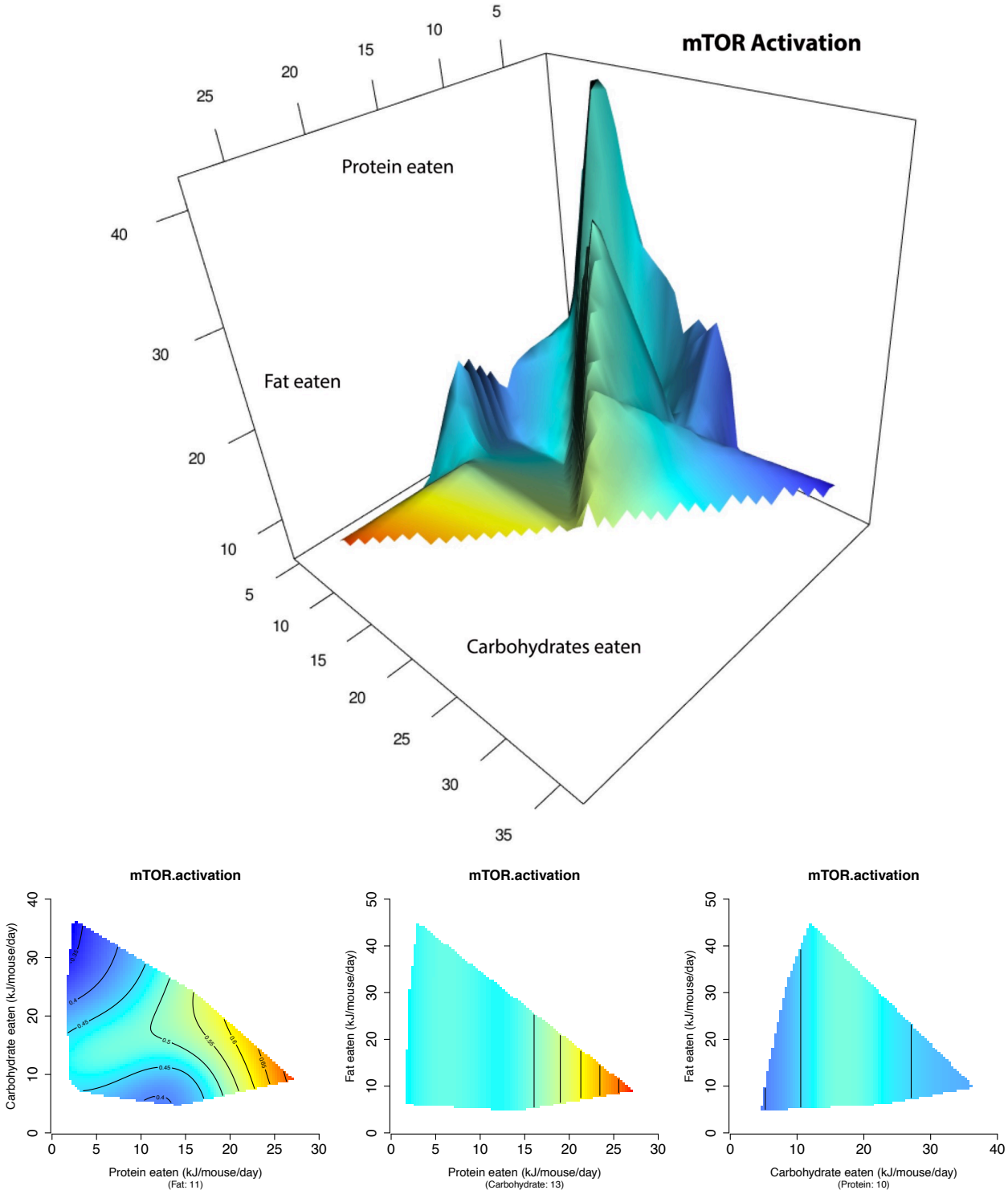
Prkaa1	-0.003	-0.087	0.034	-0.065						
Adcy6	0.064	-0.225	-0.002	-0.194			**1.37E-02**			
Adcy7	-0.126	0.307	-0.010	0.223			**4.62E-02**	**2.86E-03**		
Kat2b	0.127	-0.074	-0.032	-0.010						
Akt1s1	0.148	-0.111	-0.044	-0.044				**4.02E-02**		**9.90E-03**
Pik3r1	-0.157	0.271	-0.095	0.081		**5.07E-03**		**1.06E-02**		
Sirt2	0.196	0.191	-0.098	0.270		**4.25E-02**				
Kras	0.135	-0.222	-0.111	-0.236						
Eif4ebp2	0.021	0.091	-0.119	0.003						
Irs2	-0.159	0.057	-0.127	-0.181		**7.34E-03**				
Irs2	-0.159	0.057	-0.127	-0.181		**7.34E-03**				
Gpr1	0.229	-0.353	-0.163	-0.352						
Igf1	-0.069	0.407	-0.175	0.223		**2.87E-02**				
Cryab	0.076	0.507	-0.191	0.430		**3.67E-06**		**3.16E-02**		
Prkag1	-0.028	0.450	-0.199	0.278		**9.55E-03**				
Hdac11	0.256	-0.087	-0.220	-0.097						
Adcy3	-0.081	0.075	-0.226	-0.193						
Itpka	0.210	0.062	-0.248	0.001						
Clpb	0.123	-0.050	-0.248	-0.189						
Insig2	0.323	0.128	-0.250	0.160		**4.07E-03**				
E4f1	0.284	-0.110	-0.312	-0.185						
Frap1	0.432	0.058	-0.357	0.071		**5.59E-03**			**4.58E-02**	
Sod1	0.101	0.033	-0.388	-0.246				**8.66E-03**		
Hras1	0.228	0.232	-0.449	0.012	**1.90E-02**	**1.15E-03**		**2.54E-02**	**4.48E-03**	**7.85E-04**

mTOR activation in the liver

Given that the majority of the genes in the liver that were significantly altered by diet were primarily influenced by protein intake, mTOR was further analysed, as its activation is known to be regulated by circulating amino acids, through its role as a dietary protein sensor. As mTOR gene expression was assayed through both microarray and PCR, a western blot was performed to look at the ratio of phosphorylated mTOR to total mTOR, to study the dynamics of mTOR activation. Here, it was shown that protein intake had a significant effect ($p=0.0281$) on mTOR activation, with mTOR positively correlated to protein intake [See Figure 4.15].

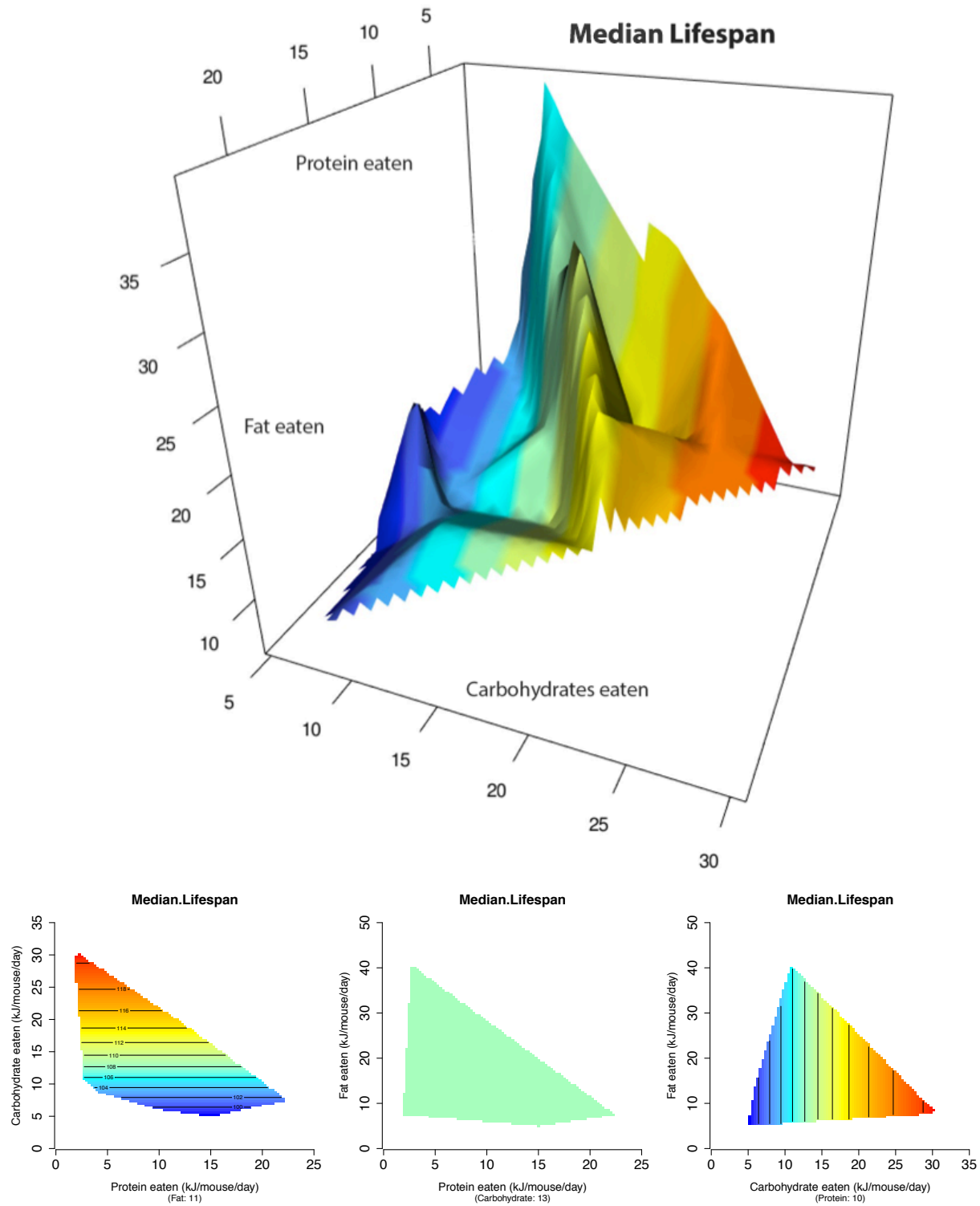
While protein is shown to be the key driver of mTOR, high protein diets were the ones with the highest P:C ratio. Similarly with the lifespan data shown in Figure 4.16, the statistically significant driver is carbohydrate intake ($p=0.0132$), however the diets highest in carbohydrate are those low in P:C ratio [Table 4.15]. Therefore, while there isn't direct alignment of mTOR activation and lifespan along a single macronutrient axis, the ratio of protein to carbohydrates plays an important role in both these responses in opposite directions. The diet group with the highest lifespan was aligned with a local minimum in the mTOR activation response surface. The highest median lifespan was actually seen in a relatively high P:C ratio group, however when looking at the trend across groups, increasing P:C ratio was negatively correlated with both median and maximum lifespan. Given that mTOR is generally considered as a pro-ageing growth pathway, these findings are in line with the literature.

Interestingly, mTOR activation was not significantly correlated with mTOR gene expression ($r=-0.052$, $p=0.74$ [Figure 4.17]. This raises the perennial question as to whether gene expression should be interpreted as a separate entity to protein activation. It should be noted that both mTOR activation and gene expression, are significantly correlated to protein intake, but in different directions, as shown in the GF plots in the next section.



p-value	P	C	F	P:C	P:F	C:F
mTOR	**2.8E-02**	0.48	0.53	0.08	0.70	0.54

Figure 4.15. GF response surfaces correlating hepatic mTOR activation to macronutrient intake.



p-value	P	C	F	P:C	P:F	C:F
Median Lifespan	0.50	**1.3E-02**	1	0.99	0.97	0.62

Figure 4.16. GF response surfaces correlating median lifespan to macronutrient intake. Data presented in Table 4.15.

Table 4.15. Median and maximum lifespan in weeks. Maximum lifespan was determined as the average of the longest lived 10% (n=2-3) of each cohort. Table from (Solon-Biet et al. 2014).

Energy Density	Protein (%)	Carb (%)	Fat (%)	Protein: Carb ratio	Median lifespan	Maximum lifespan
Medium	5	75	20	0.07	121.86	157.43
High	5	20	75	0.25	106.43	154.21
High	5	75	20	0.07	119.43	151.79
Medium	14	57	29	0.25	123	151.57
High	42	29	29	1.45	138.86	151.14
Medium	42	29	29	1.45	122.57	148
Medium	14	29	57	0.48	113.86	147.36
High	5	48	48	0.1	124.43	146.21
Medium	33	48	20	0.69	122.57	145.71
Medium	23	38	38	0.61	123.86	143.07
High	33	48	20	0.69	98.29	141
High	14	57	29	0.25	117.43	140.07
High	33	20	48	1.65	107.14	136.86
Low	33	48	20	0.69	126.57	134.14
Medium	33	20	48	1.65	106.57	133.79
High	14	29	57	0.48	108	133.71
Medium	60	20	20	3	108	129.5
High	60	20	20	3	99.57	127.57
High	23	38	38	0.61	100	124.57
Low	14	57	29	0.25	98.57	119.43
Low	33	20	48	1.65	78.57	116.36
Low	14	29	57	0.48	88.71	115.07
Low	42	29	29	1.45	85.85	104
Low	60	20	20	3	84.29	102.86
Low	23	38	38	0.61	89.29	100.36

The relationships between nutrient sensing pathways and phenotype in the liver

Based on published literature, there are several canonical pathways that link diet and ageing. These include the sirtuin pathways, mTOR, AMPK, IIS and FGF21 (Solon-Biet et al. 2015b). This study measured parameters related to several of these pathways at 15 months of age. It was not possible to correlate these pathways directly with life expectancy in the same animals, however, it was possible to perform indirect correlations via diet, i.e. the measures of the pathways measured at 15 months in mice on a particular diet was correlated with the lifespan of mice maintained on the same diet. Collating all the information collected on growth pathways in these animals, it is apparent that the majority of pathways which are significantly responsive to diet are affected by protein intake [See Table 4.16]. These results support the hypothesis that macronutrient intake, particularly protein, is responsible for modulating nutrient sensing intracellular growth pathways, whose downstream effects could impact ageing and health.

Here, insulin, IGF-1 and FGF21 were measured in plasma from blood samples, and median lifespan was correlated from animals in the same dietary groups. The remainder of the assays; PCR, microarray and western blots; were performed from liver samples to ensure consistency of the sample tissue. These results were also compared to median lifespan in the same cohort of animals, however, since the lifespan data is from different animals, the results are only correlated via diet, limiting the conclusions that can be drawn. While mTOR and IIS pathways are inversely correlated with SIRT, FGF21 and AMPK pathways as expected, interestingly, only energy intake and FGF21 were significantly correlated with median lifespan [See Figure 4.17]. However it is important to note that this analysis was performed by simple Pearson correlations, and therefore less meaningful given the multidimensional nature of this nutritional study compared to the GF plots, shown in Figure 4.18.

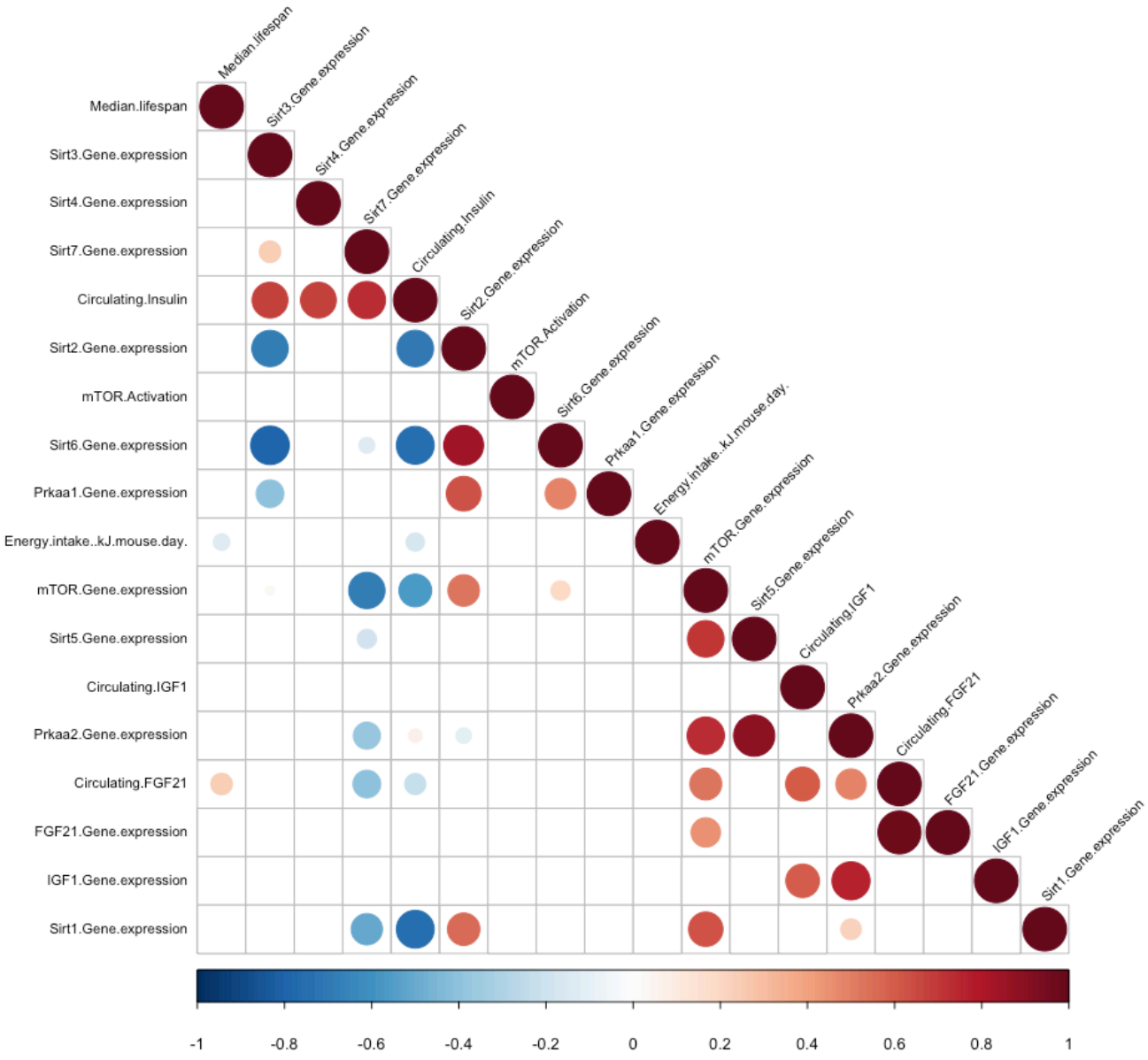
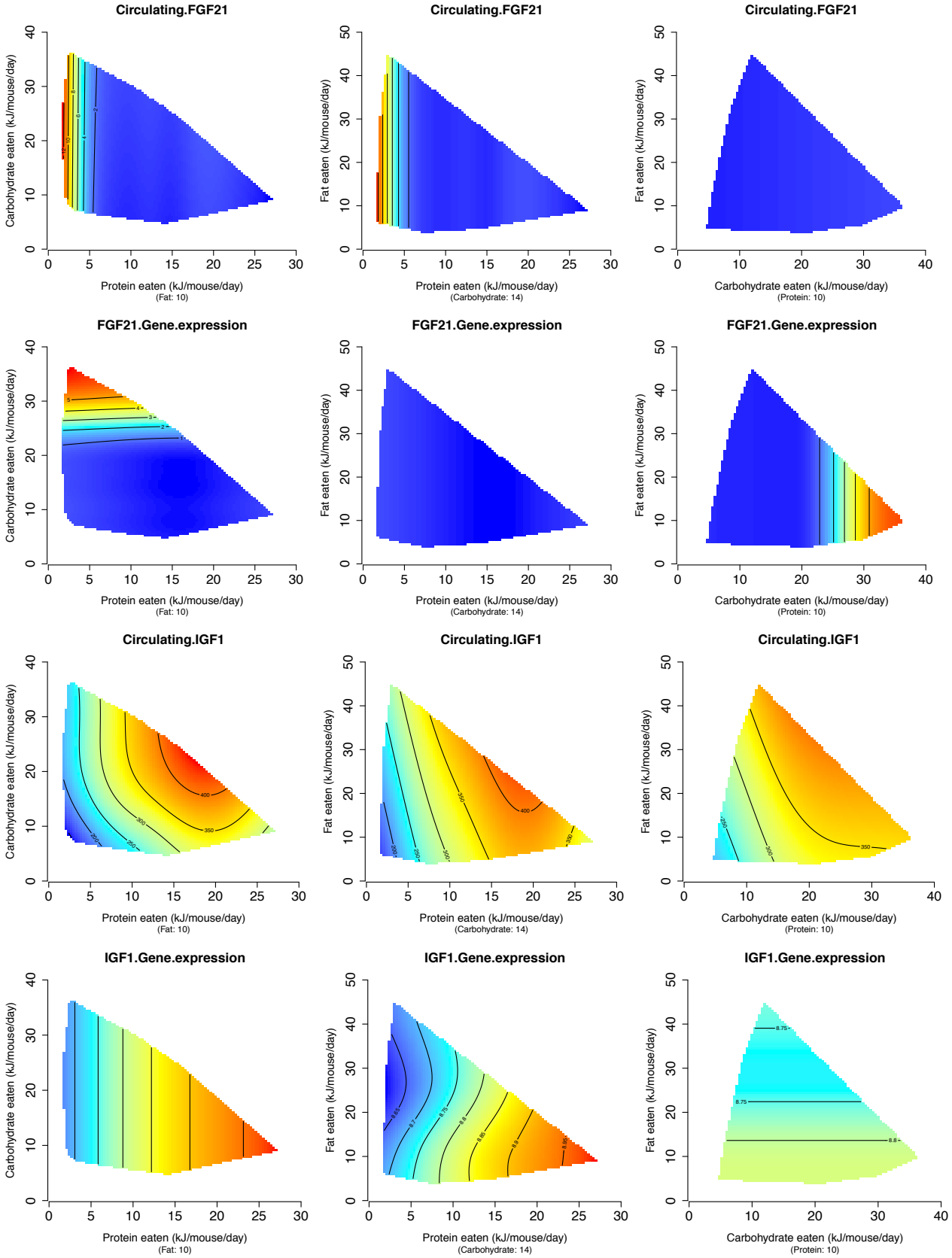


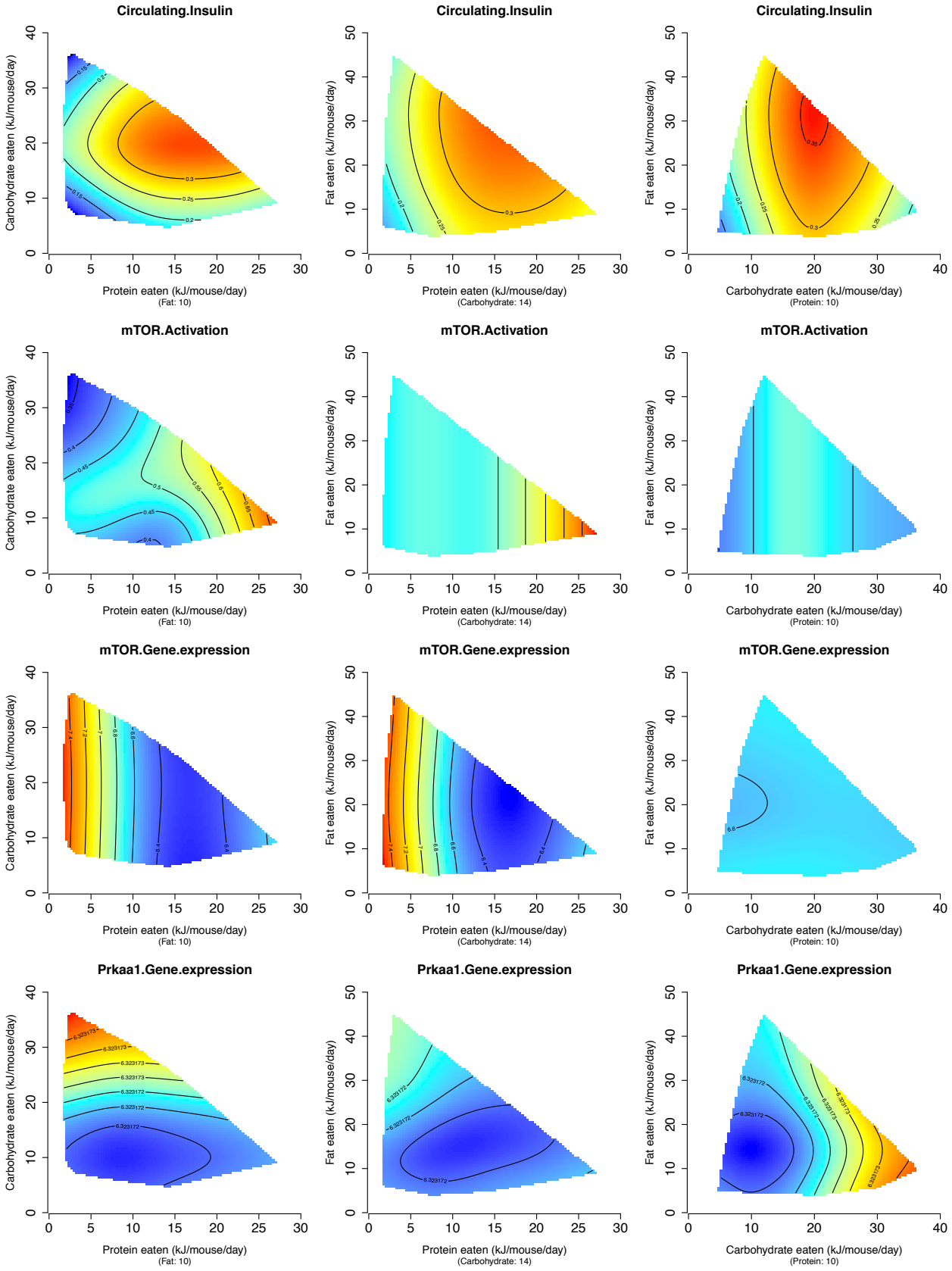
Figure 4.17. Nutrient sensing pathway correlations to each other and to median lifespan (liver gene expression and blood levels). Blank results indicate non-significant correlations ($p>0.05$). The correlations show that SIRT/FGF21/AMPK expression is opposite to mTOR/IIS, in line with the literature given that the latter set are considered pro-ageing, while the former set are considered pro-longevity (Solon-Biet et al. 2015b).

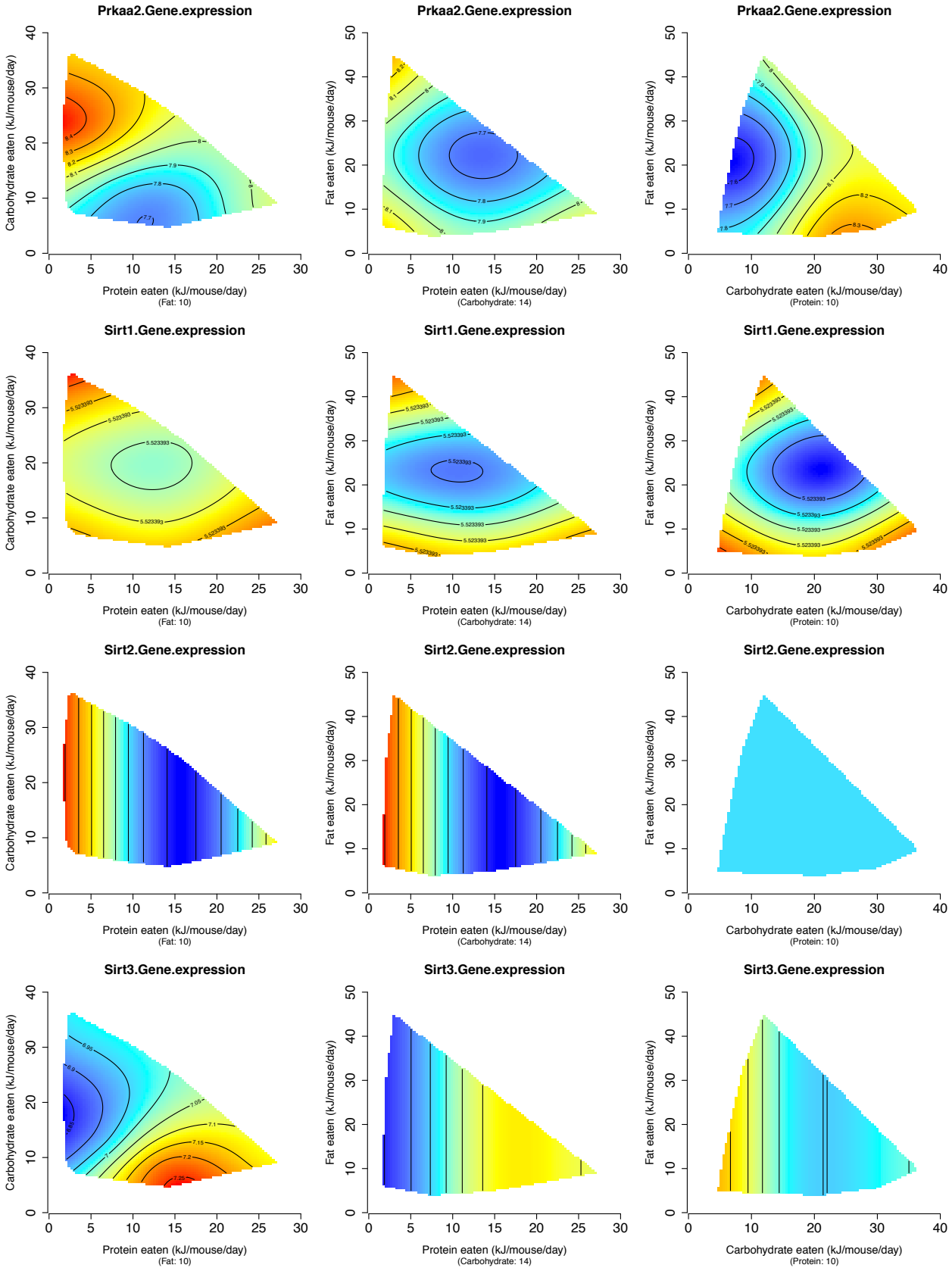
Table 4.16. Nutrient sensing pathway in liver analysis associated with macronutrient intake using GAMs. The surfaces are shown in Figure 4.18.

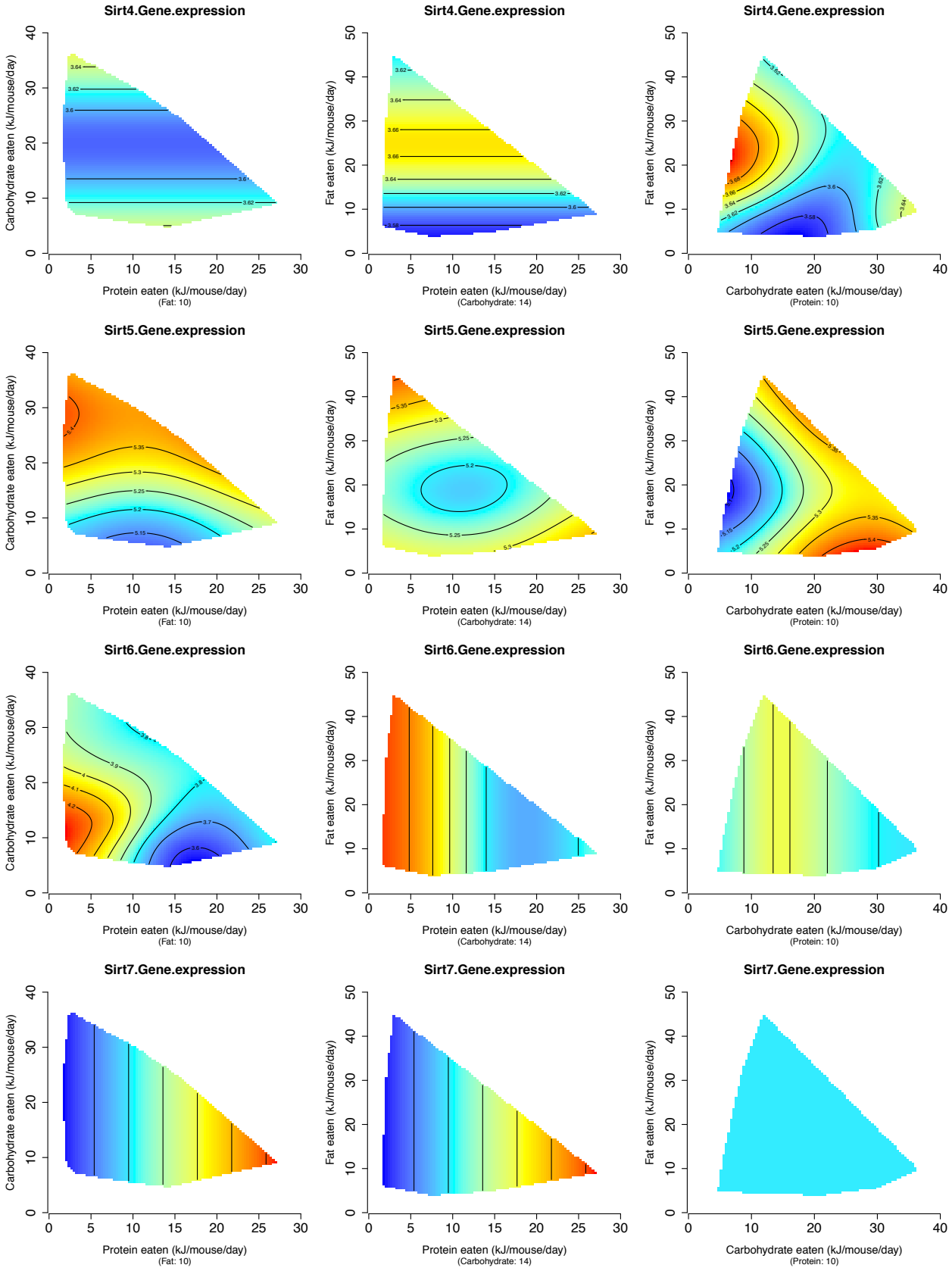
Pathway	Assay	P	C	F	P:C	P:F	C:F
FGF21	Plasma (protein)	**1.41E-29**	0.24	0.81	0.41	0.60	0.44
FGF21	PCR (gene)	**1.91E-10**	0.36	1	1	0.648	0.67
IGF1	Plasma (protein)	**2.76E-04**	**3.91E-02**	0.08	0.08	0.48	1.00
IGF1	PCR (gene)	**1.93E-02**	0.35	0.57	1	0.15	0.62
Insulin	Plasma	**7.74E-04**	**6.66E-05**	0.06	0.16	0.17	0.28
Median lifespan	Correlated measure	0.50	**1.30E-02**	1	0.99	0.97	0.62
mTOR	Western blot (protein)	**2.81E-02**	0.48	0.53	0.08	0.70	0.54
mTOR	PCR (gene)	**1.45E-05**	0.51	0.77	0.36	0.29	0.29
Prkaa1	PCR (AMPK α-subunit 1)	0.82	0.50	1.00	0.73	0.71	0.68
Prkaa2	PCR (AMPK α-subunit 2)	0.55	0.61	0.54	**1.05E-03**	0.32	0.08
SIRT1	PCR	0.66	0.68	0.69	0.77	0.79	0.71
SIRT2	PCR	**7.74E-03**	0.59	0.66	0.34	0.40	0.40
SIRT3	PCR	0.20	0.21	1.00	0.06	0.72	0.91
SIRT4	PCR	0.53	0.98	0.46	0.91	0.97	0.22
SIRT5	PCR	0.78	0.09	0.39	0.41	0.22	0.35
SIRT6	PCR	0.10	0.97	0.68	**1.28E-02**	0.56	0.69
SIRT7	PCR	**2.19E-03**	0.47	0.73	0.80	0.53	0.81

Figure 4.18. 2D GF plots for hepatic nutrient sensing pathway assays, including circulating and tissue protein levels, and gene expression in the liver (statistical analysis provided in Table 4.16).




Circulating.Insulin
Carbohydrate eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Fat: 10)
Circulating.Insulin
Fat eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Carbohydrate: 14)
Circulating.Insulin
Fat eaten (kJ/mouse/day)
Carbohydrate eaten (kJ/mouse/day)
(Protein: 10)
mTOR.Activation
Carbohydrate eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Fat: 10)
mTOR.Activation
Fat eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Carbohydrate: 14)
mTOR.Activation
Fat eaten (kJ/mouse/day)
Carbohydrate eaten (kJ/mouse/day)
(Protein: 10)
mTOR.Gene.expression
Carbohydrate eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Fat: 10)
mTOR.Gene.expression
Fat eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Carbohydrate: 14)
mTOR.Gene.expression
Fat eaten (kJ/mouse/day)
Carbohydrate eaten (kJ/mouse/day)
(Protein: 10)
Prkaa1.Gene.expression
Carbohydrate eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Fat: 10)
Prkaa1.Gene.expression
Fat eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Carbohydrate: 14)
Prkaa1.Gene.expression
Fat eaten (kJ/mouse/day)
Carbohydrate eaten (kJ/mouse/day)
(Protein: 10)


Prkaa2.Gene.expression
Carbohydrate eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Fat: 10)
Prkaa2.Gene.expression
Fat eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Carbohydrate: 14)
Prkaa2.Gene.expression
Fat eaten (kJ/mouse/day)
Carbohydrate eaten (kJ/mouse/day)
(Protein: 10)
Sirt1.Gene.expression
Carbohydrate eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Fat: 10)
Sirt1.Gene.expression
Fat eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Carbohydrate: 14)
Sirt1.Gene.expression
Fat eaten (kJ/mouse/day)
Carbohydrate eaten (kJ/mouse/day)
(Protein: 10)
Sirt2.Gene.expression
Carbohydrate eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Fat: 10)
Sirt2.Gene.expression
Fat eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Carbohydrate: 14)
Sirt2.Gene.expression
Fat eaten (kJ/mouse/day)
Carbohydrate eaten (kJ/mouse/day)
(Protein: 10)
Sirt3.Gene.expression
Carbohydrate eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Fat: 10)
Sirt3.Gene.expression
Fat eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Carbohydrate: 14)
Sirt3.Gene.expression
Fat eaten (kJ/mouse/day)
Carbohydrate eaten (kJ/mouse/day)
(Protein: 10)


Sirt4.Gene.expression
Carbohydrate eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Fat: 10)
Sirt4.Gene.expression
Fat eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Carbohydrate: 14)
Sirt4.Gene.expression
Fat eaten (kJ/mouse/day)
Carbohydrate eaten (kJ/mouse/day)
(Protein: 10)
Sirt5.Gene.expression
Carbohydrate eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Fat: 10)
Sirt5.Gene.expression
Fat eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Carbohydrate: 14)
Sirt5.Gene.expression
Fat eaten (kJ/mouse/day)
Carbohydrate eaten (kJ/mouse/day)
(Protein: 10)
Sirt6.Gene.expression
Carbohydrate eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Fat: 10)
Sirt6.Gene.expression
Fat eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Carbohydrate: 14)
Sirt6.Gene.expression
Fat eaten (kJ/mouse/day)
Carbohydrate eaten (kJ/mouse/day)
(Protein: 10)
Sirt7.Gene.expression
Carbohydrate eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Fat: 10)
Sirt7.Gene.expression
Fat eaten (kJ/mouse/day)
Protein eaten (kJ/mouse/day)
(Carbohydrate: 14)
Sirt7.Gene.expression
Fat eaten (kJ/mouse/day)
Carbohydrate eaten (kJ/mouse/day)
(Protein: 10)

Discussion

Nutrient sensing pathways have been widely studied in ageing research studies, with a variety of results, largely depending on experimental paradigm (DR versus CR versus macronutrient restriction versus pharmacological intervention), species studied, tissue type used, and sex of the animal (Garratt et al. 2016). Research over the past few decades has shown that protein in particular is an important regulator of these effects on lifespan, however given the aforementioned variation, the debate remains ongoing (Nakagawa et al. 2012).

Transcriptional modulation by diet and age have been studied through the use of microarrays in the past, however the majority of these have focused on single interventions, for example ad libitum versus CR (Wanders et al. 2014). Here, the GF was harnessed to disentangle the effects of diet on transcriptional responses by evaluating relationships with specific macronutrient intakes and ratios. Genome wide changes were assessed both by unbiased methods, as well as through specific interrogation of nutrient sensing pathways, and other intracellular pathways thought to be involved with ageing. These results were then validated using quantitative real-time PCR, and correlated various other assays performed on the same cohort of mice. Together, these form a set of pathways, which can be correlated to lifespan in the same cohort of animals, to provide insights into the mechanisms underlying ageing.

Impacts of diet on the hepatic gene regulation and mTOR activation

In a review of 172 publications on the effects of nutrients on the hepatic transcriptome, (Osada 2013) reported that (1) protein intake influenced genes involved with lipogenesis, fatty acid uptake, oxidative stress and DNA methylation; (2) carbohydrate intake influenced genes involved with oxidative stress, cell proliferation, ammonium and as well as *Pparga* and *Fxr*; and (3) fat intake influenced

inflammation, beta-oxidation and several genes including *A2m, Slc13a5, Nrep, Cyp3a* and *Scd1*. The studies in the review were mostly short-term over days and weeks and used heterogeneous dietary interventions. By contrast, the long-term study examining the effects of 25 different diets varying in macronutrients over 15 months showed that protein intake was the most powerful driver of gene expression, while the intake of carbohydrates and fat had very limited effects which were in the opposite direction to changes seen with protein intake. It appears that either aging and/or very long-term dietary exposure, only protein intake remains a potent regulator of hepatic gene expression, which presumably reflects a greater biological imperative to regulate long-term protein metabolism.

There have been a few other studies that have examined the effects of differing amounts of macronutrients on hepatic gene expression over longer-term periods, and these reported similar effects to those observed with dietary protein. Diaz-Rua and colleagues (Díaz-Rúa et al. 2017) fed rats two diets differing in protein content (protein:carbohydrate:fat 20:70:10 vs 45:45:10) and determined effects on hepatic transcriptome after four months. They identified 30 genes that were the most relevant genes affected by a high protein diet in the liver, of which 14 were also identified in this study (*Slc7a2, Slc43a1, Agxt, Got1, Gpt, Hpd, Sds, Asl, Cps1, Nnmt, Slc4a2, Kcnma1, Pgam2, Dgat1*). By comparison, Schwarz et al (Schwarz et al. 2012) fed mice one of three diets (protein:carbohydrate:fat 15:75:10, 15:50:35 or 50:15:35) for 12 weeks and then measured hepatic gene expression. They identified 154 genes where expression was increased by dietary protein, and these were involved with amino acid and nitrogen metabolism or energy and oxidative metabolism and included 12 of these same genes listed above.

Gene pathway analysis of these genes (using Enrichr and the KEGG database) shows that they are involved with the biosynthesis of amino acids and the metabolism of alanine, aspartate and glutamate, while a biological process analysis (using Enrichr and the GO Biological Process 2017) shows that

they are involved with the urea cycle and amino acid biosynthetic processes. These pathways and biological processes have obvious implications for the metabolism of dietary protein. It should be noted that the quality (i.e. source) of dietary protein has also been reported to influence hepatic transcriptome (Endo et al. 2002; Song et al. 2016). It was also shown that LPHC diets are associated with longer lifespan in ad libitum-fed mice, a finding which is supported by numerous insect studies (Solon-Biet et al. 2014, Le Couteur et al. 2016). Most previous studies on nutrition and aging have shown that caloric restriction reduces aging and increases lifespan (Mercken et al. 2012; Ingram & de Cabo 2017), and there have been many studies investigating the effects of caloric restriction on gene expression (Swindell 2008; Plank et al. 2012).

Therefore gene expression results from this study were compared with published data on genes whose expression is influenced by aging or caloric restriction. When compared to the KEGG longevity regulating pathways only seven out of 59 genes were found to be common (*Atg5, Mtor, Pik3r2, Igf1r, Prkab2, Hsap1b, Adcy3*) which suggests that low protein, high carbohydrate diets might influence lifespan by alternative pathways. However, it should be noted that these seven genes include pathways that are critical to the response of aging to nutrients including autophagy, mTOR, IGF-1 and AMPK. Gene expression in this study was also compared with published data on caloric restriction from a meta-analysis (Plank et al. 2012) and only found a few genes in common (6-17% depending upon analytic method). Again this lack of overlap suggests that low protein, high carbohydrate diets influence aging via different pathways.

Therefore this study interrogated the data for pathways that are generally believed to be crucial for mediating the effects of nutrition on aging: mTOR, AMPK, SIRT1, IGF-1 and FGF21 [See Table 4.16]. Low protein intake was associated with increased *Ffg21* and *Prkab2* expression and reduced *Igf-1* expression, which reflect the direction of change seen with caloric restriction. However, surprisingly

Mtor expression was increased with low protein intake, despite the observation that mTOR phosphorylation was decreased with low protein, high carbohydrate diets [See Figure 4.18].

No significant correlation was found between mTOR phosphorylation and *Mtor* gene expression so this disparity probably reflects the differences between transcription, translation and activation [Figure 4.17]. Overall, it appears that the effects of macronutrients on gene expression in the liver do not substantially overlap with effects that have previously been found to be associated with caloric restriction or regulation of longevity, however there are some key pathways that are concordant including IGF-1, AMPK and FGF21. It should be noted that a high protein *ad libitum*-fed diet will lead to reduced energy intake through protein leverage and vice versa (Simpson and Raubenheimer 2005), therefore a high protein intake may induce changes in gene expression similar to those seen in caloric restriction because of concomitant reduction in calorie intake.

Interestingly, Pearson correlation between these pathways and median lifespan only showed a significant result for circulating FGF21 and energy intake. There are two potential explanations for this; first, direct correlations are likely inadequate given the complex nature of intake-response dynamics in many of these pathways (as highlighted by the use of the GF above), and second, the median lifespan values used here were correlations within diet groups, not actual lifespan in the mice undergoing the phenotype/genotype assays. These results are well aligned with the literature, with protein restriction, particularly BCAA restriction, being implicated partly with the effects of DR on ageing (Wanders et al. 2014; Park & Prolla 2005; Solon-Biet et al. 2014; Solon-Biet et al. 2016). Further investigation of individual subcomplexes of mTOR (mTORC1, mTORC2) would be the next important step in understanding the modulation of mTOR through diet, and its effects on ageing. Many individual genes of interest were found due to their statistical association with macronutrients and their involvement metabolic responses and aging, and some of these are discussed below. For

example, Velazquez-Villegas et al found that high ratios of dietary protein to carbohydrates (P:C:F of 50:33:17 vs 20:63:17 vs 5:77:18) fed to mice over 8 days increased the hepatic expression of glutaminase 2 (*Gls2*) (Velázquez-Villegas et al. 2016).

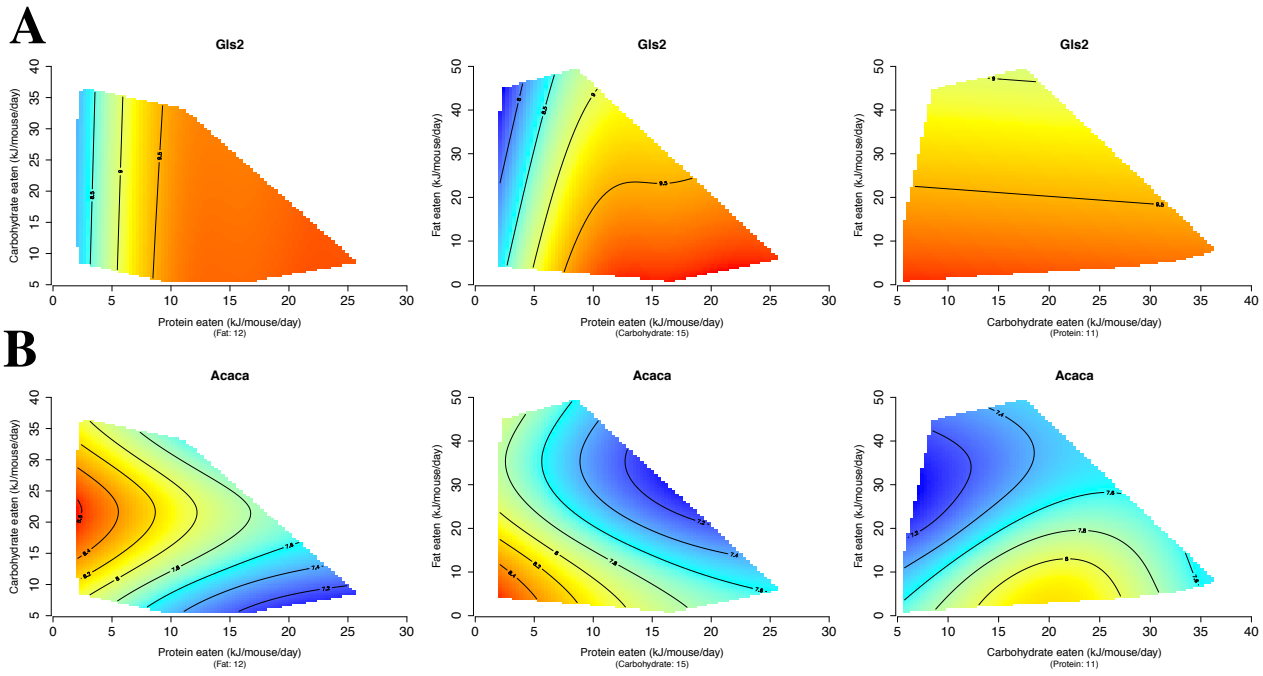


Figure 4.19. Three dimensional response surfaces created using the GF. The relationship between the macronutrients and hepatic genes (A *Gls2*, B *Acaca*) are demonstrated. Each graph shows the effects of two of macronutrients at the median point of the third macronutrient (shown in parenthesis below the X axis label). The response surfaces vary from red, which is the most negative value to blue which is the most positive value. The GAM statistics are shown in Table 4.17.

Likewise Schwarz et al found that high protein diets increased expression of *Gls2* in mice after 1 and 12 weeks (Schwarz et al. 2012). *Gls2* is the main glutaminase expressed in the liver and increases protein catabolism via the urea cycle. *Gls2* has been reported to be upregulated in response to high protein diets, inflammation and diabetes (Velázquez-Villegas et al. 2016). In this study, *Gls2* was also positively correlated with protein intake according to both GAMS and correlation analysis, and was amongst the top twenty genes influenced by protein intake [Figure 4.19, Table 4.17]. Analysis by the

GF showed that *Gls2* expression was mostly influenced by protein intake, with a small effect of fat intake, and no effect of carbohydrate intake. Of course it is not unexpected for a gene to be involved in protein catabolism to be upregulated in response to high dietary protein intake, and the data shows that this response is maintained long-term and into old age.

Table 4.17. Statistics for the Geometric Framework figures in Figure 4.19. GAMS data for hepatic *Gls2* and *Acaca* expression.

	Edf	df	F	P
Gls2				
Protein	2.88E+00	8	4.756	2.74E-07
Fat	8.96E-01	8	1.074	0.003
Carbohydrates	1.85E-01	8	0.028	0.26
PxF	2.96E-06	3	0	0.71
PxC	2.39E-06	3	0	0.957
FxC	6.17E-06	3	0	0.998
Acaca				
Protein	1.42E+00	8	1.379	0.001
Fat	4.04E-05	8	0	0.469
Carbohydrates	2.86E-05	8	0	0.462
PxF	2.41E-05	3	0	0.345
PxC	2.52E-01	3	0.098	0.25
FxC	1.61E+00	3	3.913	0.001

Garcia-Caraballo et al. studied the effects of seven diets varying in macronutrients (protein 11-58%, carbohydrates 0-81%, fat 8-42%) over 3 weeks on hepatic gene expression (Garcia Caraballo et al. 2017). They found that high-protein, low-carbohydrate diets reduced *Fgf21* and *Pparg* expression whereas using the GF, it was shown that these were only reduced by protein intake and not influenced by the other macronutrients. They also found that *Acaca* (acetyl-CoA carboxylase 1) was influenced by the ratio of carbohydrates to fat. On GF analysis it was also found that the interactive term between carbohydrates and fat was associated with *Acaca* expression as well as protein (Figure 4.19B, Table 4.17). The overlapping results between the two studies indicate that macronutrients individually and interactively can influence the expression of genes involved in fatty acid and glucose metabolism, as well as the key metabolic hormone, FGF21. Unlike Garcia-Carabello, this study did not find any changes in the expression of two other key metabolic regulators, *Pck1* or *Fasn*.

Pathways analysis revealed an association between protein intake and several neurodegenerative disorders including Alzheimer's disease, while biological processes included some involved with neuronal projections, dendrite morphogenesis and axon guidance. Protein intake was shown to be negatively associated with the expression of *App*. Polymorphisms in *App* are associated with Alzheimer's disease, while APP has crucial functions during the development of the brain, and in neuronal plasticity, memory and neuroprotection in the aging brain (Müller & Deller 2017). This might provide a mechanism linking diet to cognitive impairment, noting that many of the diets associated with better cognitive function are low in protein and animal-based protein (van de Rest et al. 2015). In this study, macronutrients did not influence the expression of the other important dementia gene, *Apoe*. High protein intake was associated with increased expression in *Nnmt* (Figure 4.5A), which might be of interest with another neurodegenerative disease, Parkinson's disease. Pathways analysis showed an association between protein intake and pathways linked to Parkinson's disease. Higher levels of NNMT have been detected in the cerebrospinal fluid of patients of patients

with Parkinson's disease (Aoyama et al. 2001), and low protein, high carbohydrate diets are associated with delayed onset of signs in a Drosophila model of Parkinson's disease (Bajracharya & Ballard 2016).

There are limitations to this study. Only a subset of 46 mice of the total of 183 euthanised at 15 months of age were used for gene expression studies, although they did span the full range of diets. Only a single tissue and single strain of mouse were analysed, and the data were not robust enough to study the effects of sex. There is not any established statistical method to evaluate gene expression across a large range of diets, so this study used two methods: correlation analysis with intake of each macronutrient, and a GAMS analysis where all the macronutrients and their interactions are assessed within a single statistical model. The advantage of correlation analysis is that the direction of change is immediately apparent. However the use of GAMS and GF is preferable because it allows interactive terms to be assessed. Encouragingly both methods generated similar conclusions in terms of the predominant effect of protein on gene expression, and the pathways, processes and genes that were identified. This adds additional robustness to the conclusions drawn here.

In summary, protein was the strongest driver of gene expression in the livers of old mice. Protein intake was associated with pathways involved with energy and amino acid metabolism. Although there was only some overlap with genes known to be associated with regulation of longevity and caloric restriction, protein intake was associated with changes in several key nutrient sensing pathways that influence aging including AMPK, mTOR, IGF-1 and FGF21.

Impacts of diet on the hypothalamic gene regulation

While multiple studies have looked at the effects of caloric restriction and intermittent fasting on the central nervous system, the impact of individual macronutrients, and intake ratios have not been thoroughly studied (Moretto et al. 2017). In this study, the GF was used to evaluate the effects of diet on hypothalamic gene expression. Similar to the results in the hepatic transcriptome in the same cohort of animals, as well as the overall hypothalamic gene expression changes, the longevity regulating pathways were affected in opposite directions by fat and protein. This may be due to the fact that since fat and P:C ratio had the greatest overall effects on overall gene expression, it may be the fat vs non-fat component that regulates gene expression in the hypothalamus, with protein being the main driver of the non-fat component, as shown above, and seen in previous studies (Solon-Biet et al. 2014; Solon-Biet et al. 2015b).

In both correlation and GF analysis, fat was the macronutrient that was most influential on gene expression, consistent with previous studies on the hypothalamic transcriptome (Jang et al. 2017; Yamamoto et al. 2009). Expression of *Npy*, *Pomc* and *Agrp* have been demonstrated to respond to short-term dietary changes in mice, particularly with changes in composition of dietary fat and caloric intake (Jang et al. 2017). While fat and energy were the primary drivers of gene expression changes in this study, *Npy* was most strongly correlated with carbohydrate intake (7x fold change, -0.3248 Pearson's correlation coefficient, p=0.0068), while *Pomc* and *Agrp* expression was not captured in the microarray [Figure 4.20A, Table 4.18].

On the other hand, NF-kB regulation, which has previously been described as being important in the control of systemic ageing (G. Zhang et al. 2013), was identified as one of the key pathways linked with protein intake through the gene ontology biological process. Given that protein intake was correlated with NF-kB import into the nucleus and sequestration of NF-kB, this points to a potential

mechanistic link between protein and ageing. *Nfkbia* gene expression was negatively correlated with protein (3.5x fold change, -0.366 Pearson's correlation coefficient, p= 3.34E-04) [See Figure 4.20B, Table 4.18].

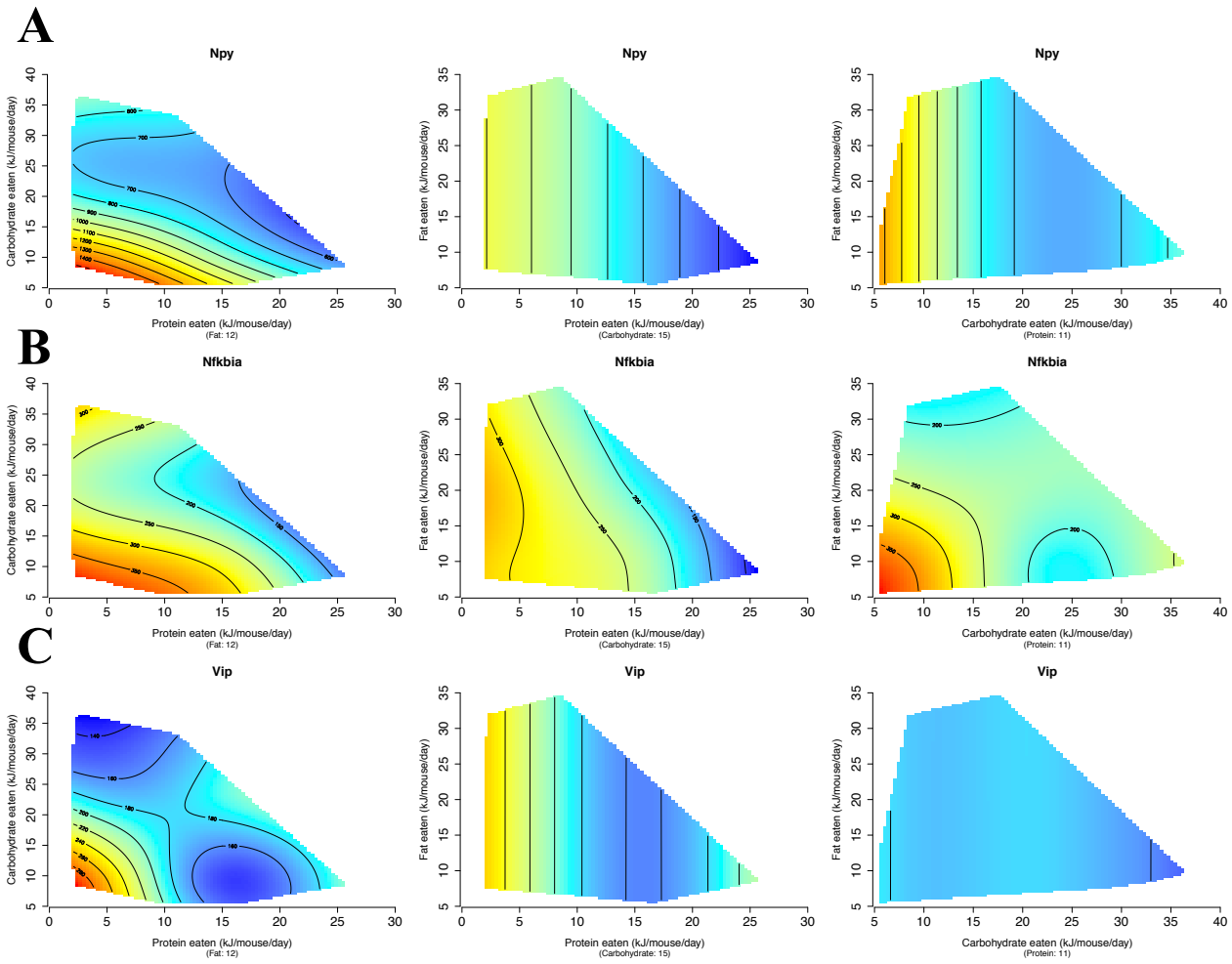


Figure 4.20. Representative three dimensional response surfaces created using the GF. The relationships between the macronutrients and the hypothalamic genes are demonstrated using the colour maps, with red being the most positive response, and blue being the most negative response. Slices are taken through the median of each of the axes (shown in parenthesis below the x-axis label). Figure 4.20A is the surface for the *Npy* gene. Figure 4.20B is the surface for the *Nkfbia* gene, and Figure 4.20C is the surface for the *Vip* gene. GAM statistics are provided in Table 4.18.

Table 4.18. Statistics for the Geometric Framework figures in Figure 3. GAMS data for hypothatlamic expression of *Npy, Nfkbia,* and *Vip*.

	edf	df	F	p-value
Npy				
Protein	8.03E-01	8	2.990	2.79E-02
Carbohydrates	1.39E+00	8	3.789	1.68E-02
Fat	9.93E-05	8	0.000	3.14E-01
P x C	1.40E+00	3	0.000	4.52E-02
P x F	7.54E-06	3	0.000	8.26E-01
C x F	7.57E-06	3	0.000	1.00E+00
Nfkbia				
Protein	9.35E-01	8	5.295	3.34E-04
Carbohydrates	8.81E-01	8	5.063	4.23E-03
Fat	5.69E-05	8	0.000	5.65E-01
P x C	1.01E+00	3	0.000	1.02E-01
P x F	1.09E+00	3	0.000	1.29E-01
C x F	2.80E-05	3	0.000	3.64E-01
Vip				
Protein	6.26E-01	8	0.380	1.98E-01
Carbohydrates	6.65E-01	8	0.000	8.46E-01
Fat	4.15E-01	8	1.982	3.62E-02
P x C	1.44E+00	3	0.000	2.06E-02
P x F	1.59E-05	3	0.000	4.89E-01
C x F	4.82E-05	3	0.000	8.16E-01

A review by Satoh et al. identified multiple key genes involved with lifespan extension in C57BL/6 mice, including *Ucp2*, *Irs2, Igf1r, Ikbk, Sirt1,* as well as *Vip*, which while not involved in lifespan extension, were shown to be altered in ageing (Satoh et al. 2017). In the present study, *Vip* was the 19th most highly affected gene by fold change, and was most significantly influenced by fat (9.89x fold change, 0.375 Pearson's correlation coefficient, p=0.0206) [See Figure 4.20C, Table 4.18]. The longevity data from the same cohort of mice showed that LPHC diets were the most effective in extending lifespan in mice, and interestingly, these same diets corresponded the most significantly to the KEGG longevity regulating pathways, with 13 of the 43 genes being influenced by the P:C ratio in the diet, 12 genes being influenced by carbohydrate intake, and 6 genes being influenced by protein intake [Figure 4.14, Table 4.14].

Despite the interesting correlations found in this study that supports previous work in the field, it should be noted that only 1 animal per diet group was used for this microarray analysis so this is considered a pilot study. While this did cover all groups in the study, the use of only male mice limited the ability to correlate gene expression results to phenotypic results. In addition, sex is known to inherently alter gene regulation, and have variable effects in relation to metabolism, dietary macronutrients and longevity pathways (Satoh et al. 2017; Michán et al. 2010). Another limitation to the study was the use of analytical tools. As there is no established statistical methodology to correlate gene expression results over multiple diets, this study used a combination of correlation analysis and GF analysis, as previously described. The fact that both methods revealed similar patterns of change with relation to macronutrients provides some support for the validity of the results, however a larger trial with great numbers would increase the statistical power of such a study.

General remarks

Overall, this set of work provides analysis of the effects of dietary macronutrients and their interactions on nutrient sensing pathways, and on gene expression globally. Nutrient sensing pathways in both the liver and the hypothalamus were heavily influenced by diet, albeit in different ways, with hepatic gene expression being most strongly modulated by protein intake, and hypothalamic gene expression being most strongly modulated by fat and total energy intake. While many studies have previously outlined similar findings, this is the first study of its kind to systematically analyse the impact of an array of macronutrient intake ratios on gene expression, and anlyse the results through a multidimensional geometric framework. In particular, the inverse relationship between macronutrients and gene expression (protein vs non-protein in the liver, and fat vs non-fat in the hypothalamus) are novel findings, and highlight critical differences between the roles of the same pathways in different tissues. Further research samples collected from various organ types could reveal more detailed insights as to modulation of tissue specific pathways. Broadly, this study has shown that dietary macronutrients play an important role in transcription in both the liver and hypothalamus, with many of the genetic pathways being significantly altered by macronutrient intake. Given the interplay between protein and carbohydrates, and their documented effects on lifespan, this study provides evidence that modulation of these nutrient-sensing pathways are a likely mechanism linking diet and ageing.

Mechanistically, it can be argued that the impact of total energy intake on longevity-related gene expression in the hypothalamus supports previous work suggesting that caloric restriction itself, regardless of macronutrient balance, may be a key driver for longevity. However, while longevity-related genes in the hypothalamus were largely driven by fat and total energy intake, these results did not correlate with an increase in lifespan in the same cohorts of mice. Given that the hypothamlus is known to be involved in multiple physiological feedback loops, the role of fat and energy intake on

hypothalamic gene expression must be interpreted in the context of the impact of these same diets on both other tissue types, as well as on wider outcomes such as health and lifespan. On the other hand, protein restriction, which was shown to increase lifespan in the cohort studied here, is mechanistically tied to modulation of gene expression in nutrient sensing pathways related with metabolism in the liver. Therefore, these results support the hypothesis that caloric restriction and protein restriction, while both important influencers of health and lifespan, may have distinct, albeit somewhat overlapping mechanisms of action. Tissue-specific activation of nutrient sensing pathways, complexities in measuring intake and energy derived from different macronutrient sources, and compensatory feeding responses such as protein leverage, may also contribute to the differences in pathway activation between the liver and hypothalamus; a topic which is discussed in greater detail in Chapter 6. While a comprehensive analysis from gene to protein was performed for mTOR, further work should be done in the other nutrient sensing pathways to determine the relationship between transcription and protein activation. Furthermore, the data shows differential activation of these pathways between the brain and liver, suggesting that there is likely tissue specific variation. Given that other tissue types such as brown adipose tissue (BAT), white adipose tissue (WAT), and skeletal muscle, also play important roles in metabolism and energy storage, further studies using similar experimental methods could be performed in these tissues to better understand their roles in the ageing process.

Finally, it was also shown that the GF is a useful analytical paradigm for complex, multifactorial data, especially when used in combination with simple correlation methods, such as Pearson product-moment coefficients. The generation of analytical tools through R scripts is therefore an important step in improving data processing, and providing outputs, which can be easily interpreted through visualisation.

Chapter 5: The Impact of Diet on Age-Related End Points

To date, nutritional interventions such as caloric restriction (CR) and intermittent fasting have been the most robust non-genetic interventions to delay the onset of age-related disease and increase lifespan (Longo et al. 2015; Finkel 2015; Kowald & Kirkwood 2016). The benefits of CR and intermittent fasting have been demonstrated in a variety of species ranging from yeast to non-human primates thus indicating that diet is a critical and conserved intervention for increasing health span and lifespan (Heilbronn & Ravussin 2003; Fontana & Partridge 2015).

Recently it was demonstrated in a large dietary study, that a low-protein, high-carbohydrate (LPHC) ad libitum-fed diet was also beneficial in extending healthspan and lifespan (Solon-Biet et al. 2014). This result has now been demonstrated in a variety of species, however the mechanisms which mediate the effects of a LPHC diet on longevity and health remain unclear (Mirzaei et al. 2014; Nakagawa et al. 2012; Grandison et al. 2009; Soultoukis & Partridge 2016; Le Couteur et al. 2015).

In Chapter 4, the effects of macronutrients on the nutrient sensing pathways that underpin ageing are presented, and it was shown that macronutrients and macronutrient balance have significant impacts on gene regulation, and activity of these pathways, particularly in the liver. Given that augmentation of these pathways has been shown to link nutrition with ageing, the next step is to study the downstream effects on age-related phenotypic end points. These so called 'Hallmarks of Ageing' (Chapter 1), encapsulate many cellular processes influenced by ageing and most, if not all of these processes are influenced in some way by nutrient sensing pathways (López-Otín et al. 2016).

Therefore, in this Chapter, the effects of nutrition and nutrient sensing pathways are examined on three age-related endpoints:

1) Telomere length – Chapter 5.1

2) Mitochondrial number and function – Chapter 5.2

3) Inflammation – Chapter 5.3

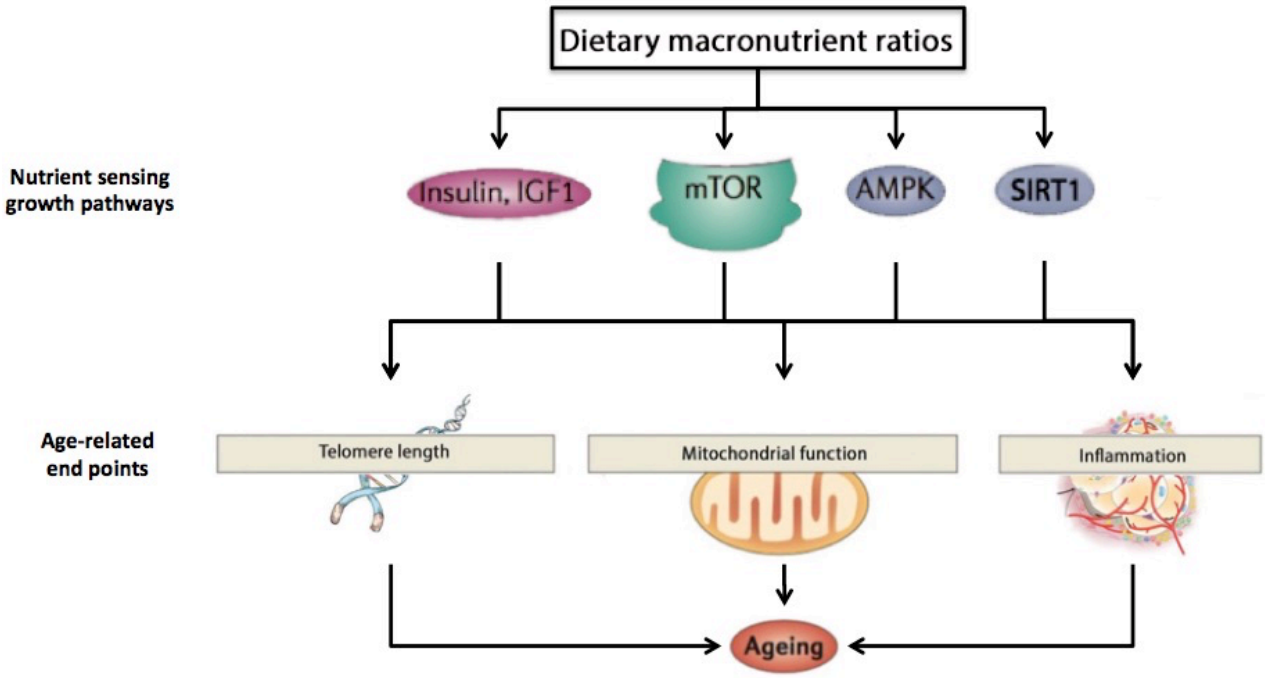


Figure 5.0. Proposed end points to by studied in this chapter.

Chapter 5.1: The relationship between dietary macronutrients and hepatic telomere length

A version of this chapter has been published in (Gokarn et al. 2017).

Background

Aging can be delayed by a variety of nutritional interventions, of which caloric restriction has been the most extensively studied (Mercken et al. 2012; Lee & Longo 2011). Studies utilising the principles of nutritional geometry have found that dietary macronutrients also influence lifespan, with most studies in insects and mice reporting that LPHC diets maximise lifespan in ad libitum feeding paradigms (Lee et al. 2008; Raubenheimer et al. 2016; Le Couteur et al. 2015; Solon-Biet et al. 2014). In a recent study, mice maintained on diets with a protein to carbohydrate ratio of nearly 1:10 had the longest lifespan and best late-life health despite increased fat mass (Solon-Biet et al. 2014). These diets were associated with optimisation of various nutrient sensing pathways that influence aging including mTOR, insulin, FGF21 and IGF-1 (Solon-Biet et al. 2014; Solon-Biet et al. 2016).

A large number of biological processes underlie aging and some of these have been termed the 'hallmarks of aging' (López-Otín et al. 2013): amongst these processes is telomere attrition. Telomeres form the ends of eukaryotic chromosomes and comprise repetitive stretches of DNA (TTAGGG) bound to specific proteins. Telomeres play a critical role in longevity due to their role in protecting the ends of chromosomes and prevention of chromosome fusion. A key enzyme, telomerase, is critical in the maintenance of telomere length, and this enzyme has been shown to be attenuated by the same nutrient sensing pathways affected by dietary intervention (Blasco 2005). It has been suggested that the "molecular clock" – the shortening of telomeres throughout an organism's life,

might eventually signal growth arrest, leading to replicative senescence (McLean & Le Couteur 2004). This phenomenon has been observed both in cultured non-immortalised cells (Endo et al. 2002; Song et al. 2016), as well as in live animal studies (Rudolph et al. 1999). Although the results are variable, a number of epidemiological studies have suggested that leukocyte telomere length (LTL) decreases with age in humans (Vidaček et al. 2017; Mons et al. 2017). Thus, telomere length has been proposed to be a biomarker of aging, with short telomeres contributing to aging by causing cellular senescence (Coppé et al. 2010; Blasco 2005; Vidaček et al. 2017).

In addition, mouse models of certain age-related diseases such as Werner syndrome and ataxia telangiectasia have also been used to study telomere length. Interestingly, these models only phenotypically manifest common pathologies when there is premature telomere shortening, thus highlighting the critical role of telomere integrity in attenuation of disease manifestation (Sahin & DePinho 2012). Taken together, these results suggest that telomeres are a critical factor in the maintenance of healthspan and improved lifespan in both wild-type organisms and models of disease. Given that a LPHC diet was effective in promoting healthspan and extending lifespan in a large cohort of mice, even more so than 20% calorie restriction by dilution (Solon-Biet et al. 2014), this study aimed to investigate the effect of these dietary regimes on telomere length. The relationship between telomere length and various markers of nutrition (nutrient sensing pathways, circulating amino acids and fatty acids) and mitochondrial function were also explored as potential mechanistic links between nutrition, telomeres and aging.

Methods

Methods on animal husbandry and diet are outlined in full in Chapter 2. The lifespan, metabolomics and signaling pathway data from this mouse study have been published previously (Solon-Biet et al. 2014; Solon-Biet et al. 2016).

Briefly, liver tissue (n=183) was harvested for evaluation of telomere length. Blood levels of insulin, FGF21 and IGF-1 were measured by ELISA, hepatic mitochondrial function using Seahorse XF Extracellular Flux Analyzer, hepatic mTOR and p-mTOR by western blotting. In 22 of the samples, genomic DNA quality did not pass QC thresholds, and were excluded from the analysis. Average telomere length was measured from total genomic mouse liver DNA by using a real-time quantitative PCR method (Cawthon 2002). Normalisation was done using the acidic ribosomal phosphoprotein PO (36B4) gene. Standard curves for 36B4 and telomeric repeats were used for absolute quantitation. Telomere concentrations were normalised to the 36B4 control gene concentration of the same sample.

Data obtained in this manner from replicates is considered to be the average telomere length ratio (ATLR), as previously described (Callicott & Womack 2006). Data were analysed using Pearson's correlation coefficient. The Geometric Framework (GF) was used to visualise the relationship between macronutrients and ATLR as described in detail in Chapter 3. P-values less than 0.05 were considered statistically significant and a Bonferroni correction was applied for multiple comparisons.

Results

Telomere length was able to be determined in DNA extracted from 161 livers collected from 183 mice. The average ATLR was 6.8 ± 18.2, the median 2.0, and the majority of the values were between 1-4 [Figure 5.1.1A]. There was no difference between females (n=85, 7.1 ± 16.6) and males (n=76, 6.4 ± 14.5) so they were pooled. The telomere length in kbp was estimated from the ATLR using an empiric formula derived in C57Bl/6 mice previously published (Callicott & Womack 2006). Using this estimation, the telomere length across all diets averaged 16.6 ± 23.3 kbp and the distribution of telomere length was skewed to the right [Figure 5.1.1A-B].

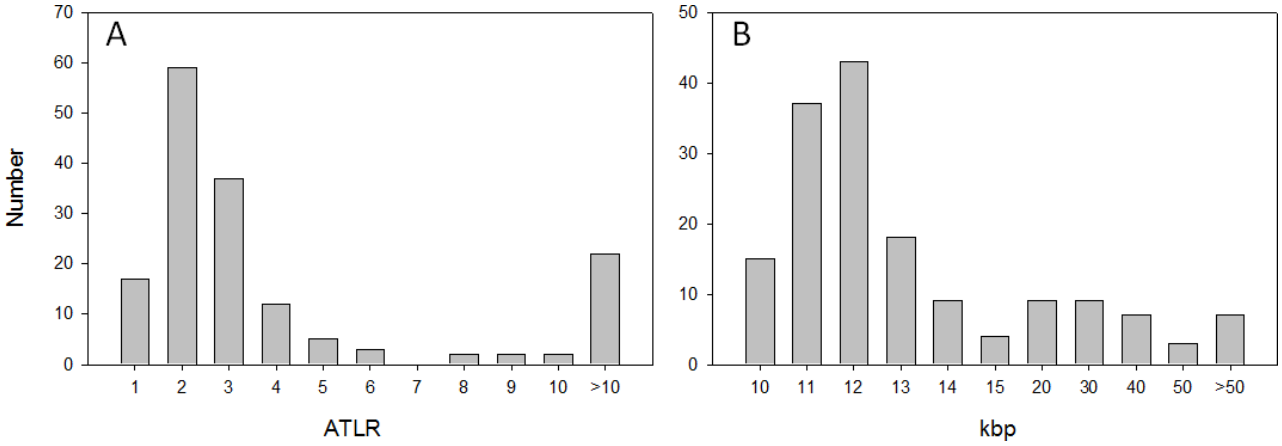


Figure 5.1.1. The distribution of telomere lengths in DNA extracted from mouse livers, determined by ATLR (A) and converted to kbp (B) according to the empiric equation of (Callicott & Womack 2006). The Y-axis refers to the number of mice.

There was a significant positive correlation between carbohydrate intake and ATLR (r=0.18, P=0.02) and a significant negative correlation with protein intake (r=-0.16, P=0.04). There were no correlations between ATLR and fat intake or total energy intake [Figure 5.1.2A-D].

Analysis of the full, 3-macronutrient response surface using GF and GAMS revealed that carbohydrate intake was the main driver of ATLR (P=0.04), with the longest telomeres occurring in mice on the low protein, high carbohydrate diets. The pattern of distribution of ATLR across the dietary surfaces matched the changes previously reported for lifespan, such that the longest telomeres and lifespan were seen in mice maintained on low protein high, carbohydrate ad libitum-fed diets [Figure 5.1.3A-B, Table 5.1.1]. Direct comparison between ATLR and lifespan was not possible in the same animals because ATLR was measured in animals sacrificed at 15 months of age.

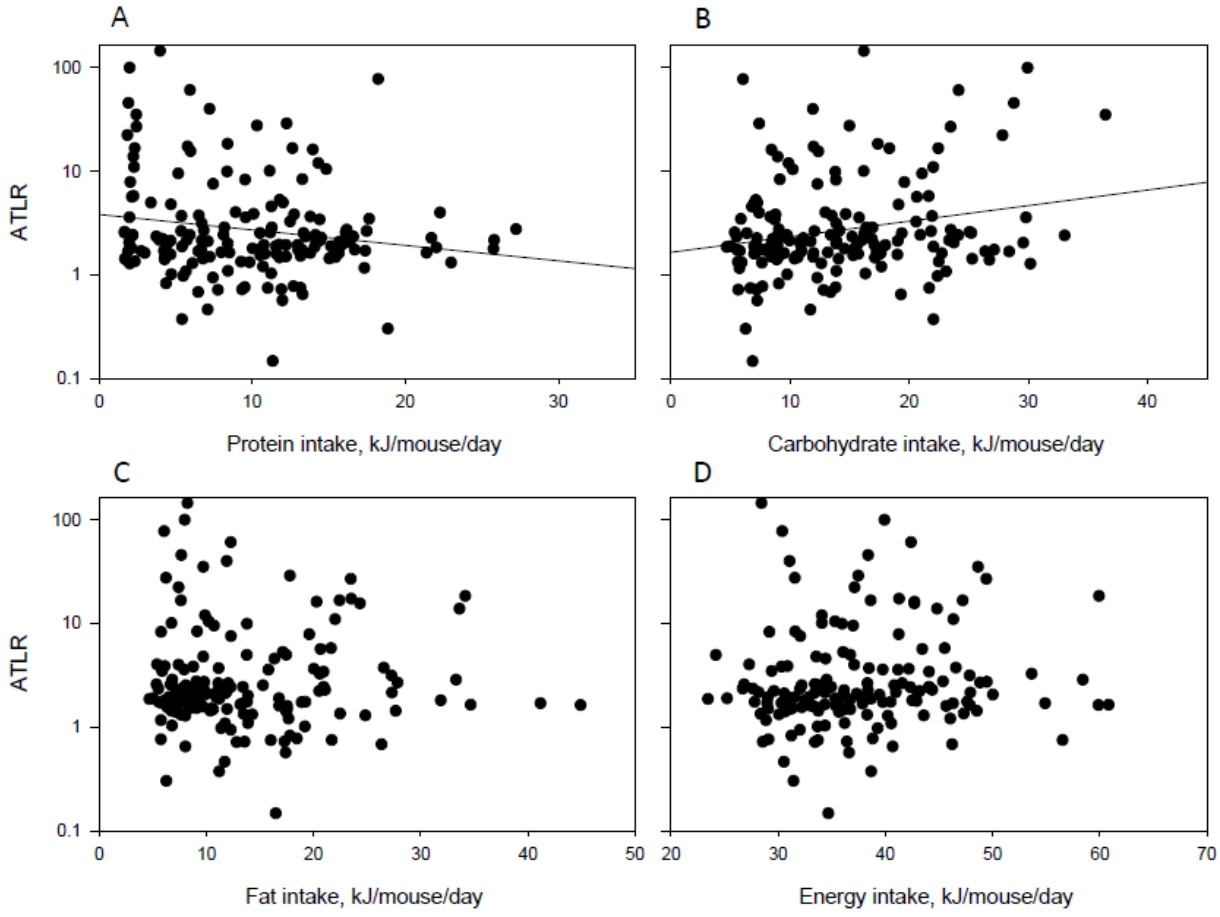


Figure 5.1.2. The relationship between the intake of dietary macronutrients and energy with ATLR (average telomere length ratio) in DNA from livers of mice aged 15 months. There were significant correlations with protein (5.1.2A, r=-0.16, P=0.04) and carbohydrates (5.1.2B, r-=0.18, P=0.02) but not fat (C) or total energy (D).

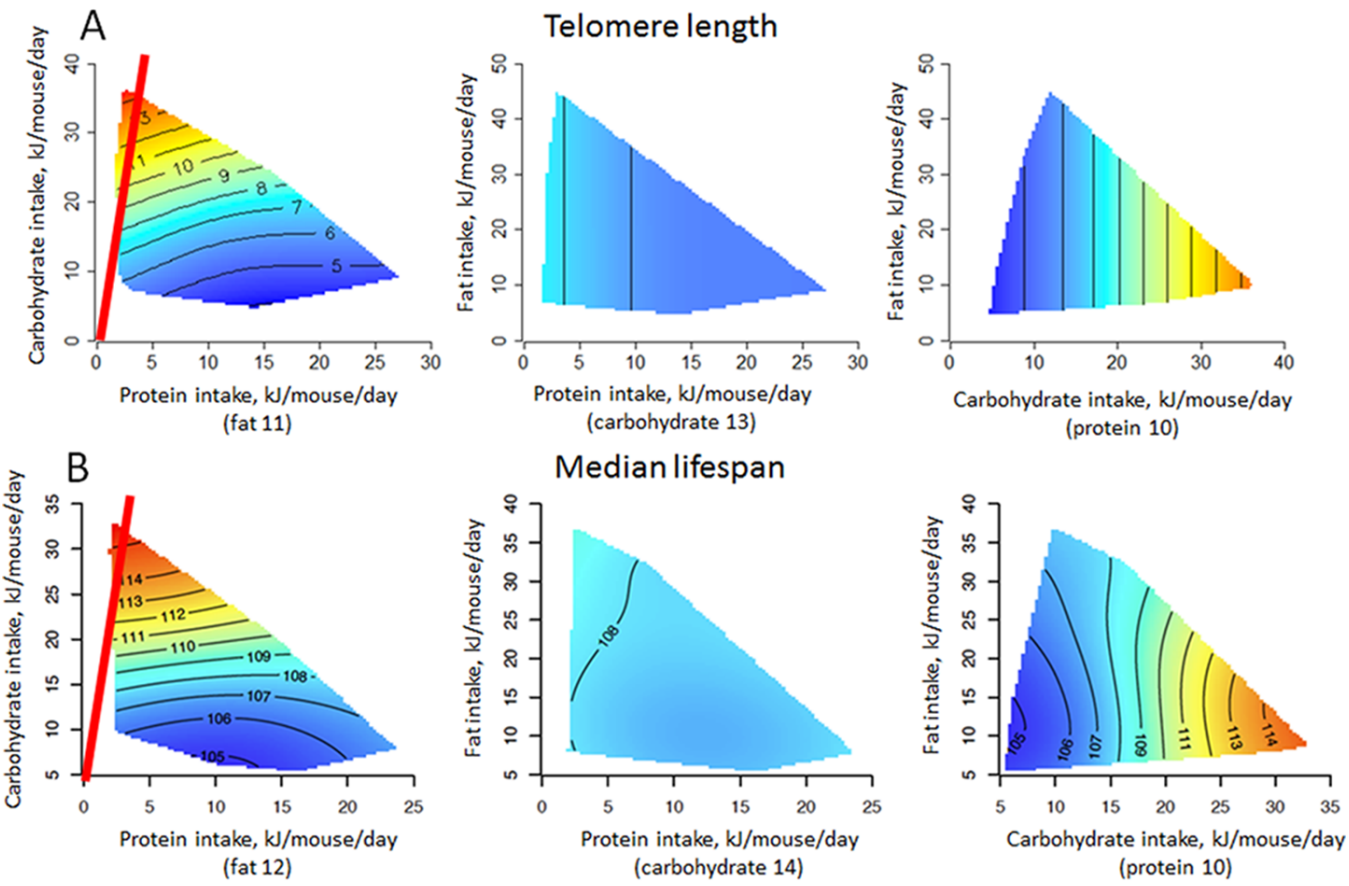


Figure 5.1.3. Representation of the relationship between macronutrients and ATLR (A) and median lifespan (B) using the Geometric Framework. Three 2D slices are given to show all three nutrient dimensions (protein, carbohydrate, fat). For each 2D slice, the third factor is at its median (shown below the x-axis in parentheses). In all surfaces, red indicates the highest value, while blue indicates the lowest value with the colors standardised across the three slices. The diet with the longest telomere length and lifespan is demonstrated with the red line, and this was a low protein, high carbohydrate diet in both cases. There is a very similar response of both ATLR and median lifespan to dietary macronutrients. For 3D representation, see Figure 5.1.5.

Table 5.1.1. GAM statistics related to Figure 5.1.3 and Figure 5.1.5. The results for median lifespan are from Solon-Biet et al (2014).

	Edf	**df**	**F**	**P**
ATLR				
Protein (P)	0.30	8	0.05	0.20
Carbohydrate (C)	0.69	8	0.28	0.04
Fat (F)	0.00	8	0.00	0.45
P × C	0.43	3	0.18	0.24
P × F	0.00	3	0.00	0.59
C × F	0.00	3	0.00	0.58
P × C × F	0.00	7	0.00	0.48
Median lifespan				
Protein (P)	0.00	4	0.00	0.50
Carbohydrate (C)	1.12	4	1.68	0.01
Fat (F)	0.00	4	0.00	1.00
P × C	0.00	2	0.00	0.10
P × F	0.00	2	0.00	0.10
C × F	0.00	2	0.00	0.62

The correlations between ATLR, circulating amino acids (n=120) and fatty acids (n=128) were assessed. There were significant positive correlations between ATLR and asparagine (P=0.01), glutamate (P=0.0006) and taurine (P=0.008) [Figure 5.1.4A-D], but not with other amino acids or fatty acids. Only glutamate remained significant after Bonferroni correction for analysis of 26 amino acids [Table 5.1.2]. There was a weak association with one fatty acid C21.0 (P=0.04).

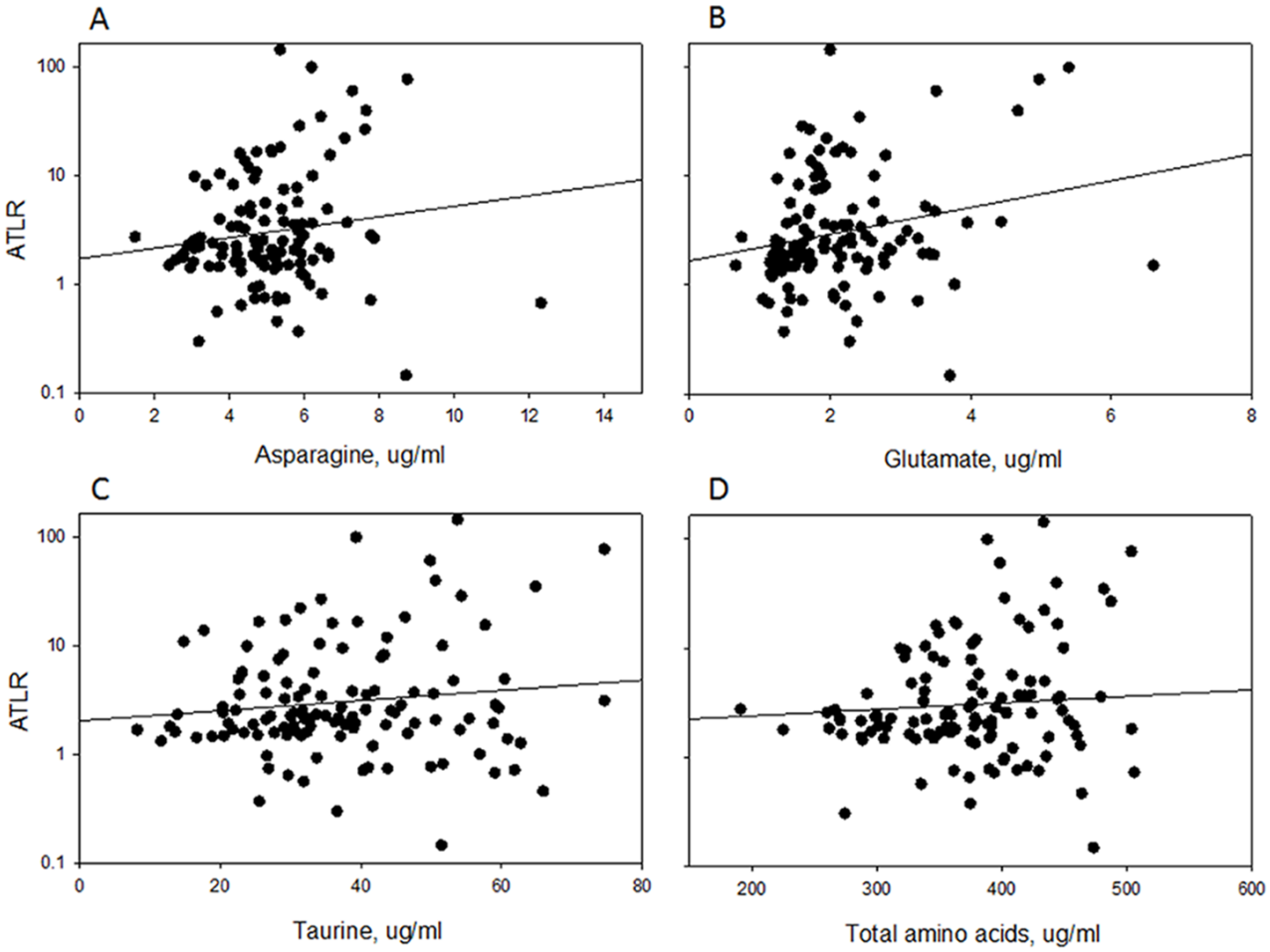


Figure 5.1.4. The relationship between circulating amino acids and ATLR. There were significant correlations with asparagine (3A, r=0.23, P=0.01), glutamate (3B, r=0.31, P=0.0006), taurine (3C, r=0.24, P=0.008) and a weak correlation with total amino acids (3D, r=0.18, P=0.05) but not with the other amino acids.

There were no statistically significant correlations between ATLR and hepatic pmTOR/mTOR, measures of hepatic mitochondrial function determined by Seahorse, or circulating levels of FGF21, insulin or IGF-1 [Table 5.1.2].

Table 5.1.2. Pearson's correlation coefficient and P value for the relationship between ATLR and amino acids, fatty acids, hepatic mitochondrial function and nutrient sensing pathways (*FGF21 was insignificant when 1 outlier was removed from analysis). The bold font identifies statistically significant values prior to Bonferroni correction.

Parameter	**r**	**P**	**Parameter**	**r**	**P**
Amino acids					
Alanine	0.06	0.49	Isoleucine	0.00	0.97
alpha Aminoadipic acid	0.01	0.95	Leucine	-0.04	0.64
alpha Aminobutyric acid	0.10	0.28	Lysine	0.07	0.43
Arginine	0.18	0.05	Methionine	0.10	0.27
Asparagine	**0.23**	**0.01**	Ornithine	0.07	0.43
Aspartic acid	0.12	0.20	Phenylalanine	0.09	0.35
Citrulline	0.17	0.07	Proline	0.13	0.16
Cysteine	0.02	0.84	Serine	0.13	0.16
Glutamate	**0.31**	**0.0006**	**Taurine**	**0.24**	**0.008**
Glutamine	0.17	0.07	Threonine	0.12	0.21
Glutathione	0.07	0.43	Tryptophan	0.09	0.31
Glycine	0.02	0.80	Tyrosine	0.15	0.11
Histidine	0.09	0.34	Valine	-0.05	0.57
Fatty acids					
C9.0	0.03	0.77	C18.2	0.08	0.40

C10.0	-0.15	0.09	C18.1.cis	0.07	0.41
C11.0	-0.06	0.52	C18.3	0.03	0.74
C12.0	-0.14	0.11	C18.1.trans	0.06	0.50
C13.0	0.01	0.92	C18.0	0.04	0.66
C14.0	0.01	0.95	C19.0	-0.10	0.26
C15.0	-0.05	0.57	C20.4	0.06	0.51
C16.1	0.10	0.26	C20.0	-0.03	0.72
C16.0	0.03	0.74	**C21.0**	**-0.18**	**0.04**
C17.0	0.04	0.67	C22.1	-0.17	0.06
			C22.0	-0.07	0.42
Hepatic mitochondrial function					
beta-hydroxyacyl-CoA dehydrogenase	-0.02	0.74	H2O2 glutamate	0.03	0.70
aspartate aminotransferase	-0.01	0.87	State III palmitoyl	-0.01	0.92
State III pyruvate	-0.03	0.71	State IV palmitoyl	-0.01	0.87
State IV pyruvate	-0.02	0.77	H2O2 palmitoyl	-0.03	0.77
H2O2 pyruvate	-0.03	0.72	RCR pyruvate	0.09	0.26
State III succinate	-0.03	0.68	RCR succinate	0.00	0.96
State IV succinate	-0.03	0.74	RCR glutamate	0.04	0.61
H2O2 succinate	-0.03	0.69	RCR palmitoyl carnitine	0.02	0.82
State III glutamate	-0.03	0.70	Citrate synthase	-0.08	0.33
State IV glutamate	-0.03	0.69			
Signaling and nutrient sensing pathways					
Insulin	-0.07	0.43	IGF-1	-0.12	0.17
FGF21*	0.12	0.22	pmTOR/mTOR	0.02	0.83

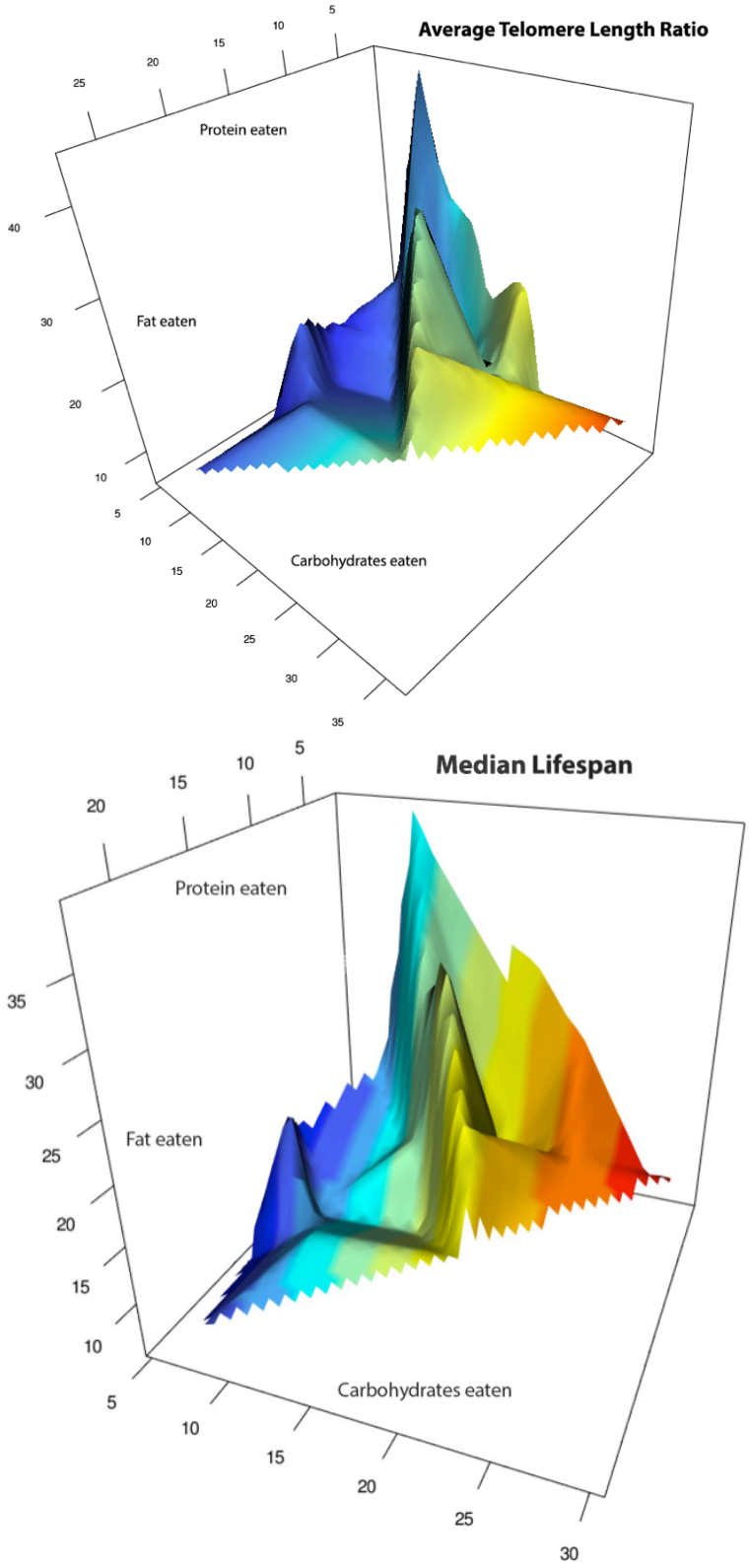


Figure 5.1.5 3D Representation of the relationship between macronutrients and ATLR and median lifespan using the Geometric Framework from Figure 5.1.3, Table 5.1.1. Differences in shape reflect differences in specific nutritional intake points between the two sets of mice.

Discussion

Many observational studies in human populations have found an association between various diet constituents and leukocyte telomere length (LTL). Consumption of anti-oxidant rich, plant-derived foods and the Mediterranean diet are linked with longer LTL, while shorter LTL have been associated with dietary fats, refined cereals, meat products and sugar-sweetened beverages (Vidaček et al. 2017; Freitas-Simoes et al. 2016; Rafie et al. 2016). In a prospective study of young humans, change in telomere length over time was inversely associated with total energy intake as well as that of each macronutrient (Kark et al. 2012). A number of environmental and lifestyle factors including diet appear to influence telomere length, which may provide a mechanism for their effects on aging and age-related health (Vidaček et al. 2017; Freitas-Simoes et al. 2016).

There have been fewer studies of nutrition and telomeres in mice and rats. Telomeres are longer and shorten more rapidly with ageing in rodents than in humans (Vera et al. 2012; Tanrikulu-Kucuk & Ademoglu 2012). Caloric restriction in mice was associated with longer LTL at 15 months (Vera et al. 2013) while 40% protein restriction maintained telomere length in livers of rats aged 16 months, compared with a decrease in telomere length in those rats on standard chow (Tanrikulu-Kucuk & Ademoglu 2012). In this study, in which mice had ad libitum access to food, telomere length assessed by ATLR was positively correlated with carbohydrate intake, and negatively correlated with protein intake, while fat and total energy intake had no effect. Analysis using the Geometric Framework showed that ATLR positively correlated with carbohydrate intake, and highest ATLR was achieved in mice maintained on low protein, high carbohydrate, low fat diets that were ad libitum-fed over a lifetime. The surface generated by Geometric Framework analysis reveals that ATLR doubled from approximately 6 in mice on high protein, low carbohydrate diets to about 13 in those on low protein high, carbohydrate diets. These values are approximately equivalent to 16 and 27 kbp (using an empiric conversion equation (Callicott & Womack 2006)), which are similar to values of 19 and 23

kbp seen in the livers of 16 month old rats maintained on standard chow or protein restriction respectively (Tanrikulu-Kucuk & Ademoglu 2012). In the mouse caloric restriction study (Vera et al. 2013), liver telomere length was not reported, however, caloric restriction was associated with longer telomeres in kidney (94 kbp vs 62 kbp), lung (55 vs 21 kbp) and muscle (14 vs 11 kbp) at 15 months of age. Together, the data in humans and rodents are consistent with a robust effect of nutrition on telomere length in older age, thereby providing a plausible mechanistic link for the effects of nutrition on ageing and age-related health.

Telomere length provides a biomarker and a mechanism for aging. The ability of telomere length to predict aging and lifespan has been reported both in aged mice (Vera et al. 2012; Cherif et al. 2003; Ludlow et al. 2012) and humans (Mons et al. 2017; Cawthon et al. 2003). This relationship has mostly been studied using LTL (Blasco 2005). However one study in humans found that telomere length in the liver was 12.9 kbp in newborns compared to 8.3 kbp in a centenarian (Takubo et al. 2000), while there is a reduction in the percentage of longer telomeres in livers of rats by 15 months of age (Cherif et al. 2003).

In the present study, it was not possible to directly compare telomere length and lifespan in the same mice, because the telomeres were evaluated in the livers of mice sacrificed at 15 months of age. However, responses of both ATLR and median lifespan to macronutrients in mice could be compared to corresponding data from the same experimental cohort. As shown in Figure 5.1.3 and Figure 5.1.5, the surfaces demonstrating the relationship between macronutrients and median lifespan versus ATLR are almost identical. Mice maintained on low protein, high carbohydrate diets had the longest median lifespan and the longest ATLR. The results provide indirect evidence that is consistent with the concept that long telomere length is associated with longer lifespan. In addition, the rankings by diet of the median lifespan were compared to the telomere length, and there was a significant

positive relationship, such that diets associated with longest median lifespan were also associated with longest telomeres [Figure 5.1.6].

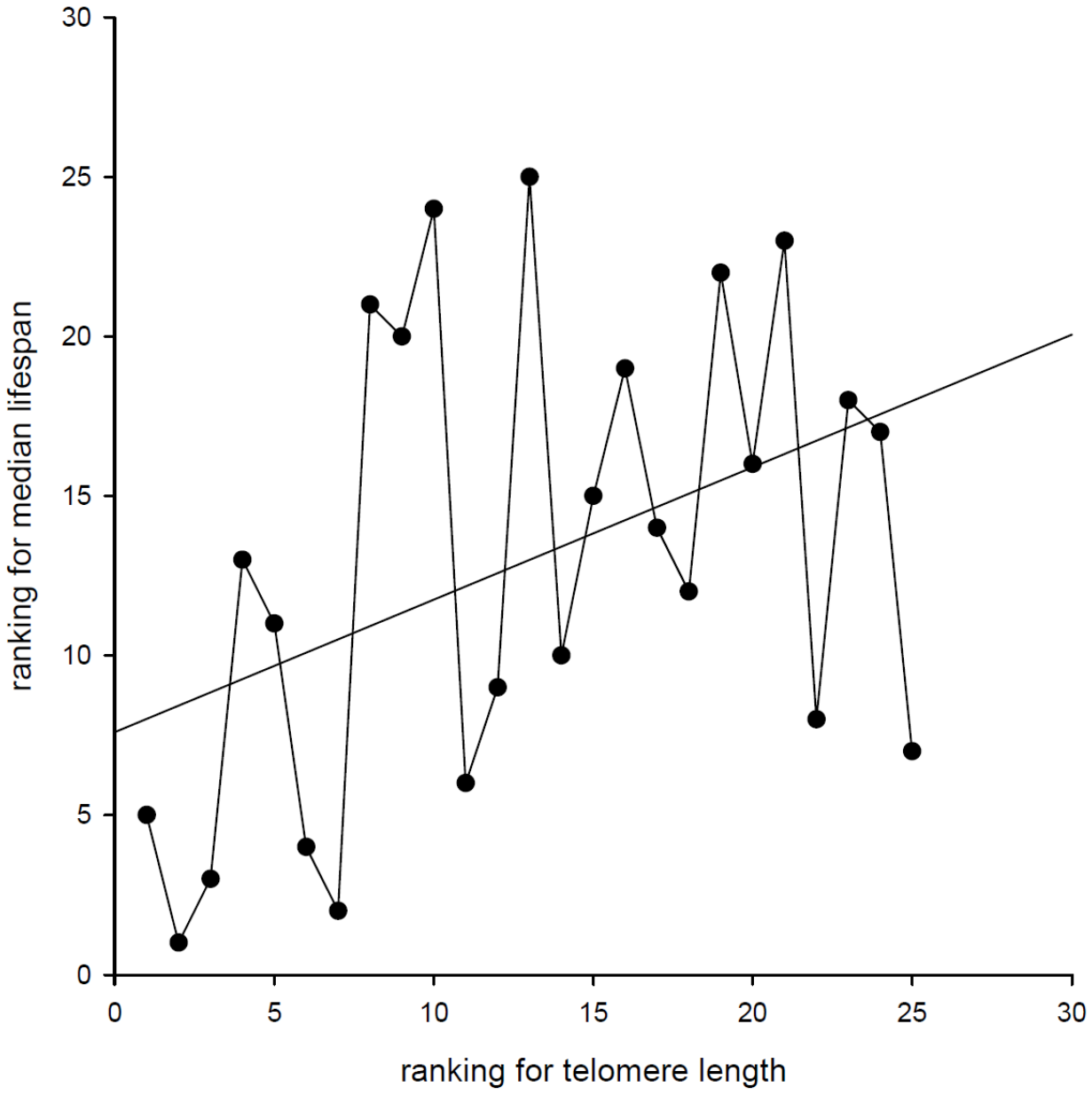


Figure 5.1.6. The relationship between the telomere length at 15 months of age and median lifespan. The average median lifespan and telomere length were determined for each diet, then ranked by diet. There was a significant correlation between the rankings (Pearson's coefficient = 0.42, P = 0.04).

Next, this study explored whether the association between telomeres, nutrition and lifespan is mediated by several mechanisms that are thought to link nutrition with aging (Solon-Biet et al. 2015b). There was no association with ATLR and circulating levels of insulin, FGF21 or IGF1, hepatic mTOR activation, nor hepatic mitochondrial activity, although this may reflect the power of the study. However, there was an association between ATLR and blood levels of amino acids including asparagine, glutamate and taurine. Previously it has been shown that the chronic circulating levels of most amino acids (except branched chain amino acids) are inversely correlated with protein intake (Solon-Biet et al. 2014). Therefore the positive association between some amino acids and ATLR in this study may simply reflect the effects of a low protein diet, although only glutamate remained significant after correction for multiple comparisons. On the other hand, taurine is an antioxidant which could influence telomere shortening (Ozsarlak-Sozer et al. 2010). There are few reports linking amino acids with telomere length. One report in humans studied the relationship between LTL and several circulating amino acids (alanine, glycine, histidine, phenylalanine, leucine, isoleucine, valine, and tyrosine) and found an inverse relationship with phenylalanine (Eriksson et al. 2017).

In conclusion, mice maintained on low protein, high carbohydrate diets had longer hepatic telomeres, which correlated with a longer median lifespan. Nutrition has a powerful impact on aging and age-related health, which might be mediated in part by its effects on telomere length.

Chapter 5.2: The relationship between dietary macronutrients and hepatic mitochondrial function

Data from this chapter has been published in (Solon-Biet et al. 2014).

Background

Mitochondria are essential not only for providing energy in the form of ATP, but also for their involvement in multiple functions, many of which are associated with changes in nutrient sensing pathways (Sahin & DePinho 2012; M. B. Jensen & Jasper 2014). The primary function of mitochondria is to provide cellular energy through the process of oxidative phosphorylation via four respiratory chain complexes (complexes I-IV) and ATP synthase (complex V) (Bratic & Larsson 2013). Changes in mitochondrial bioenergetics, reactive oxygen species (ROS) production, mutations in mitochondrial DNA and impaired respiratory chain function have been implicated in ageing, not only as a correlation, but these changes have even been suggested to be a potential a mechanism driving the ageing process (Sevini et al. 2014; Sahin & DePinho 2010; Scialò et al. 2016).

Mitochondrial dysfunction is generally considered a hallmark of ageing, and has been well documented in mammals (López-Otín et al. 2013; Kujoth 2005; Trifunovic et al. 2004). These changes have been linked with altered regulation of SIRT1, mTOR and AMPK through a variety of mechanisms including pathways linked with altered regulation of mitophagy, mitochondrial biogenesis, NAD^+ availability, PGC1α and p53 [Figure 5.2.1] (Kujoth 2005; Gomes et al. 2013; Green et al. 2011; Sahin & DePinho 2012). Increased ROS production as a result of mitochondrial dysfunction has been of particular interest, due to its association with oxidative stress and DNA damage (Hekimi et al. 2011; Fang et al. 2016). However, in many species, low levels of mitochondrial

dysfunction have actually been noted to extend lifespan (Ventura et al. 2009; Palikaras et al. 2015; Merkwirth et al. 2016; Munkácsy & Rea 2014), therefore suggesting that perturbations to mitochondrial function exert a hormetic effect on ageing (Hekimi et al. 2011; López-Otín et al. 2016). Mitochondrial dysfunction has also been shown influence ageing independently of ROS production through alteration of apoptotic pathways, as seen in animal studies with deficiencies caused by mitochondrial DNA mutations (Hiona et al. 2010; Edgar et al. 2009).

Mitochondrial dynamics have also been studied with relation to nutrient exposure, and there is evidence that mitochondria in nutrient-rich states are more fragmented compared to the elongated mitochondria seen in nutrient starved tissues (Liesa & Shirihai 2013; Jacobi et al. 2015). Changes in hepatic mitochondrial number and function are therefore associated with an ageing phenotype, but it is unclear which of these, if any, is more important in healthy ageing (Prolla & Denu 2014; Aon et al. 2016). In humans, CR has been shown to increase mitochondrial DNA content and mitochondrial biogenesis in skeletal muscle, and was associated with increased expression of genes associated with mitochondrial function including PGC1α, eNOS, SIRT1 and PARL (Civitarese et al. 2007). The benefits of intermittent fasting on health have also been linked to decreased mitochondrial degeneration (Castello et al. 2011; Raefsky & Mattson 2017).

It has been recently reported that specific macronutrient intake rather than CR by dilution is linked with longevity and metabolic health in ad libitum-fed mice (Solon-Biet et al. 2014). Since diet also affects mitochondrial function, examination of this interaction could provide evidence for a mitochondrial mechanism underlying the beneficial effects of CR on longevity. Here, the effects of varying protein-to-carbohydrate-to-fat (P:C:F) ratios on mitochondria in mice were investigated, through assays of mitochondrial number and function. Mitochondrial number was assessed in two ways; PCR for expression of the mitochondrial gene cytochrome b for mitochondrial DNA copy

number, and an assay of citrate synthase activity as previously described (Larsen et al. 2012; Uddin et al. 2016). Mitochondrial function and bioenergetics were tested using the Seahorse XF24 Extracellular Flux analyser, which assesses oxygen phosphorylation through oxygen consumption rates after the addition of various substrates. Hydrogen peroxide, the beta-oxidative enzyme 3-hydroxyacyl-CoA dehydrogenase (HOAD), and citrate synthase were measured through spectrophotometry. Since the same cohort of mice was used to collect longevity data, these findings were compared to lifespan to see if there was a correlation with diet and longevity.

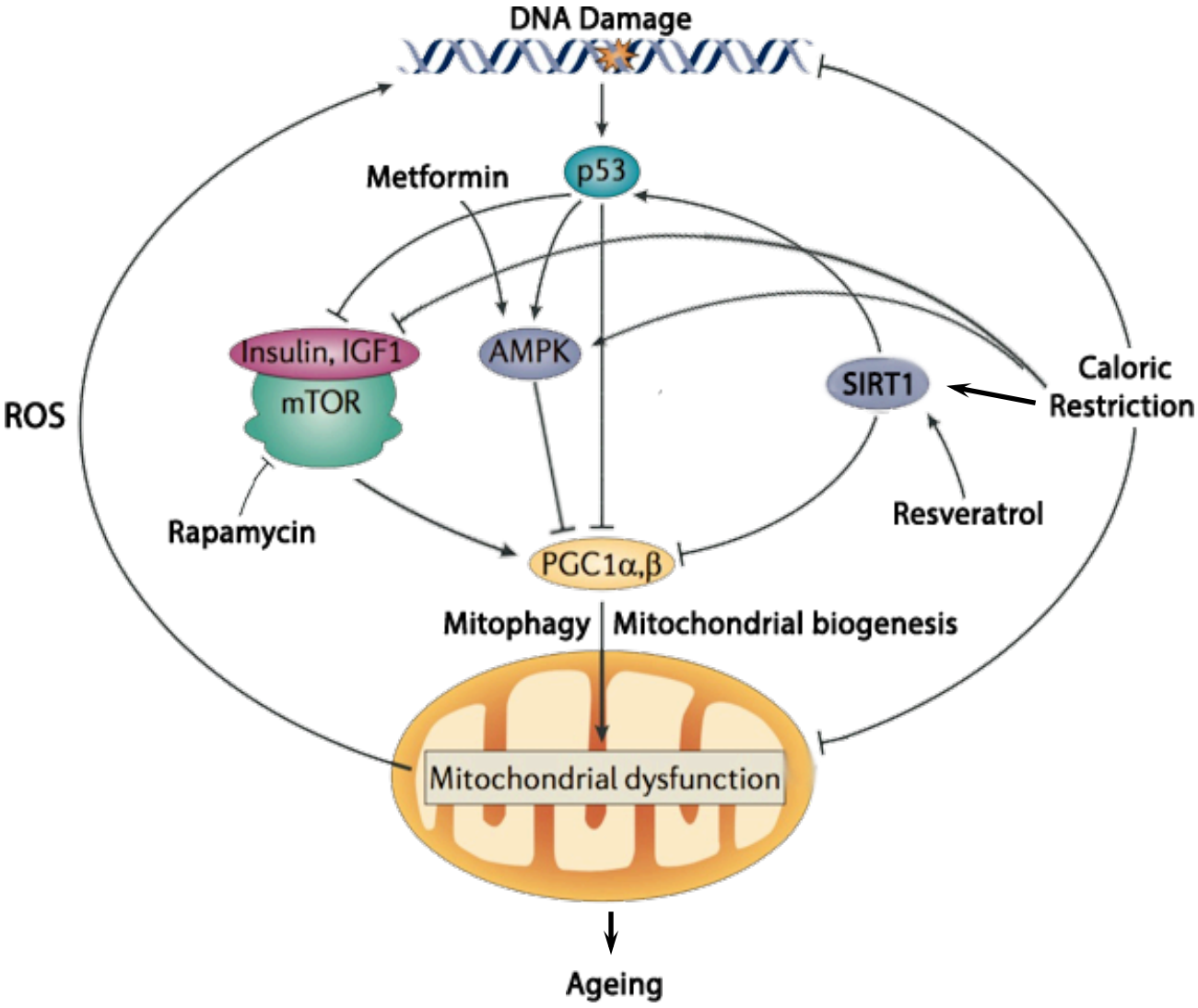


Figure 5.2.1. CR, CR mimetics and nutrient sensing pathways in mitochondrial dysfunction and ageing.

Methods

Methods on animal husbandry and diet are outlined in full in Chapter 2. Here, additional studies were conducted to investigate the effects of macronutrient balance on hepatic mitochondrial number and function, and its correlation with longevity, as previously described (Solon-Biet et al. 2014).

Briefly, mitochondrial function was studied using mitochondria isolated from liver tissue (n=170) of mice aged 15 mths, examining ATP production, basal respiration, maximum respiration and spare capacity using oxygen consumption rates after the addition of various substrates (pyruvate-malate, glutamate-malate, succinate-rotenone, palmitoyl carnitine-malate). Respiratory Control Ratios (RCR's) were defined as State III/State IVo. Hydrogen peroxide production and enzymatic activities (HOAD, aspartate aminotransferase (AST) and aconitase) were measured via spectrophotometry. Citrate synthase activity was used to normalise the results, which are therefore expressed as mitochondrial function per mitochondrion. Mitochondrial copy number was estimated from genomic mouse mtDNA using a real-time quantitative PCR method normalised to the acidic ribosomal phosphoprotein PO (36B4) gene as previously described (Uddin et al. 2016). All PCRs were done in triplicate. Standard curves for 36B4 and cytochrome b were used for absolute quantitation.

The data is displayed as 2D and 3D response surfaces generated by the Geometric Framework as described in Chapter 3, which were analysed with GAM statistics. The values reported for macronutrient intake axes are kJ/mouse/day averaged over intake during 6-15 months of age. Overall, 37 mitochondrial assays are reported here, 32 of which are related to mitochondrial function directly through measures of oxidative phosphorylation and free radical production, 3 of which are related to enzyme activity (HOAD, AST, and aconitase), and 2 related to gene expression.

Results

To assess mitochondrial copy number (CN), cytochrome b expression was examined, normalised to the control gene 36B4 as previously described (Uddin et al. 2016). Mitochondrial copy number was found to be positively correlated with protein intake (p=0.0004) [Figure 5.2.2, Table 5.2.1]. Citrate synthase (CS) activity, which has been used as a surrogate marker to quantify mitochondrial number (Larsen et al. 2012), was also positively correlated with protein intake (p=0.00003.28), however, CS was also influenced by carbohydrate and fat intake [Figure 5.2.3, Table 5.2.1]. Encouragingly, both methods produced similar results, with Pearson's correlation between these two markers was also significant (r=0.23, p=0.004) [Table 5.2.3]. However, correlation with median lifespan results in the same cohort did not produce any statistically significant findings, with correlation coefficients close to zero in both cases (CN: r=-0.058, p=0.47; CS: r=0.019, p=0.99). This indicates that while mitochondrial number is affected by diet, it may not be directly correlated with lifespan; however it is important to note that despite being in the same cohort, mitochondrial assays were performed on mice sacrificed at 15 months and are therefore the data sets are not from the same mice as the longevity data.

Table 5.2.1. GAM statistics for mitochondrial copy number (normalised cytochrome b expression) and citrate synthase activity. Corresponding GF plots are shown in Figures 5.2.2 and 5.2.3.

p-value	P	C	F	P:C	P:F	C:F
Normalised Cytochrome b Expression	**3.98E-04**	0.0831	0.3429	0.2901	0.2041	0.4106
Citrate Synthase Activity	**1.92E-05**	**1.93E-03**	**2.74E-02**	0.4640	0.2415	0.3726

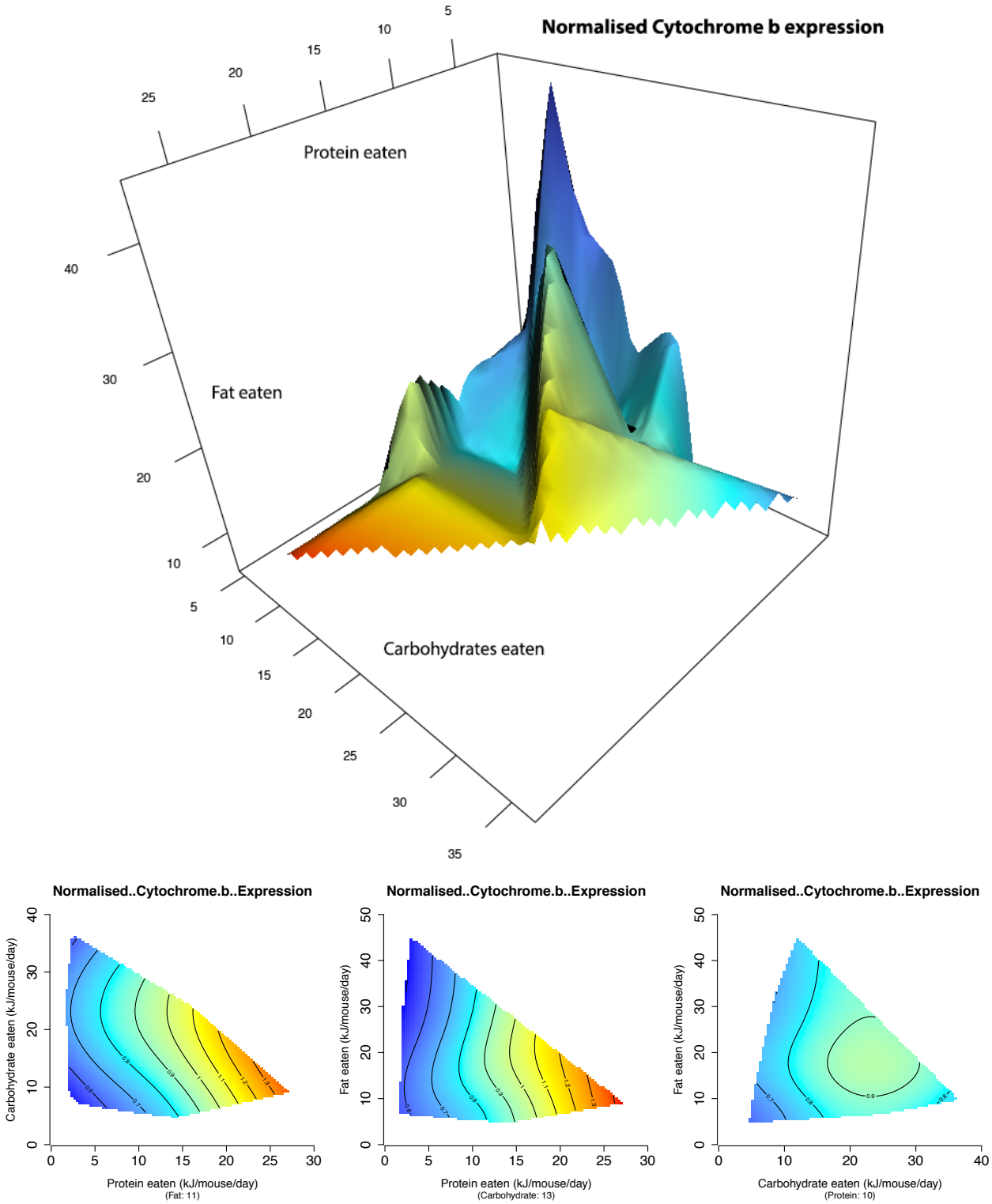


Figure 5.2.2. 2D and 3D GF plots for mitochondrial copy number (normalised cytochrome b gene expression). Corresponding GAM statistics are shown in Table 5.2.1.

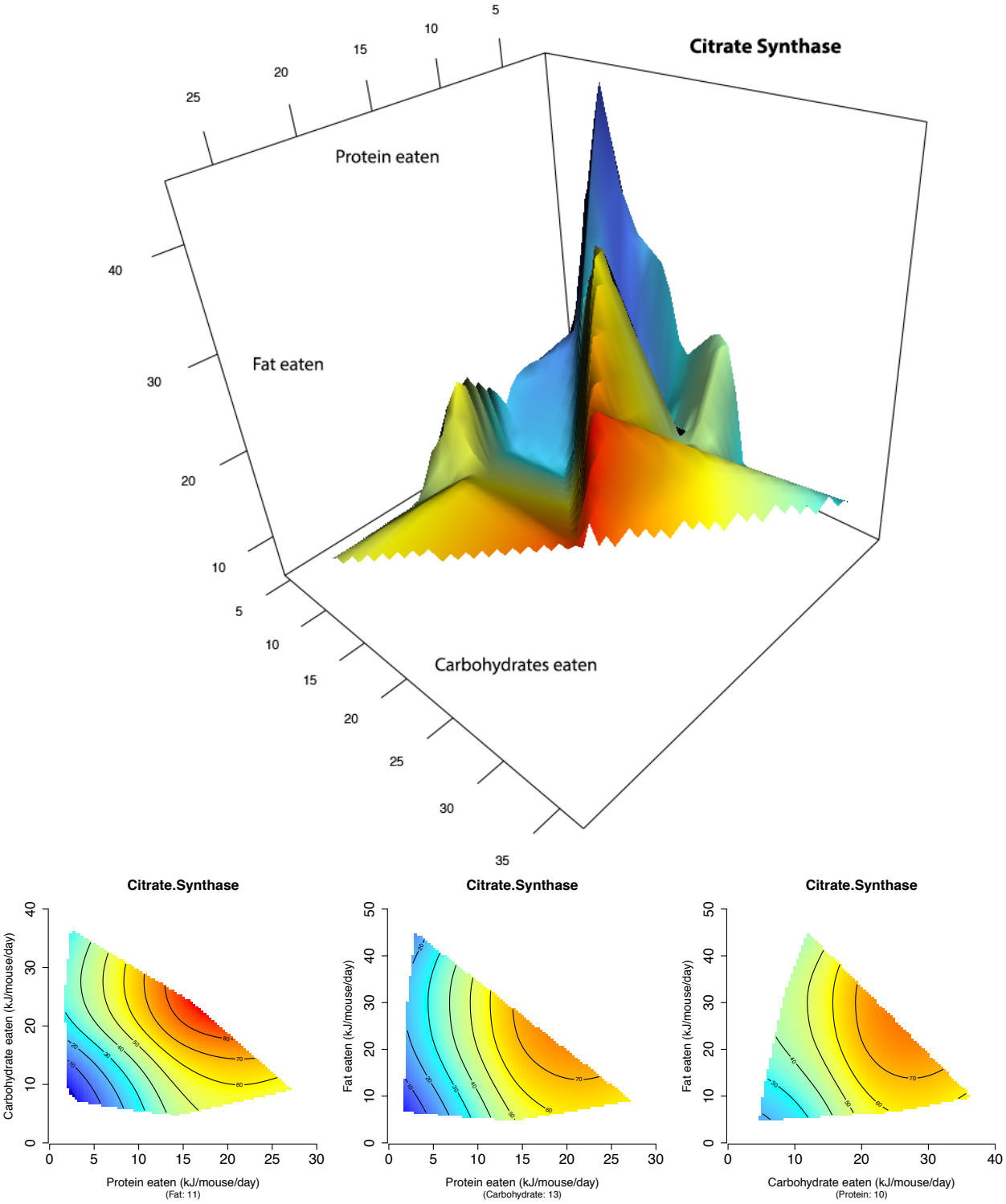


Figure 5.2.3. 2D and 3D GF plots for citrate synthase activity correlated with macronutrient intake. Corresponding GAM statistics are shown in Table 5.2.1.

Of the 35 non-genetic assays performed on mitochondrial function and enzymatic activity, 21 assays (60%) were correlated with macronutrient intake or ratios in a statistically significant manner [Table 5.2.2]. Of these, protein intake was by far the most significant factor influencing mitochondrial function (n=12). Interestingly, the general pattern of correlation with mitochondrial function was in a negative direction, with low dietary protein intake being associated with increased oxidative phosphorylation of substrates. While none of the mitochondrial functional assays were significantly correlated with directly lifespan, the diets that produced the longest lifespan were associated with increased hepatic mitochondrial activity and free radical production in the same cohort of mice.

Table 5.2.2. GAM statistics from GF analysis on mitochondrial function assays correlated with macronutrient intake.

Assay	**P**	**C**	**F**	**P:C**	**P:F**	**C:F**
Aconitase	0.44	0.31	1	0.44	0.81	0.95
AST	**1.13E-02**	0.34	0.57	0.46	0.70	0.31
Glutamate Antimycin A	0.09	0.50	1	0.36	0.97	0.16
Glutamate H2O2	**4.44E-02**	0.83	0.82	0.44	0.65	0.48
Glutamate RCR III/IVo	0.09	1	0.56	0.52	0.64	1
Glutamate Spare Capacity	**1.10E-04**	0.65	0.27	0.54	0.51	0.08
Glutamate State II	0.4	1	**2.62E-02**	0.79	0.45	0.81
Glutamate State III	0.81	0.87	0.49	0.90	0.54	0.18
Glutamate State IIIu	**1.14E-03**	0.95	0.54	0.73	0.54	0.25
Glutamate State IVo	0.52	0.78	0.76	**3.22E-02**	0.7	0.69
HOAD	**2.97E-02**	0.73	0.50	0.71	0.32	0.19
Palmitoyl carnitine Antimycin A	0.05	0.68	1	0.42	0.95	0.5
Palmitoyl carnitine RCR III/IVo	1	1	0.92	1	1	1

Palmitoyl carnitine Spare Capacity	0.14	0.34	0.85	0.86	**3.19E-02**	0.53
Palmitoyl carnitine State II	0.51	0.47	1	0.29	1	0.46
Palmitoyl carnitine State III	0.38	0.79	**1.77E-10**	0.62	**2.14E-02**	0.40
Palmitoyl carnitine State IIIu	0.65	0.2	0.25	0.62	0.1	0.83
Palmitoyl carnitine State IVo	0.61	1	**1.23E-03**	0.74	0.38	1.74E-03
Palmitoyl CoA H2O2	0.08	0.37	0.72	0.32	0.36	0.15
Pyruvate Antimycin A	0.69	0.44	0.47	0.89	0.87	0.48
Pyruvate H2O2	**2.70E-02**	0.90	0.78	0.50	0.60	0.48
Pyruvate RCR III/IVo	0.78	0.49	1	0.71	0.91	0.96
Pyruvate Spare Capacity	0.46	0.53	0.77	0.28	0.07	0.29
Pyruvate State II	**3.60E-02**	0.57	0.29	0.44	**4.70E-02**	0.74
Pyruvate State III	**1.13E-02**	0.14	0.59	0.43	0.66	0.27
Pyruvate State IIIu	0.12	0.95	0.3	0.63	0.08	0.44
Pyruvate State IVo	**2.59E-02**	0.99	0.7	0.59	0.56	0.38
Succinate Antimycin A	0.15	0.8	0.51	0.85	0.42	0.52
Succinate H2O2	**3.72E-02**	0.96	0.80	0.48	0.61	0.45
Succinate RCR III/IVo	**2.01E-02**	0.17	0.60	**1.48E-02**	0.24	0.05
Succinate Spare Capacity	**4.52E-09**	**3.31E-02**	1	0.34	0.90	0.09
Succinate State II	0.27	1	0.33	1	**1.22E-02**	0.82
Succinate State III	0.39	0.28	0.66	0.93	**1.51E-02**	0.66
Succinate State IIIu	0.36	**4.91E-02**	0.69	0.8	0.32	0.14
Succinate State IVo	1	0.49	0.73	1	**4.79E-02**	0.38

Discussion

In order to control metabolism and energy homeostasis, mitochondria adjust substrate utilisation in relation to nutrient availability (Boutant et al. 2016; Barbato & Aquilano 2016; Minamino et al. 2009). The three main nutrient sources are amino acids, glucose, and fatty acids, and under physiological circumstances, mitochondria are able to switch their fuel source between substrates freely (Muoio 2014; Pani 2010). Dysregulation of mitochondrial function on the other hand leads to metabolic inflexibility, decreased respiratory efficiency, and increased production of ROS (Payne & Chinnery 2015). While ROS have historically been thought to have a deleterious effect on ageing (Harman 1956), recent evidence has shown that these molecules may have key roles in cellular signaling pathways, and may in fact be beneficial to the ageing process in a hormetic fashion (López-Otín et al. 2016; Sharma et al. 2010). Caloric restriction, which had been initially predicted to lower basal metabolic rate (BMR) and ROS production has actually been shown to increase mitochondrial biogenesis and increase BMR (Nisoli et al. 2005; Zarse et al. 2012). Mild increases in ROS production with caloric restriction have also been shown to exert longevity benefits in many studies (Sharma et al. 2010; Rea et al. 2007; Munkácsy & Rea 2014), however these have also been shown to be accompanied by increased ROS scavenging mechanisms (Nisoli et al. 2005; Ristow et al. 2009). Here, it was shown that mitochondrial respiratory control ratios (RCR) and spare capacity, markers of mitochondrial function, were generally negatively correlated with protein intake. The notable exception was palmitoyl carnitine, where the spare capacity and RCR was driven more by the intake of fat and the P:F ratio, indicating that mitochondria adapted to increase capacity for fat utilisation as a substrate when dietary fat intake was high [Table 5.2.2]. As expected, these results are consistent with the literature where mitochondria optimise efficiency of substrate utilisation depending on nutrient availability in the cellular environment. Overall, the study shows that low protein intake was associated with increased mitochondrial activity and free radical production, while high protein intake was associated with increased citrate synthase, mitochondrial copy number. Therefore, animals on low

protein diets, which produced the greatest lifespan extension in the same cohort of mice, displayed increased mitochondrial activity, but a lower mitochondrial number. However, correlation of the results with lifespan data collected from the same cohort did not show any significant results. Given that mitochondrial number was associated with changes in pathways also influencing mitochondrial function, it can be hypothesised that the change in mitochondrial number may be a response to compensate for mitochondrial dysfunction, rather than a direct deleterious effect on longevity.

Table 5.2.3. Pearson's correlations between mitochondrial DNA copy number and nutrient sensing pathway data described in Chapter 4. *Longevity correlated via diet group in the same cohort.

Correlation with:	**R**	**p-value**
Circulating FGF21	-0.012	0.14
Circulating IGF1	0.49	0.85
Circulating Insulin	**0.87**	**0.03**
Citrate Synthase (CS)	**0.41**	**0.021**
FGF21 Gene expression	0.067	0.077
IGF1 Gene expression	0.57	0.58
Longevity*	-0.48	0.45
mTOR Activation	-0.96	0.45
mTOR Gene expression	-0.18	0.21
Prkaa1 Gene expression	**-0.67**	**0.027**
Prkaa2 Gene expression	0.59	0.2
Sirt1 Gene expression	-0.46	0.14
Sirt2 Gene expression	**-0.95**	**0.018**
Sirt3 Gene expression	**0.96**	**0.024**
Sirt4 Gene expression	0.83	0.63
Sirt5 Gene expression	0.51	0.99
Sirt6 Gene expression	-0.92	0.065
Sirt7 Gene expression	0.43	0.95

When compared with the growth pathway data from the same cohort of mice (described in detail in Chapter 4), mitochondrial DNA copy number, a marker for mitochondrial biogenesis was positively correlated with circulating insulin and gene expression SIRT3; and negatively correlated with AMPK and SIRT2 gene expression [Table 5.2.3]. Mitochondrial function on the other hand, was significantly correlated with multiple growth pathways; mTOR activation was positively correlated with RCR when pyruvate was used as a substrate, but negatively correlated with RCR when glutamate was used. Interestingly, while the geometric framework surface for mTOR activation showed a remarkably similar response pattern to that of mitochondrial copy number (i.e. positive correlations between protein intake and both mTOR activation and mitochondrial copy number), direct correlation between the two outcomes was not significant [Table 5.2.3]. While direct correlation was not statistically significant here, other studies have shown that inhibition of mTOR through gene mutations and rapamycin decrease mitochondrial copy number as well as expression of mitochondrial genes (Chen et al. 2008; Cunningham et al. 2007; Laplante & Sabatini 2009). Of the sirtuins localised to the mitochondria (SIRT3-5), the role of SIRT3, is the most well studied, and has been shown to play a role in mediating the effects of caloric restriction on lifespan (Someya et al. 2010; Houtkooper et al. 2012; Zhong & Mostoslavsky 2011). In the present study, SIRT3 gene expression was significantly correlated with 17 of the 32 mitochondrial activity assays (53%), while IGF-1 was correlated with 13 (41%), and AMPK with 10 (31%) [Figure 5.2.4, Table 5.2.4]. Interestingly, all three of these pathways were almost solely negatively correlated with mitochondrial function, except for SIRT3, which was positively correlated with mitochondrial spare capacity and H_2O_2 production when glutamate was used as a substrate. Taken together, these findings suggest that mitochondrial function is significantly influenced by diet, with alterations to IGF-1, AMPK and SIRT3 playing key roles in the regulatory process. This provides a plausible mechanism by which diet influences ageing, as diets associated with the highest longevity in the study, were also associated with changes to nutrient sensing pathways, mitochondrial function, and metabolic phenotype.

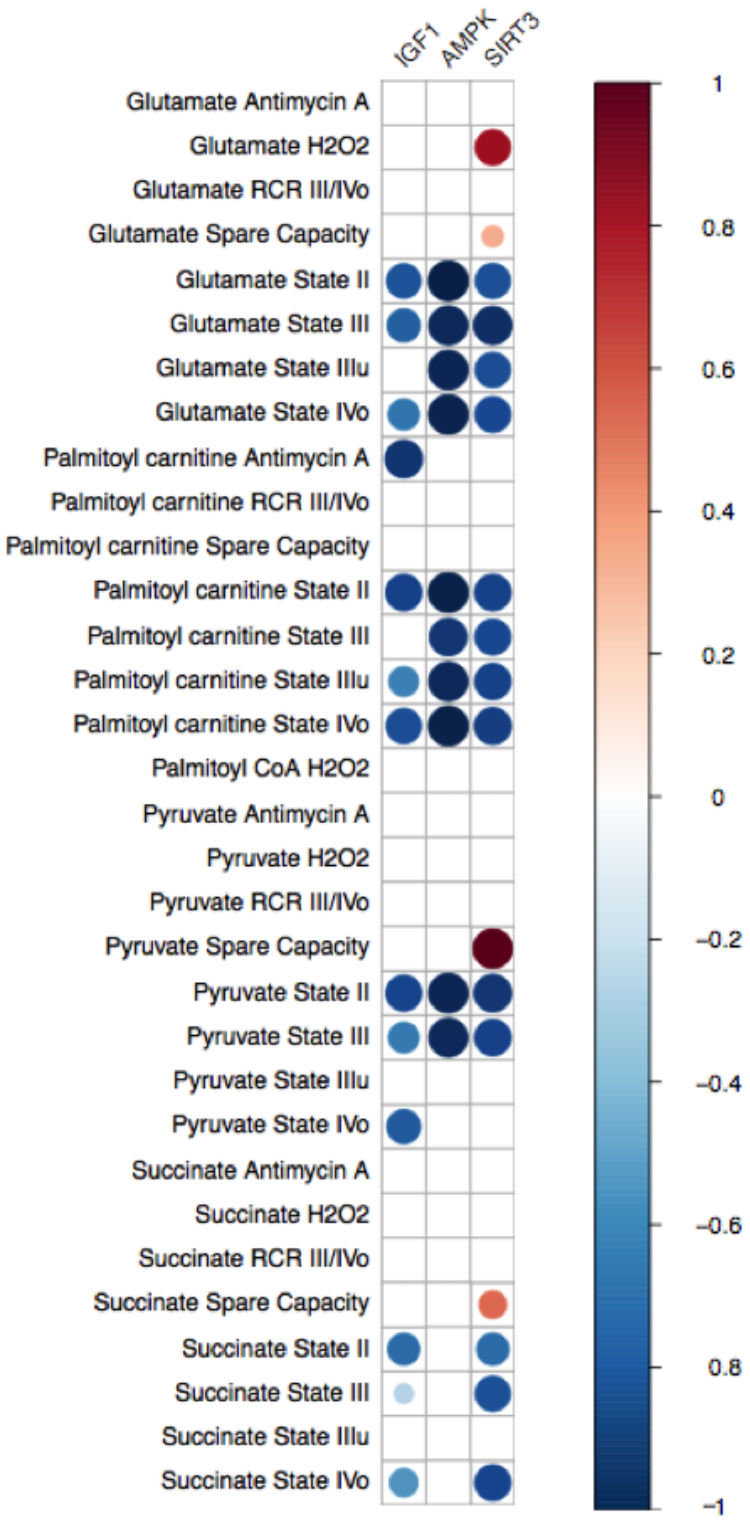


Figure 5.2.4. Pearson's correlations between mitochondrial function assays and IGF-1, AMPK & SIRT3. Corresponding data is shown in Table 5.2.4. Blank results indicate non-significant correlations ($p>0.05$).

Table 5.2.4. Pearson's correlations between mitochondrial function assays and IGF-1, AMPK & SIRT3. Values are expressed as Pearson's correlation coefficient, with p-values shown in brackets.

R (p-value)	IGF1	AMPK	SIRT3
Glutamate Antimycin A	0.44 (0.83)	-0.29 (0.21)	-0.12 (0.67)
Glutamate H2O2	0.38 (0.85)	0.24 (0.72)	**0.76 (0.024)**
Glutamate RCR III/IVo	-0.055 (0.78)	0.57 (0.59)	-0.038 (0.48)
Glutamate Spare Capacity	0.27 (0.89)	-0.35 (0.082)	**0.28 (0.0073)**
Glutamate State II	**-0.74 (0.014)**	**-0.99 (0.00053)**	**-0.76 (0.0001)**
Glutamate State III	**-0.68 (0.0081)**	**-0.94 (0.0031)**	**-0.9 (0.000023)**
Glutamate State IIIu	-0.55 (0.071)	**-0.96 (0.000079)**	**-0.77 (0.0011)**
Glutamate State IVo	**-0.6 (0.048)**	**-0.97 (0.00047)**	**-0.79 (0.00034)**
Palmitoyl carnitine Antimycin A	**-0.88 (0.033)**	-0.95 (0.1)	-0.81 (0.12)
Palmitoyl carnitine RCR III/IVo	0.79 (0.75)	0.32 (0.74)	0.1 (0.65)
Palmitoyl carnitine Spare Capacity	-0.3 (0.56)	0.0077 (0.37)	0.18 (0.45)
Palmitoyl carnitine State II	**-0.82 (0.0033)**	**-0.98 (0.0018)**	**-0.82 (0.0024)**
Palmitoyl carnitine State III	-0.43 (0.053)	**-0.87 (0.011)**	**-0.79 (0.01)**
Palmitoyl carnitine State IIIu	**-0.55 (0.033)**	**-0.94 (0.0087)**	**-0.81 (0.0044)**
Palmitoyl carnitine State IVo	**-0.77 (0.016)**	**-0.98 (0.0018)**	**-0.84 (0.002)**
Palmitoyl CoA H2O2	-0.095 (0.27)	-0.0082 (0.96)	0.54 (0.2)
Pyruvate Antimycin A	0.57 (0.65)	-0.047 (0.98)	0.47 (0.076)
Pyruvate H2O2	0.7 (0.51)	0.018 (0.45)	0.2 (0.23)
Pyruvate RCR III/IVo	0.42 (0.27)	0.47 (0.92)	-0.21 (0.39)
Pyruvate Spare Capacity	0.79 (0.81)	0.7 (0.28)	**0.95 (0.00034)**
Pyruvate State II	**-0.8 (0.0027)**	**-0.96 (0.0051)**	**-0.87 (0.0021)**
Pyruvate State III	**-0.58 (0.038)**	**-0.93 (0.017)**	**-0.82 (0.011)**
Pyruvate State IIIu	0.031 (0.49)	-0.63 (0.28)	-0.33 (0.63)
Pyruvate State IVo	**-0.71 (0.018)**	-1 (0.062)	-0.66 (0.15)
Succinate Antimycin A	-0.48 (0.25)	-0.89 (0.52)	-0.35 (0.64)
Succinate H2O2	0.078 (0.19)	0.69 (0.81)	0.59 (0.28)
Succinate RCR III/IVo	0.44 (0.82)	0.49 (0.58)	-0.17 (0.25)
Succinate Spare Capacity	0.14 (0.22)	0.62 (0.97)	**0.47 (0.023)**
Succinate State II	**-0.63 (0.019)**	-0.97 (0.091)	**-0.64 (0.032)**
Succinate State III	**-0.23 (0.047)**	-0.72 (0.093)	**-0.76 (0.014)**
Succinate State IIIu	0.037 (0.33)	-0.44 (0.42)	-0.57 (0.55)
Succinate State IVo	**-0.5 (0.037)**	-0.91 (0.06)	**-0.8 (0.022)**

Chapter 5.3: The relationship between dietary macronutrients and inflammation

Data from this chapter has been published in (Solon-Biet et al. 2015a).

Background

The liver provides a plausible mechanistic link between diet and ageing, as it plays a crucial role in nutritional metabolism and displays age-related changes such as inflammation, which can be histologically assessed (Le Couteur et al. 2008; Schmucker 2005; Popper 1986). This relationship between ageing and systemic low-grade chronic inflammation, or inflamm-ageing, is a key area of research associated with nutritional intervention, and is crucial to understanding the process of healthy ageing (Franceschi & Campisi 2014). Inflammatory markers such as IL-6, TNF-α, IL-1β and N-glycans have been studied as biomarkers of ageing and disease with varying degrees of success (Dall'Olio et al. 2013; Maggio et al. 2006; Klein et al. 2014; Wei et al. 2016; Kiecolt-Glaser et al. 2003). Diet has also been seen to be a contributor to inflammatory changes, particularly in the liver (Kim et al. 2016; Baur et al. 2006; Le Couteur et al. 2010). Age-related pseudocapillairisation of the liver involves thickening and defenestration of the sinusoidal endothelium, collagen deposition, inflammatory changes, and fat accumulation in stellate cells (Le Couteur et al. 2010; Le Couteur et al. 2008).

While the liver is known to display these changes, the endocrine pancreas has been less well studied in terms of histological correlation with diet (Harvey et al. 2014). The pancreas is known have a role in metabolism through its secretion of insulin and glucagon, and has also been shown to be affected by ageing and caloric restriction (Reaven & Reaven 1981; Okauchi et al. 1995; He et al. 2012). In fact, well-regulated glucose metabolism, low levels of insulin resistance and pancreatic β-cell maintenance

have been shown to be conserved phenotypes in centenarians, suggesting that pancreatic function is a key component to healthy ageing (Paolisso 2001). Since both CR and LPHC diets have also been shown to improve measures related with metabolic and cardiovascular health (Solon-Biet et al. 2015b), the impact of these diets on the liver and pancreas are assessed here. The effects of long-term CR or dietary macronutrient manipulation on the liver and pancreas have been established in many previous studies (Kim et al. 2016; Le Couteur et al. 2010; Valle et al. 2008; Jamieson et al. 2007; He et al. 2012; Okauchi et al. 1995), however whether these phenotypes can be induced by diet on a shorter time scale is unknown. In addition, while implementing long-term dietary regimes in humans is difficult, short-term diets are relatively common. Therefore, understanding the impact of short-term dietary manipulation and CR is useful not only to provide new insights into the mechanisms underlying the impact of nutrition on the body, but also add potential translational value to nutritional research.

Therefore, this section investigated the short-term effects of varying dietary macronutrients on hepatic steatosis, fibrosis and inflammatory infiltration, and pancreatic islet function. Data from the liver is correlated with histological data collected previously in the long-term dietary study, as described in detail in Chapter 2. The short-term experiment is therefore based on the general phenotypic findings of the larger-scale study, with maximal dietary differences used to decide the ratio of P:C used. Here, two month old mice were assigned one of three diets varying in P:C ratio, and allocated to either ad libitum (AL) or 40% CR intake regimes. Thus, both CR and macronutrient ratio were assessed in the short-term study.

Methods

Animal and dietary interventions are discussed in detail in Chapter 2. Additional methods used in the short-term dietary study are discussed here, and are adapted from (Solon-Biet et al. 2015a) where the

data has been previously published. Data from the long-term study was analysed by the Geometric Framework as discussed in detail in Chapter 3. Short-term study data is presented as mean ± SEM, and differences are considered significant when $p < 0.05$.

Male C57BL6/J mice (8 week old; n = 90; Jackson Laboratory) were grouped in cages containing five mice each at the National Institute of Aging, Baltimore, USA. All animal protocols were approved by the Gerontology Research Center Animal Care and Use Committee (352-LEG-2012) of the National Institute on Aging. Isocaloric (4kcal/g; Dyets) experimental diets were classified as low protein, high carbohydrate (5% protein; LPHC); medium protein, medium carbohydrate (33% protein; MPMC); and high protein, low carbohydrate (60% protein, HPLC). The ratio of fat was therefore fixed at 20% across the diets. Mice in each of these groups were assigned to an ad libitum (AL), or 40% calorically restricted (CR) group, where intake was limited to 60% of the caloric intake measured in the corresponding AL group. Mice were kept on these diets for 8 weeks, prior to being euthanised. Blood and tissue samples were collected for histological and biochemical analyses. On the day of the sacrifice, CR mice were not fed while AL mice were allowed to eat normally. A total of n=15 mice were in each dietary group.

Paraffin-embedded liver tissue from both short and long-term dietary studies were sectioned and stained using Haematoxylin & Eosin (H&E), Periodic acid-Schiff (PAS), and Sirius Red. Embedded pancreatic tissue was sectioned and immunohistochemistry was performed using monoclonal anti-glucagon antibody (Sigma G2654) and monoclonal anti-insulin antibody (Sigma I2018). The extent of change in the response variables (fatty infiltration, inflammation, glucagon and insulin intensity) was assessed and scored (0, 1+, 2+, 3+) by four independent observers, blinded to the tissue category, through light microscopy.

Results

While histological changes were noted between groups in the short-term study, all diets displayed normal gross liver histology, as seen in Figure 5.3.1. H&E staining of these livers showed a positive correlation between P:C ratio and portal inflammation in AL animals, but not in CR animals [Figures 5.3.2, 5.3.3, Table 5.3.1]. Sirius Red staining indicated no effect of P:C ratio on fibrosis, however there was a negative correlation between P:C ratio and steatosis in both the AL and CR animals. In the pancreas, the number of insulin-containing cells was not significantly different in CR animals compared to AL animals, however in the AL animals, there was an increase in intensity of staining for glucagon in the HPLC group compared to the other AL groups [Figure 5.3.4, Table 5.3.1]. This was accompanied by an increase in glucagon secretion, elevated blood glucose levels, and glucose intolerance, however those data sets were not analysed during the course of this thesis, and are described elsewhere (Solon-Biet et al. 2015a).

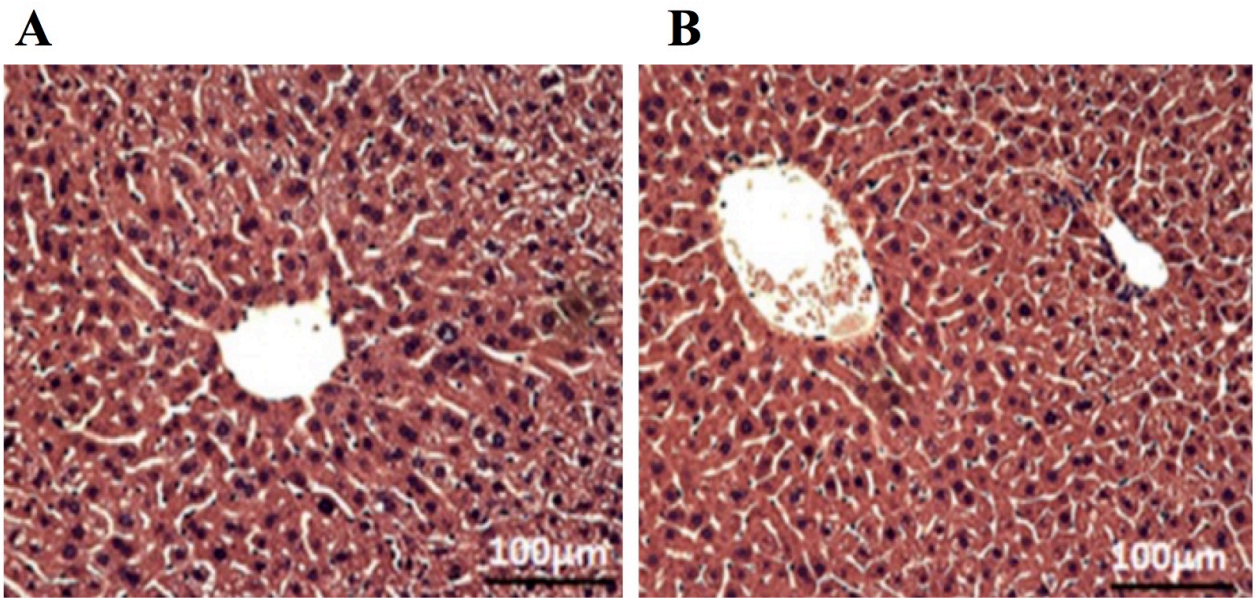


Figure 5.3.1. Representative H&E stained livers from the short-term dietary study; (A) AL group, and (B) CR group.

Table 5.3.1. Histological scores in the liver and pancreas from the short-term dietary study. Data presented as mean+/- SEM. Grey boxes indicate statistical significance on t-test compared with the corresponding LPHC group; corresponding p-values shown in Table 5.3.2.

Histology	LPHC-AL	MPMC-AL	HPLC-AL	LPHC-CR	MPMC-CR	HPLC-CR
Sinusoidal inflammation	0.8+/-0.2	1.23+/-0.23	0.78+/-0.15	1.31+/-0.21	0.85+/-0.19	0.91+/-0.25
Portal inflammation	1.1+/-0.18	1.23+/-0.17	**2.11+/-0.11**	1.62+/-0.18	1.38+/-0.18	1.91+/-0.16
Central inflammation	0.1+/-0.1	0.15+/-0.1	0.22+/-0.15	0.23+/-0.17	0.23+/-0.17	0.55+/-0.21
Steatosis	2.18+/-0.18	**1.23+/-0.23**	**1+/-0.17**	2.08+/-0.31	**1+/-0.28**	**0.64+/-0.2**
Sinusoidal fibrosis	1.45+/-0.25	1.54+/-0.24	2.11+/-0.2	1.31+/-0.13	**1.92+/-0.24**	1.82+/-0.23
Portal fibrosis	1.18+/-0.18	1.31+/-0.29	1.44+/-0.29	1.38+/-0.27	**1.77+/-0.23**	1.36+/-0.24
Central fibrosis	1+/-0.27	0.69+/-0.24	0.89+/-0.31	0.54+/-0.18	0.92+/-0.21	0.82+/-0.23
Insulin	2.1+/-0.28	1.96+/-0.24	1.56+/-0.18	1.46+/-0.23	2.54+/-0.14	1.36+/-0.23
Glucagon	1.4+/-0.22	1.43+/-0.17	**2.4+/-0.16**	0.9+/-0.18	1.33+/-0.26	1.13+/-0.13

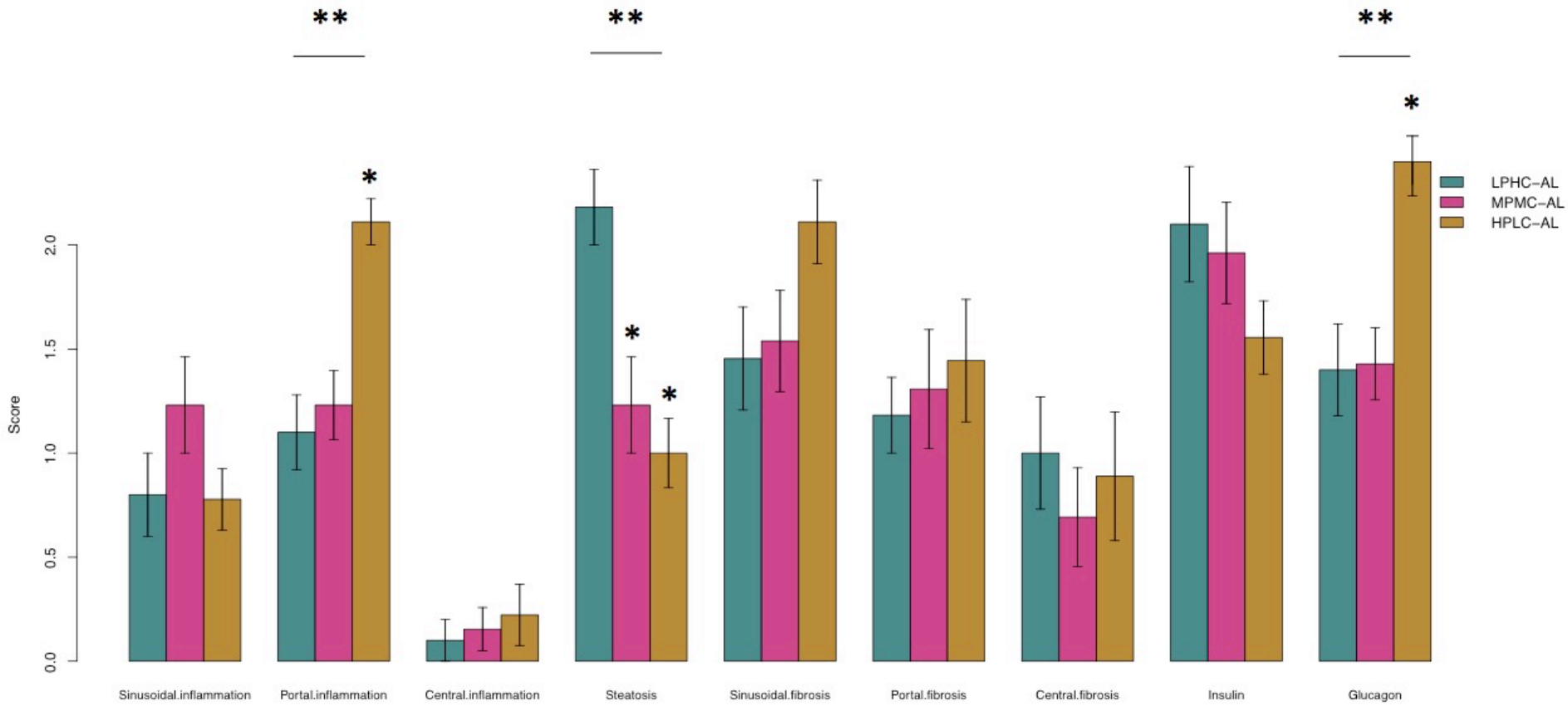


Figure 5.3.2. Histological scores for ad libitum-fed mice in the short-term dietary study. *indicates $p<0.05$ on t-test against LPHC group [Table 5.3.1]; **indicates $p<0.05$ on ANOVA [Table 5.3.2]. Corresponding data is shown in Table 5.3.1. Experimental diets were classified as low protein, high carbohydrate (5% protein; LPHC); medium protein, medium carbohydrate (33% protein; MPMC); and high protein, low carbohydrate (60% protein, HPLC).

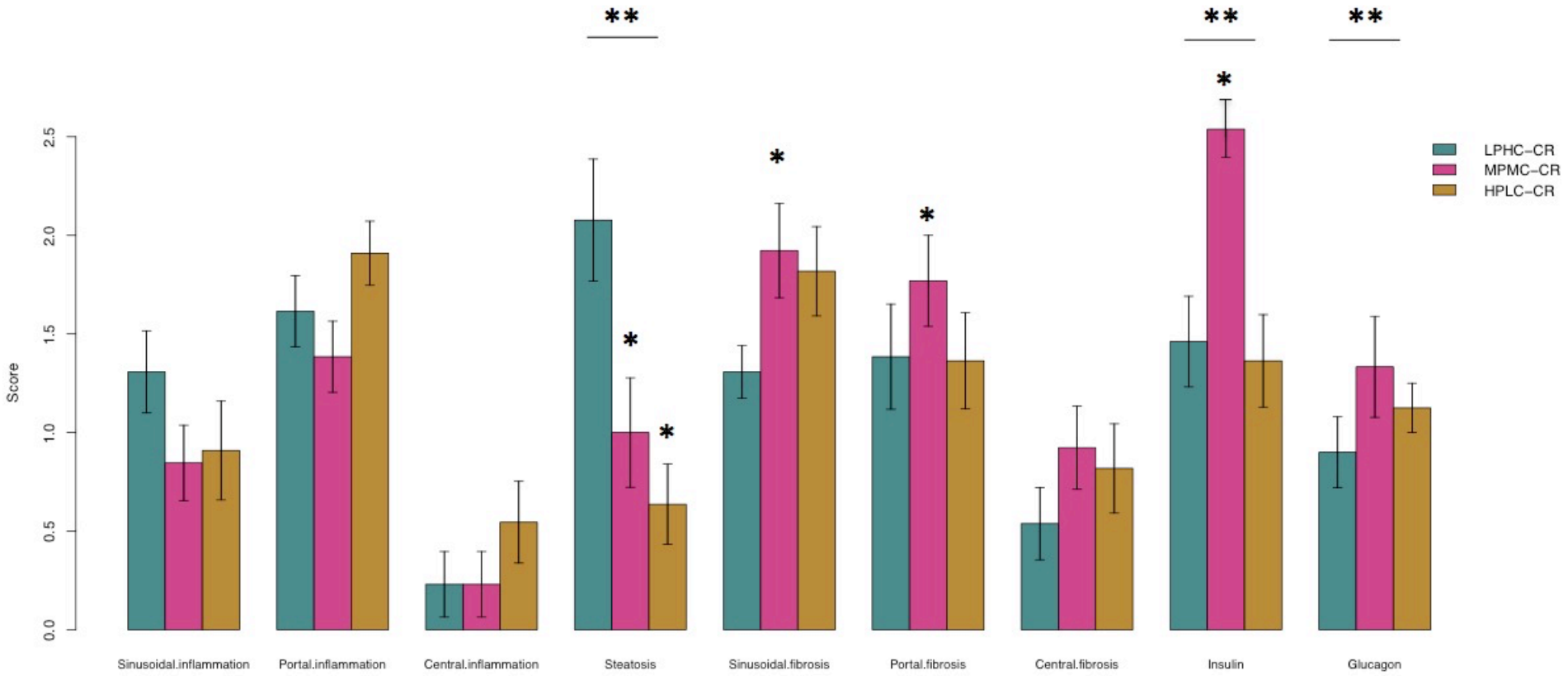


Figure 5.3.3. Histological scores for 40% calorically restricted mice in the short-term dietary study. *indicates $p<0.05$ on t-test against LPHC group [Table 5.3.1]; **indicates $p<0.05$ on ANOVA [Table 5.3.2]. Corresponding data is shown in Table 5.3.1. Experimental diets were classified as low protein, high carbohydrate (5% protein; LPHC); medium protein, medium carbohydrate (33% protein; MPMC); and high protein, low carbohydrate (60% protein, HPLC).

Table 5.3.2. Statistics for histological scores in the liver and pancreas from the short-term dietary study. Data presented as p-values. Experimental diets were classified as low protein, high carbohydrate (5% protein; LPHC); medium protein, medium carbohydrate (33% protein; MPMC); and high protein, low carbohydrate (60% protein, HPLC). The ratio of fat was fixed at 20% across the diets, and mice were assigned to either an ad libitum (AL), or 40% calorically restricted (CR) group.

Histology	T-test compared to LPHC group				ANOVA		
	MPMC-AL	**HPLC-AL**	**MPMC-CR**	**HPLC-CR**	**Combined**	**AL**	**CR**
Sinusoidal inflammation	0.73	1.00	0.24	0.40	0.31	0.17	0.33
Portal inflammation	0.68	**1.77E-02**	0.49	0.20	**9.36E-04**	**8.88E-04**	0.26
Central inflammation	1.00	1.00	1.00	0.10	0.45	0.17	0.67
Steatosis	**4.31E-02**	**6.60E-03**	**2.51E-02**	**3.53E-02**	**6.39E-05**	**2.25E-03**	**6.28E-03**
Sinusoidal fibrosis	0.22	0.06	**4.55E-02**	0.08	0.11	0.27	0.05
Portal fibrosis	0.47	0.56	**2.51E-02**	0.80	0.67	0.78	0.21
Central fibrosis	0.22	0.76	0.10	0.22	0.74	0.86	0.42
Insulin	0.71	0.08	**5.76E-03**	0.63	**1.93E-03**	0.50	**5.60E-04**
Glucagon	0.73	**4.27E-05**	0.36	0.61	**1.23E-04**	**1.03E-03**	**3.99E-02**

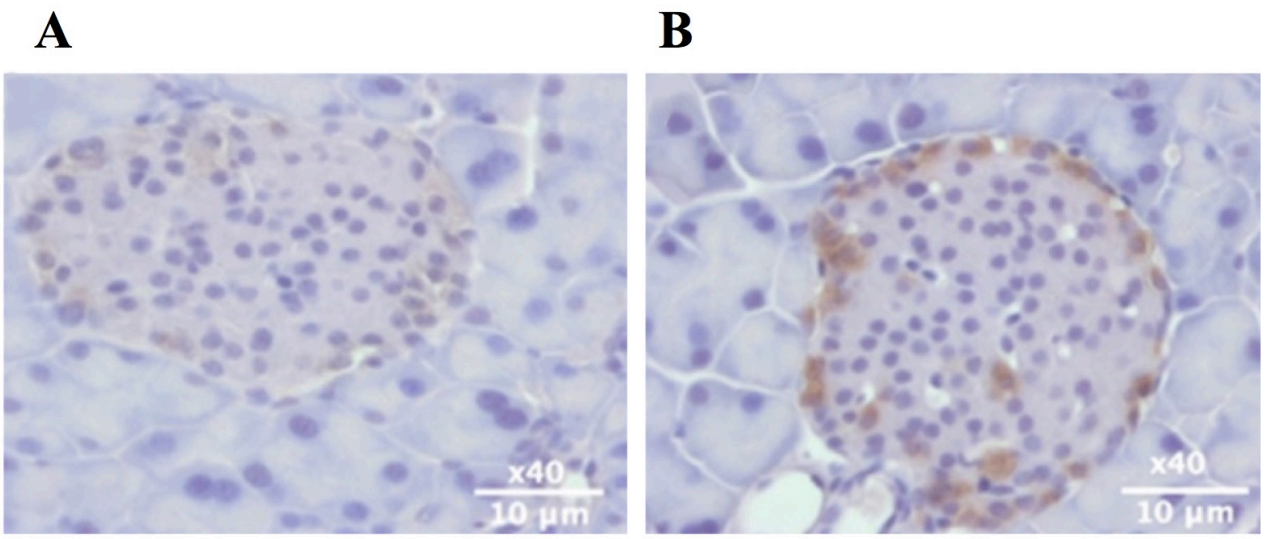


Figure 5.3.4. Representative glucagon stained pancreas samples from the short-term dietary study; (A) AL group, and (B) CR group.

In the long-term studies, hepatic inflammation, both through fibrotic changes, as well as fatty infiltration was associated with the P:F ratio in the diet ($p=0.00218$ and $p=0.006.86$ respectively) [Figure 5.3.5, Table 5.3.3]. On the other hand, glycogen content, which is a marker for energy stores, was affected by C:F ratio, and was only mildly negatively correlated with steatosis, implying that this change was likely unrelated to inflammation. Histology in the long-term diets was influenced heavily by the ratio of fat in the diet, a factor which was not assessed in the short-term study.

Table 5.3.3. GAM statistics for Geometric Framework analysis of the phenotypic results from the long-term mouse study. Corresponding surfaces are shown in Figure 5.3.5.

Hepatic histology	P	C	F	P:C	P:F	C:F
Steatosis (Sirius Red)	**4.08E-02**	**8.67E-03**	**3.51E-03**	0.17	**2.18E-03**	0.83
Fibrosis (H&E)	0.14	0.33	0.18	0.10	**6.86E-03**	0.44
Glycogen (PAS)	1.00	0.28	0.46	0.54	0.63	**2.13E-02**

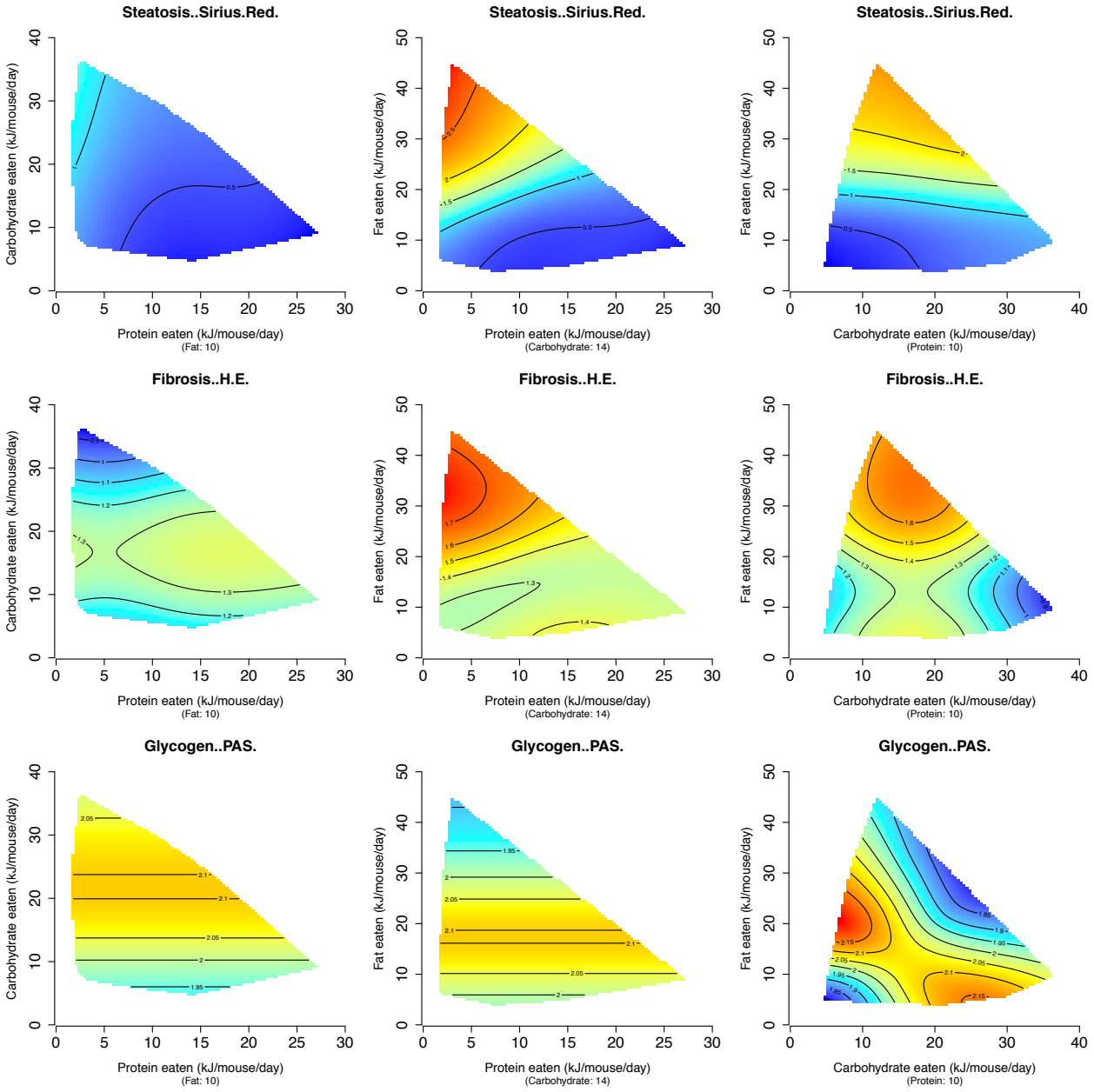


Figure 5.3.5. 2D GF surfaces of hepatic histology from the long-term dietary study. Corresponding statistics are shown in Table 5.3.3.

Discussion

Chronic overnutrition is linked with increasing instances of obesity and metabolic disorders such as diabetes, cardiovascular disease and non-alcoholic fatty liver disease (Buettner et al. 2007). The amount of protein in the diet is one of the key drivers of food intake, as organisms are known to regulate their protein intake more heavily compared with other macronutrients; a concept known as protein leverage (Simpson & Raubenheimer 2005; Gosby et al. 2013; Lee et al. 2008), and this result is seen in both cohorts of mice used in this study (Solon-Biet et al. 2014; Solon-Biet et al. 2015a). While the results of the diets used here on metabolic outcomes have been discussed in the aforementioned publications, it is interesting to note that manipulation of macronutrient ratios did not provide additional benefits to 40% CR animals in the short-term, but did influence the outcomes in ad libitum fed animals (Solon-Biet et al. 2015a). This seems to indicate that food intake, particularly the ratio of P:C in the diet, is one of the key factors influencing metabolic outcomes; a result that has been shown throughout this thesis, and elsewhere (Fanson & Taylor 2012; Le Couteur et al. 2015; Jensen et al. 2015; Simpson et al. 2003).

In the long-term studies, hepatic steatosis was shown to be significantly correlated with protein and P:F ratio, while fibrosis was shown to be significantly correlated with the P:F ratio. These results are consistent with studies in both humans and mice, where fat plays a major role in hepatic histological changes (Benard et al. 2016; Buettner et al. 2007; Hill et al. 2000). In the short-term studies, protein and the P:C ratio were the key drivers of inflammation, with high P:C ratios being linked with increased portal inflammation, and low P:C ratios are linked with hepatic fat accumulation. However, it should be noted that the ratio of fat in the diet, which was a significant fact driver of histology in the long-term study, was not assessed in the short-term study, where the ratio of fat was fixed across all diets at 20%. This limitation makes it difficult to compare the results across the studies.

Intensity of insulin staining was not significantly different between CR and AL animals, nor linked directly to macronutrient intake. Glucagon on the other hand, was significantly influenced by protein in the diet, and when correlated with blood glucose levels and oral glucose tolerance tests in the same mice, suggest that high protein intake is associated with increased glucagon secretion, higher circulating glucose levels, and glucose intolerance, as previously reported (Solon-Biet et al. 2015a).

Taken together, these findings imply that in the short-term, dietary protein is the key driver of inflammatory changes, however fat intake seems to drive hepatic inflammation in the long-term, consistent with findings in the literature. CR animals displayed less inflammation than AL animals in the short-term, suggesting that protein restriction may be beneficial for healthy liver function. It is important however to note that macronutrient effects may vary with age, due to adaptation, and changes in metabolic and cellular function (Brown-Borg & Buffenstein 2016; Simpson et al. 2015). Thus, the results are consistent with the idea that high protein and fat intake is associated with inflammatory liver changes, while low protein intake (either low P:C ratio or CR) is hepato-protective. When combined with the larger panel of metabolic findings in these animals, it was shown that macronutrient balance, particularly LPHC diets, can mimic the benefits of CR on phenotypic end points even in the short-term (Solon-Biet et al. 2015a). Interestingly, short-term CR and intermittent fasting have also been shown to be equivalent to, or in some cases better than chronic caloric restriction for weight loss and indicators of chronic disease (Chen et al. 2016; Harvie & Howell 2012; Heilbronn et al. 2012). This study therefore represents an important area of research, as understanding the relationship between diet, ageing and age-related end points may help form guidelines to promote healthy ageing without the issues associated with long-term caloric restriction.

Chapter 6: General Discussion

Nutrition has profound influences on aging. The major nutritional intervention that has been studied is caloric restriction (CR), whereby lifelong reduction in energy intake by 10-50% has been reported to increase lifespan and delay biological changes associated with aging in many species (Mercken et al. 2012; Ingram & de Cabo 2017). Although CR has been a foundation of aging research for nearly a century, more recent studies have questioned whether it is CR per se that is responsible for the effects on health and lifespan (Brown-Borg & Buffenstein 2016; Fanson & Taylor 2012; Solon-Biet et al. 2015b; Solon-Biet et al. 2014; Le Couteur et al. 2015). The effects of CR on aging have been shown to vary by species, strain and sex (Mitchell et al. 2016; Baar et al. 2015) and while reduced energy intake is a key contributor to its benefits (Speakman et al. 2016; Gibbs & Smith 2016), other factors such as intermittent fasting (Liao et al. 2010; Y. Chen et al. 2016; C. Lee & Longo 2011) and reduced intake of macronutrients, especially protein (Lee et al. 2008, Solon-Biet et al. 2015) may also be important. Recent studies in insects and mice have increasingly focused on the ratio of macronutrients in the diet using the Geometric Framework, with most studies reporting that diets composed of low protein combined with high carbohydrates increase lifespan even in ad libitum-fed organisms (Lee et al. 2008, Solon-Biet et al. 2014, Le Couteur et al. 2016). From a translational perspective, this is important because ad libitum feeding is unavoidable in most humans.

While the role of diet on life extension has been relatively well established in simple model organisms, apparently contradictory evidence has been seen when long-term CR was applied to rhesus monkeys at the National Institutes of Aging (NIA) (Ingram et al. 1990; Lane et al. 1992), compared to the University of Wisconsin-Madison (UW) (Ramsey et al. 2000). At the NIA, long-term 30% CR was not shown to increase lifespan, however did demonstrate significant impacts on disease prevention,

particularly metabolic disease and cancer (Mattison et al. 2012). On the other hand, the 30% CR diet instituted at UW resulted in a reduction in age-related mortality, with a hazard ratio of 2.9 in the control group compared to CR, and a hazard ratio of 1.78 for all-cause mortality on Cox regression (Colman et al. 2014). A joint initiative from the institutions was recently published (Mattison et al. 2017), presenting a comparison of longitudinal data from both studies. Taken together, the data confirmed that CR does indeed confer health and survival benefits in non-human primates, and that differences in intrinsic trial designs could account for the differences in lifespan outcomes between the studies (Mattison et al. 2017). In particular, differences in diet (UW: semi-purified diet more similar to Western diet; NIA: minimally processed plant based diet more similar to Mediterranean diet), and macronutrient composition (UW: 13.13% P : 58.31% C : 10.6% F ; NIA: 17.3% P : 56.9% C : 5.0% F) led to all animal cohorts at the NIA, both control and CR, to consume calories at a similar rate to the CR group at UW, thereby implying that both groups at the NIA animals were likely calorically restricted, which impaired the ability to observe differences between the groups (Mattison et al. 2017). Supporting this, is the fact that both control and CR cohorts at the NIA had an average lifespan 45% longer than Rhesus monkeys kept in captivity (Rizza et al. 2014), thus highlighting the importance of both dietary intake and macronutrient composition.

While similar trials to assess the impact of diet on longevity are not feasible in humans, correlation between data in primates and mice, with short-term dietary interventions in humans, for example the CALERIE study (Ravussin et al. 2015; Heilbronn et al. 2006), shows good concordance in health-related outcomes. This suggests strong evidence to support conserved mechanisms across species, and a potential for translatable therapies to impact at the very least, age-related health, if not lifespan (Most et al. 2017; de Cabo et al. 2014). Given the importance of macronutrient balance, and the difficulty in disentangling the impact of dietary composition in isolation, here the Geometric Framework (GF) was used to facilitate interpretation of the effects of macronutrients and their

interactions on ageing and age-related end points (Simpson and Raubenheimer 2007, Simpson et al. 2015). This thesis therefore combined two bodies of work to investigate the mechanisms that underlie ageing, and the ability of nutrition to influence this process.

The first was the development of analytical methods as discussed in Chapter 3. These build upon the Geometric Framework to allow efficient and automated data collection, analysis, and visualisation across a variety of experimental structures. Two major issues were addressed here: 1) the discord between GAMs analysis and 2D visualisation, which was addressed by the development of a 3D visualisation method, and; 2) automation of the GF to large data sets, allowing multiple data types to be simultaneously analysed. These developments allowed the GF to be used as the primary analytical method across this Thesis, and be applied to various novel data sets such as PCR and microarray analysis of the transcriptome. The GF is an invaluable research tool, and further work in this area could help broaden the horizons of the GF beyond nutritional and ageing research, to a much more widely encompassing experimental paradigm.

The second component was primary experimental work performed on nutrient sensing pathways, and investigation into their roles in ageing, lifespan and phenotypic age-related end points using the paradigm of a dietary study in mice. In Chapter 4, macronutrient balance was shown to play a significant role in the regulation of nutrient sensing pathways in both the liver and the hypothalamus, and produced patterns of change in both gene regulation, as well as signaling pathway activation, that were associated with improvements in health and longevity, in concordance with the literature (Evans et al. 2011; Lamming et al. 2013; Vaiserman et al. 2016; Wanders et al. 2014). In the liver, all major nutrient sensing pathways (mTOR, IIS, AMPK, sirtuins) were altered by dietary macronutrient intake ratios, with dietary protein in particular shown to be the main driver of gene expression. LPHC diets were shown to decrease mTOR activity, reduce circulating FGF-21 levels, and increase circulating

IGF-1, as previously reported (Solon-Biet et al. 2014; Solon-Biet et al. 2016). By contrast, intake of carbohydrates and fat had limited effects on gene regulation, most of which were in the opposite direction to changes seen with protein intake. In the hypothalamus, overall gene expression changes was most influenced by fat intake, a result that is consistent with previous studies (Jang et al. 2017; Yamamoto et al. 2009). Once again, protein was demonstrated to influence the gene expression in opposite directions compared to carbohydrates and fat.

This is interesting because altering the concentration of one macronutrient in the diet also influences the ratio of the other intakes, as well as total energy density (Le Couteur et al., 2016). Given this interaction, the use of the GF allows differentiation between the effects of macronutrient intake, ratios and total energy, which are difficult to using univariate analysis. Furthermore, the difference between ratios in diet versus ratios of actual macronutrient intake must also be pointed out, particularly given the fact that low protein diets are known to increase food consumption due to titration of intake to protein targets, a concept known as protein leverage (Simpson and Raubenheimer, 2005). Discrepancies in analytical methodology and experimental design in this regard have led to seemingly diametrically opposite results. In particular, debate exists as to whether the effects of CR on longevity are primarily a result of a reduction of calories (Speakman, Mitchell, & Mazidi, 2016; Hu et al. 2018), or protein (Nakagawa et al. 2012; Solon-Biet et al. 2014; Fontana et al. 2016).

This discussion has been extensively reviewed previously (Ingram & Cabo, 2017; Picca, Pesce, & Lezza, 2017; Simpson et al. 2017b), which collectively conclude that while both CR and protein restriction lead to health benefits, and perhaps even modulate overlapping pathways, they are mechanistically different. Indeed, Speakman et al. also acknowledge that protein restriction can produce longevity benefits relative to reference diets independent of CR (Speakman, Mitchell, &

Mazidi, 2016). In addition, taking a nutritional geometry approach to these same sets of data has been shown to reconcile the apparently disparate findings seen on conventional analysis (Simpson et al. 2017b). Therefore, while debate still exists however around the degree of importance of CR versus protein restriction, the GF seems to be the most appropriate analytical tool available to evaluate study data given the complexity between macronutrient and interactions, compensatory feeding responses and interpretation of outcomes (Le Couteur et al., 2016; Simpson et al., 2017b).

Given the differences in gene regulation between the liver and hypothalamus, further analysis using multiple tissue types could play an important role in consolidating the relationship between diet, gene regulation and ageing. Indeed, the variance in response between different tissue types raises the mechanistic questions of which pathways are responsible for driving outcome related changes, and how they are coordinated. While protein was seen to be the main driver for changes in gene expression in the liver, fat and total energy dominated the transcriptomic changes in the hypothalamus. Coordination of these pathways through feedback loops; both on the level of transcription vs. translation vs. post-translational modification, as well as the HPA and other endocrine axes; add another degree of complexity in interpreting results. One approach to disentangle these complexities is the use of a systems-level approach, which, when applied across a variety of species, has shown that age-related genes are highly expressed across most tissue types, and co-expressed with essential tissue-specific functional genes, often acting as intermediaries for pathway cross-talk (Zhang et al. 2016). These findings imply that activation of the same gene pathways in different tissues can have very different effects, with higher degrees of complexity and interconnectedness amongst more complex organisms.

A second approach is to look at functional enrichment analysis using platform databases such the KEGG longevity gene set and GO biological processes gene set, both of which were performed in

our studies. Here, it is shown that in both the liver and the hypothalamus, dietary manipulation influences gene expression in nutrient sensing pathways, however the complex and elaborate mechanisms of their regulation make it difficult to mechanistically describe the coordination between these two systems. Again, the literature suggests that while network characteristics are not typically tissue specific, their roles in each tissue may functionally differ (Jia et al. 2018). For example, longevity-related genes in the brain may be associated with neuronal functionality and neurogenesis (Zhang et al. 2016), associated with immunocompetency in haematopoetic cells (Passtoors et al., 2015), and with metabolic processes in the liver (Newgard & Pessin, 2014).

While both these approaches help expand our mechanistic understanding of the role of these pathways, it is important to frame these in the context of the larger question being studied here, namely the impact of these pathways on lifespan. Perhaps the most striking set of studies with regards to this are gene knockout/overexpression studies in model organisms, which have showed that alterations to nutrient sensing pathways can lead to dramatic changes in lifespan (Lamming et al. 2013; Johnson et al. 2013; Jia 2004; Kapahi et al. 2004; Junnila et al. 2013). Therefore, while a great deal of work is required to better understand the intricacies of mechanism, understanding variations in activation of nutrient pathways across a range of tissue sets under different nutritional conditions plays a crucial first step in better characterising the role of these pathways in the ageing process.

The effects of diet on age-related end points were also studied. Here, hepatic telomere length was shown to be significantly influenced by dietary intake, with diets high in carbohydrates on a low P:C background being the most significant factor correlating with longer telomere lengths. The striking similarities between the response surfaces of telomere length and median lifespan is perhaps one of the finest results discovered in this thesis, with patterns of telomere length almost exactly mirroring median lifespan in the same cohort of mice. These results are consistent with the concept that long

telomere length is associated with longer lifespan, which has been reported both in aged mice (Vera et al. 2012; Cherif et al. 2003; Ludlow et al. 2012) and humans (Mons et al. 2017; Cawthon et al. 2003). Since telomere length was shown to be sensitive to nutrition, further investigation of telomere length across various tissue types could provide important evidence supporting its use as a biomarker of ageing. Mitochondrial number and function were also investigated, and it was found that LPHC diets, which produced the greatest lifespan extension in the same cohort of mice, were correlated with increased mitochondrial activity, but a lower mitochondrial number. Mitochondrial function was also correlated with changes to gene expression of IGF-1, AMPK and SIRT3. These findings suggest that mitochondrial function is significantly influenced by diet, with nutrient sensing pathways playing key roles in the regulatory process. Finally, inflammatory phenotypes that were seen in the long-term cohort were investigated in a second cohort of mice, to see if these changes could be replicated in short-term studies. Here, it was shown that HPLC diets in ad libitum-fed animals were correlated with hepatic inflammation, and LPHC diets correlated with hepatic fat deposition. Manipulation of macronutrient ratios did not provide additional benefit to 40% CR in the short-term, implying that a combination of caloric restriction, and specific macronutrient restriction are important in healthy ageing of the liver.

This body of work therefore shows that nutrient sensing pathways, key modulators of cellular function, display differential activation patterns as a result of varying dietary macronutrient intake. In particular, protein intake was seen to be a key regulator of nutrient sensing pathway regulation and downstream effects on age-related end points. These results, when correlated with lifespan and health data in the same cohort of mice, show that modulation of nutrient sensing pathways is a key mechanism mediating the effects of dietary restriction on ageing. This study therefore represents an important step in understanding the mechanisms by which macronutrient intake influences the ageing process, and provides support for the role of nutrition in promoting healthy ageing in humans.

References

Aguilaniu, H. 2003. Asymmetric Inheritance Of Oxidatively Damaged Proteins During Cytokinesis. *Science,* 299**,** 1751-1753.

Akima, H. 1978. A Method Of Bivariate Interpolation And Smooth Surface Fitting For Irregularly Distributed Data Points. *Acm Transactions On Mathematical Software (TOMS),* 4**,** 148-159.

Aon, M. A., Cortassa, S., Juhaszova, M. & Sollott, S. J. 2016. Mitochondrial Health, The Epigenome And Healthspan. *Clinical Science,* 130**,** 1285-1305.

Aoyama, K., Matsubara, K., Kondo, M., Murakawa, Y., Suno, M., Yamashita, K., Yamaguchi, S. & Kobayashi, S. 2001. Nicotinamide-N-Methyltransferase Is Higher In The Lumbar Cerebrospinal Fluid Of Patients With Parkinson's Disease. *Neuroscience Letters*, 298, 78-80.

Armanios, M., Alder, J. K., Parry, E. M., Karim, B., Strong, M. A. & Greider, C. W. 2009. Short Telomeres Are Sufficient To Cause The Degenerative Defects Associated With Aging. *The American Journal Of Human Genetics,* 85**,** 823-832.

Arriola Apelo, S. I. & Lamming, D. W. 2016. Rapamycin: An Inhibitor Of Aging Emerges From The Soil Of Easter Island. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences*.

Ashapkin, V. V., Kutueva, L. I. & Vanyushin, B. F. 2017. Aging As An Epigenetic Phenomenon. *Current Genomics,* 18**,** 385-407.

Astrup, A., Gøtzsche, P. C., Van De Werken, K., Ranneries, C., Toubro, S., Raben, A. & Buemann, B. 1999. Meta-Analysis Of Resting Metabolic Rate In Formerly Obese Subjects. *The American Journal Of Clinical Nutrition,* 69**,** 1117-1122.

Atamna, H., Cheung, I. & Ames, B. N. 2000. A Method For Detecting Abasic Sites In Living Cells: Age-Dependent Changes In Base Excision Repair. *Proceedings Of The National Academy Of Sciences,* 97**,** 686-691.

Aubert, G. & Lansdorp, P. M. 2008. Telomeres And Aging. *Physiological Reviews,* 88**,** 557-579.

Austriaco, N. R. & Guarente, L. P. 1997. Changes Of Telomere Length Cause Reciprocal Changes In The Lifespan Of Mother Cells In Saccharomyces Cerevisiae. *Proceedings Of The National Academy Of Sciences,* 94**,** 9768-9772.

Baar, E. L., Carbajal, K. A., Ong, I. M. & Lamming, D. W. 2015. Sex- And Tissue-Specific Changes In Mtor Signaling With Age In C57bl/6j Mice. *Aging Cell,* 15**,** 155-166.

Bahar, R., Hartmann, C. H., Rodriguez, K. A., Denny, A. D., Busuttil, R. A., Dollé, M. E. T., Calder, R. B., Chisholm, G. B., Pollock, B. H., Klein, C. A. & Vijg, J. 2006. Increased Cell-To-Cell Variation In Gene Expression In Ageing Mouse Heart. *Nature,* 441**,** 1011-1014.

Bajracharya, R. & Ballard, J. W. O. 2016. Low Protein To Carbohydrate Ratio Diet Delays Onset Of Parkinsonism Like Phenotype In Drosophila Melanogaster Parkin Null Mutants. *Mechanisms Of Ageing And Development,* 160**,** 19-27.

Barbato, D. L. & Aquilano, K. 2016. Feast And Famine: Adipose Tissue Adaptations For Healthy Aging. *Ageing Research Reviews,* 28**,** 85-93.

Bartke, A. 2005. Minireview: Role Of The Growth Hormone/Insulin-Like Growth Factor System In Mammalian Aging. *Endocrinology,* 146**,** 3718-3723.

Barzilai, N., Crandall, J. P., Kritchevsky, S. B. & Espeland, M. A. 2018. Metformin As A Tool To Target Aging. *Cell Metabolism,* 23**,** 1060-1065.

Barzilai, N., Kritchevsky, S. B., Espeland, M. A. & Crandall, J. P. 2016. Targeting Aging With Metformin (Tame): A Study To Target Aging In Humans. *The Gerontologist,* 56**,** 199-199.

Bass, T. M., Weinkove, D., Houthoofd, K., Gems, D. & Partridge, L. 2007. Effects Of Resveratrol On Lifespan In Drosophila Melanogaster And Caenorhabditis Elegans. *Mechanisms Of Ageing And Development,* 128**,** 546-552.

Baur, J. A. 2010. Resveratrol, Sirtuins, And The Promise Of A DR Mimetic. *Mechanisms Of Ageing And Development,* 131**,** 261-269.

Baur, J. A. & Sinclair, D. A. 2006. Therapeutic Potential Of Resveratrol: The In Vivo Evidence. *Nature Reviews Drug Discovery,* 5**,** 493-506.

Baur, J. A., Ungvari, Z., Minor, R. K., Le Couteur, D. G. & De Cabo, R. 2012. Are Sirtuins Viable Targets For Improving Healthspan And Lifespan? *Nature Reviews Drug Discovery,* 11**,** 443-461.

Baur, J. A., Pearson, K. J., Price, N. L., Jamieson, H. A., Lerin, C., Kalra, A., Prabhu, V. V., Allard, J. S., López-Lluch, G., Lewis, K., Pistell, P. J., Poosala, S., Becker, K. G., Boss, O., Gwinn, D., Wang, M., Ramaswamy, S., Fishbein, K. W., Spencer, R. G., Lakatta, E. G., Le Couteur, D., Shaw, R. J., Navas, P., Puigserver, P., Ingram, D. K., De Cabo, R. & Sinclair, D. A. 2006. Resveratrol Improves Health And Survival Of Mice On A High-Calorie Diet. *Nature Publishing Group,* 444**,** 337-342.

Benard, O., Lim, J., Apontes, P., Jing, X., Angeletti, R. H. & Chi, Y. 2016. Impact Of High-Fat Diet On The Proteome Of Mouse Liver. *The Journal Of Nutritional Biochemistry,* 31**,** 10-19.

Benayoun, B. A., Pollina, E. A. & Brunet, A. 2015. Epigenetic Regulation Of Ageing: Linking Environmental Inputs To Genomic Stability. *Nature Publishing Group,* 16**,** 593-610.

Bhatt, J. K., Thomas, S. & Nanjan, M. J. 2012. Resveratrol Supplementation Improves Glycemic Control In Type 2 Diabetes Mellitus. *Nutrition Research,* 32**,** 537-541.

Bhullar, K. S. & Hubbard, B. P. 2015. Lifespan And Healthspan Extension By Resveratrol. *Biochimica Et Biophysica Acta (Bba) - Molecular Basis Of Disease,* 1852**,** 1209-1218.

Bishop, N. A. & Guarente, L. 2007. Two Neurons Mediate Diet-Restriction-Induced Longevity In C. Elegans. *Nature,* 447**,** 545-549.

Blagosklonny, M. V. 2013a. Immunosuppressants In Cancer Prevention And Therapy. *Oncoimmunology,* 2**,** E26961.

Blasco, M. A. 2005. Telomeres And Human Disease: Ageing, Cancer And Beyond. *Nature Reviews Genetics,* 6**,** 611-622.

Bo, S., Ciccone, G., Castiglione, A., Gambino, R., De Michieli, F., Villois, P., Durazzo, M., Cavallo-Perin, P. & Cassader, M. 2013. Anti-Inflammatory And Antioxidant Effects Of Resveratrol In Healthy Smokers A Randomised, Double-Blind, Placebo-Controlled, Cross-Over Trial. *Current Medicinal Chemistry,* 20**,** 1323-1331.

Bonawitz, N. D., Chatenay-Lapointe, M., Pan, Y. & Shadel, G. S. 2007. Reduced Tor Signaling Extends Chronological Life Span Via Increased Respiration And Upregulation Of Mitochondrial Gene Expression. *Cell Metabolism,* 5**,** 265-277.

Bonkowski, M. S. & Sinclair, D. A. 2016. Slowing Ageing By Design: The Rise Of Nad+And Sirtuin-Activating Compounds. *Nature Reviews Molecular Cell Biology,* 17**,** 679-690.

Bonnard, C., Durand, A., Peyrol, S., Chanseaume, E., Chauvin, M.-A., Morio, B., Vidal, H. & Rieusset, J. 2008. Mitochondrial Dysfunction Results From Oxidative Stress In The Skeletal Muscle Of Diet-Induced Insulin-Resistant Mice. *Journal Of Clinical Investigation,* 118**,** 789-800.

Boonekamp, J.J., Briga, M. & Verhulst, S., 2015. The heuristic value of redundancy models of aging. *Experimental Gerontology*, pp.1–8.

Bordone, L., Cohen, D., Robinson, A., Motta, M. C., Van Veen, E., Czopik, A., Steele, A. D., Crowe, H., Marmor, S., Luo, J., Gu, W. & Guarente, L. 2007. Sirt1 Transgenic Mice Show Phenotypes Resembling Calorie Restriction. *Aging Cell,* 6**,** 759-767.

Boutant, M., Kulkarni, S. S., Joffraud, M., Raymond, F., Métairon, S., Descombes, P. & Cantó, C. 2016. Sirt1 Gain Of Function Does Not Mimic Or Enhance The Adaptations To Intermittent Fasting. *Cell Reports,* 14**,** 2068-2075.

Bratic, A. & Larsson, N.-G. 2013. The Role Of Mitochondria In Aging. *Journal Of Clinical Investigation,* 123**,** 951-957.

Brestoff, J. R. & Artis, D. 2015. Immune Regulation Of Metabolic Homeostasis In Health And Disease. *Cell,* 161**,** 146-160.

Broer, L. & Van Duijn, C. M. 2015. Gwas And Meta-Analysis In Aging/Longevity. *Advances In Experimental Medicine And Biology,* 847**,** 107-125.

Brown, V. A., Patel, K. R., Viskaduraki, M., Crowell, J. A., Perloff, M., Booth, T. D., Vasilinin, G., Sen, A., Schinas, A. M., Piccirilli, G., Brown, K., Steward, W. P., Gescher, A. J. & Brenner, D. E. 2010. Repeat Dose Study Of The Cancer Chemopreventive Agent Resveratrol In Healthy Volunteers: Safety, Pharmacokinetics, And Effect On The Insulin-Like Growth Factor Axis. *Cancer Research,* 70**,** 9003-9011.

Brown-Borg, H. M. & Buffenstein, R. 2016. Cutting Back On The Essentials: Can Manipulating Intake Of Specific Amino Acids Modulate Health And Lifespan? *Ageing Research Reviews***,** 1-9.

Buettner, R., Schölmerich, J. & Bollheimer, L. C. 2007. High-Fat Diets: Modeling The Metabolic Disorders Of Human Obesity In Rodents. *Obesity,* 15**,** 798-808.

Burkewitz, K., Zhang, Y. & Mair, W. B. 2014. Ampk At The Nexus Of Energetics And Aging. *Cell Metabolism***,** 1-16.

Burt, M. G. & Ho, K. K. Y. 2003. Comparison Of Efficacy And Tolerability Of Somatostatin Analogs And Other Therapies For Acromegaly. *Endocrine,* 20**,** 299-306.

Cabreiro, F., Au, C., Leung, K.-Y., Vergara-Irigaray, N., Cochemé, H. M., Noori, T., Weinkove, D., Schuster, E., Greene, N. D. E. & Gems, D. 2013. Metformin Retards Aging In C. Elegans By Altering Microbial Folate And Methionine Metabolism. *Cell,* 153**,** 228-239.

Callicott, R. J. & Womack, J. E. 2006. Real-Time Pcr Assay For Measurement Of Mouse Telomeres. *Comparative Medicine,* 56**,** 17-22.

Cantó, C. & Auwerx, J. 2011. Calorie Restriction: Is Ampk A Key Sensor And Effector? *Physiology,* 26**,** 214-224.

Cantó, C., Jiang, L. Q., Deshmukh, A. S., Mataki, C., Coste, A., Lagouge, M., Zierath, J. R. & Auwerx, J. 2010. Interdependence Of Ampk And Sirt1 For Metabolic Adaptation To Fasting And Exercise In Skeletal Muscle. *Cell Metabolism,* 11**,** 213-219.

Carboni, J. M., Wittman, M., Yang, Z., Lee, F., Greer, A., Hurlburt, W., Hillerman, S., Cao, C., Cantor, G. H., Dell-John, J., Chen, C., Discenza, L., Menard, K., Li, A., Trainor, G., Vyas, D.,

Kramer, R., Attar, R. M. & Gottardis, M. M. 2009. Bms-754807, A Small Molecule Inhibitor Of Insulin-Like Growth Factor-1r/Ir. *Molecular Cancer Therapeutics,* 8**,** 3341-3349.

Carrano, A. C., Liu, Z., Dillin, A. & Hunter, T. 2009. A Conserved Ubiquitination Pathway Determines Longevity In Response To Diet Restriction. *Nature,* 77**,** 727.

Castello, L., Maina, M., Testa, G., Cavallini, G., Biasi, F., Donati, A., Leonarduzzi, G., Bergamini, E., Poli, G. & Chiarpotto, E. 2011. Alternate-Day Fasting Reverses The Age-Associated Hypertrophy Phenotype In Rat Heart By Influencing The Erk And Pi3k Signaling Pathways. *Mechanisms Of Ageing And Development,* 132**,** 305-314.

Cava, E. & Fontana, L. 2013. Will Calorie Restriction Work In Humans? *Aging,* 5**,** 507-514.

Cawthon, R. M. 2002. Telomere Measurement By Quantitative Pcr. *Nucleic Acids Research,* 30**,** E47.

Cawthon, R. M., Smith, K. R., O'brien, E., Sivatchenko, A. & Kerber, R. A. 2003. Association Between Telomere Length In Blood And Mortality In People Aged 60 Years Or Older. The Lancet, 361, 393-395.

Cevenini, E., Monti, D. & Franceschi, C. 2013. Inflamm-Ageing. *Current Opinion In Clinical Nutrition And Metabolic Care,* 16**,** 14-20.

Chen, C., Liu, Y., Liu, R., Ikenoue, T., Guan, K.-L., Liu, Y. & Zheng, P. 2008. Tsc–Mtor Maintains Quiescence And Function Of Hematopoietic Stem Cells By Repressing Mitochondrial Biogenesis And Reactive Oxygen Species. *Journal Of Experimental Medicine,* 205**,** 2397-2408.

Chen, H. X. & Sharon, E. 2013. Igf-1r As An Anti-Cancer Target--Trials And Tribulations. *Chinese Journal Of Cancer,* 32**,** 242-252.

Chen, Y., Ling, L., Su, G., Han, M., Fan, X., Xun, P. & Xu, G. 2016. Effect Of Intermittent Versus Chronic Calorie Restriction On Tumor Incidence: A Systematic Review And Meta-Analysis Of Animal Studies. *Scientific Reports,* 6**,** 33739.

Cherif, H., Tarry, J. L., Ozanne, S. E. & Hales, C. N. 2003. Ageing And Telomeres: A Study Into Organ- And Gender-Specific Telomere Shortening. *Nucleic Acids Research,* 31**,** 1576-1583.

Chondrogianni, N., Voutetakis, K., Kapetanou, M., Delitsikou, V., Papaevgeniou, N., Sakellari, M., Lefaki, M., Filippopoulou, K. & Gonos, E. S. 2015. Proteasome Activation: An Innovative Promising Approach For Delaying Aging And Retarding Age-Related Diseases. *Ageing Research Reviews,* 23**,** 37-55.

Civitarese, A. E., Carling, S., Heilbronn, L. K., Hulver, M. H., Ukropcova, B., Deutsch, W. A., Smith, S. R. & Ravussin, E. 2007. Calorie Restriction Increases Muscle Mitochondrial Biogenesis In Healthy Humans. *Plos Medicine,* 4**,** E76.

Cogger, V. C., Mitchell, S. J., Warren, A., De Cabo, R. & Le Couteur, D. G. 2014a. Age-Related Loss Of Responsiveness To 2,5-Dimethoxy-4-Iodoamphetamine In Liver Sinusoidal Endothelial Cells. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences,* 69**,** 514-518.

Cogger, V. C., Svistounov, D., Warren, A., Zykova, S., Melvin, R. G., Solon-Biet, S. M., O'reilly, J. N., Mcmahon, A. C., Ballard, J. W. O., De Cabo, R., Le Couteur, D. G. & Lebel, M. 2014b. Liver Aging And Pseudocapillarization In A Werner Syndrome Mouse Model. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences*, 69, 1076-1086.

Colman, R. J., Beasley, T. M., Kemnitz, J. W., Johnson, S. C., Weindruch, R. & Anderson, R. M. 2014. Caloric Restriction Reduces Age-Related And All-Cause Mortality In Rhesus Monkeys. *Nature Communications,* 5**,** 1-5.

Coperchini, F., Leporati, P., Rotondi, M. & Chiovato, L. 2015. Expanding The Therapeutic Spectrum Of Metformin: From Diabetes To Cancer. *Journal Of Endocrinological Investigation,* 38**,** 1047-1055.

Coppé, J.-P., Desprez, P.-Y., Krtolica, A. & Campisi, J. 2010. The Senescence-Associated Secretory Phenotype: The Dark Side Of Tumor Suppression. *Annual Review Of Pathology,* 5**,** 99-118.

Coste, A., Louet, J. F., Lagouge, M., Lerin, C., Antal, M. C., Meziane, H., Schoonjans, K., Puigserver, P., O'malley, B. W. & Auwerx, J. 2008. The Genetic Ablation Of Src-3 Protects Against Obesity And Improves Insulin Sensitivity By Reducing The Acetylation Of Pgc-1. *Proceedings Of The National Academy Of Sciences*, 105, 17187-17192.

Cottart, C.-H., Nivet-Antoine, V. & Beaudeux, J.-L. 2013. Review Of Recent Data On The Metabolism, Biological Effects, And Toxicity Of Resveratrol In Humans. *Molecular Nutrition & Food Research,* 58**,** 7-21.

Crandall, J. P., Oram, V., Trandafirescu, G., Reid, M., Kishore, P., Hawkins, M., Cohen, H. W. & Barzilai, N. 2012. Pilot Study Of Resveratrol In Older Adults With Impaired Glucose Tolerance. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences,* 67**,** 1307-1312.

Cunningham, J. T., Rodgers, J. T., Arlow, D. H., Vazquez, F., Mootha, V. K. & Puigserver, P. 2007. Mtor Controls Mitochondrial Oxidative Function Through A Yy1–Pgc-1α Transcriptional Complex. *Nature,* 450**,** 736-740.

Da Costa, J. P., Vitorino, R., Silva, G. M., Vogel, C., Duarte, A. C. & Rocha-Santos, T. 2016. A Synopsis On Aging − Theories, Mechanisms And Future Prospects. *Ageing Research Reviews***,** 1-88.

Dall'olio, F., Vanhooren, V., Chen, C. C., Slagboom, P. E., Wuhrer, M. & Franceschi, C. 2013. N-Glycomic Biomarkers Of Biological Aging And Longevity: A Link With Inflammaging. *Ageing Research Reviews,* 12**,** 685-698.

Dang, W., Steffen, K. K., Perry, R., Dorsey, J. A., Johnson, F. B., Shilatifard, A., Kaeberlein, M., Kennedy, B. K. & Berger, S. L. 2009. Histone H4 Lysine 16 Acetylation Regulates Cellular Lifespan. *Nature,* 459**,** 802-807.

Daniali L, Benetos A, Susser E, Kark JD, Labat C, Kimura M, Desai K, Granick M, Aviv A., 2013. Telomeres shorten at equivalent rates in somatic tissues of adults. *Nature Communications*, 4(1), p.1597.

Darwin, C. 1859. *On The Origin Of Species By Means Of Natural Selection, Or The Preservation Of Favoured Races In The Struggle For Life,* London, John Murray.

Davis, T. A., Bales, C. W. & Beauchene, R. E. 1983. Differential Effects Of Dietary Caloric And Protein Restriction In The Aging Rat. *Experimental Gerontology,* 18**,** 427-435.

De Cabo, R., Carmona-Gutierrez, D., Bernier, M., Hall, M. N. & Madeo, F. 2014. The Search For Antiaging Interventions: From Elixirs To Fasting Regimens. *Cell,* 157**,** 1515-1526.

De Haes, W., Frooninckx, L., Van Assche, R., Smolders, A., Depuydt, G., Billen, J., Braeckman, B. P., Schoofs, L. & Temmerman, L. 2014. Metformin Promotes Lifespan Through Mitohormesis Via The Peroxiredoxin Prdx-2. *Proceedings Of The National Academy Of Sciences Of The United States Of America,* 111**,** E2501-9.

Deblon, N., Bourgoin, L., Veyrat-Durebex, C., Peyrou, M., Vinciguerra, M., Caillon, A., Maeder, C., Fournier, M., Montet, X., Rohner-Jeanrenaud, F. & Foti, M. 2012. Chronic Mtor Inhibition By

Rapamycin Induces Muscle Insulin Resistance Despite Weight Loss In Rats. *British Journal Of Pharmacology,* 165**,** 2325-2340.

Denzel, M. S., Storm, N. J., Gutschmidt, A., Baddi, R., Hinze, Y., Jarosch, E., Sommer, T., Hoppe, T. & Antebi, A. 2014. Hexosamine Pathway Metabolites Enhance Protein Quality Control And Prolong Life. *Cell,* 156**,** 1167-1178.

Dhahbi, J. M., Mote, P. L., Fahy, G. M. & Spindler, S. R. 2005. Identification Of Potential Caloric Restriction Mimetics By Microarray Profiling. *Physiological Genomics,* 23**,** 343-350.

Dillin, A., Gottschling, D. E. & Nyström, T. 2014. The Good And The Bad Of Being Connected: The Integrons Of Aging. *Current Opinion In Cell Biology,* 26**,** 107-112.

Díaz-Rúa, R., Keijer, J., Palou, A., Van Schothorst, E. M. & Oliver, P. 2017. Long-Term Intake Of A High-Protein Diet Increases Liver Triacylglycerol Deposition Pathways And Hepatic Signs Of Injury In Rats. *The Journal Of Nutritional Biochemistry,* 46**,** 39-48.

Edgar, D., Shabalina, I., Camara, Y., Wredenberg, A., Calvaruso, M. A., Nijtmans, L., Nedergaard, J., Cannon, B., Larsson, N.-G. & Trifunovic, A. 2009. Random Point Mutations With Major Effects On Protein-Coding Genes Are The Driving Force Behind Premature Aging In Mtdna Mutator Mice. *Cell Metabolism,* 10**,** 131-138.

Efeyan, A., Comb, W. C. & Sabatini, D. M. 2015. Nutrient-Sensing Mechanisms And Pathways. *Nature,* 517**,** 302-310.

Endo, Y., Fu, Z., Abe, K., Arai, S. & Kato, H. 2002. Dietary Protein Quantity And Quality Affect Rat Hepatic Gene Expression1. *The Journal Of Nutrition,* 132**,** 3632-3637.

Eriksson, J. G., Guzzardi, M.-A., Iozzo, P., Kajantie, E., Kautiainen, H. & Salonen, M. K. 2017. Higher Serum Phenylalanine Concentration Is Associated With More Rapid Telomere Shortening In Men. *The American Journal Of Clinical Nutrition,* 105**,** 144-150.

Erjavec, N., Larsson, L., Grantham, J. & Nyström, T. 2007. Accelerated Aging And Failure To Segregate Damaged Proteins In Sir2 Mutants Can Be Suppressed By Overproducing The Protein Aggregation-Remodeling Factor Hsp104p. *Genes & Development,* 21**,** 2410-2421.

Evans, D. S., Kapahi, P., Hsueh, W.-C. & Kockel, L. 2011. Tor Signaling Never Gets Old: Aging, Longevity And Torc1 Activity. *Ageing Research Reviews* 10**,** 225.

Fang, E. F., Scheibye-Knudsen, M., Chua, K. F., Mattson, M. P., Croteau, D. L. & Bohr, V. A. 2016. Nuclear Dna Damage Signalling To Mitochondria In Ageing. *Nature Publishing Group***,** 1-14.

Fanson, B. G. & Taylor, P. W. 2012. Protein:Carbohydrate Ratios Explain Life Span Patterns Found In Queensland Fruit Fly On Diets Varying In Yeast:Sugar Ratios. *Age (Dordrecht, Netherlands),* 34**,** 1361-1368.

Fehrmann, S., Paoletti, C., Goulev, Y., Ungureanu, A., Aguilaniu, H. & Charvin, G. 2013. Aging Yeast Cells Undergo A Sharp Entry Into Senescence Unrelated To The Loss Of Mitochondrial Membrane Potential. *Cellreports,* 5**,** 1589-1599.

Feser, J., Truong, D., Das, C., Carson, J. J., Kieft, J., Harkness, T. & Tyler, J. K. 2010. Elevated Histone Expression Promotes Life Span Extension. *Molecular Cell,* 39**,** 724-735.

Finkel, T. 2015. The Metabolic Regulation Of Aging. *Nature Medicine,* 21**,** 1416-1423.

Fischer, K. E. & Riddle, N. C. 2018. Sex Differences In Aging: Genomic Instability. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences,* 73**,** 166-174.

Flachsbart, F., Croucher, P. J., Nikolaus, S., Hampe, J., Cordes, C., Schreiber, S. & Nebel, A. 2006. Sirtuin 1 (Sirt1) Sequence Variation Is Not Associated With Exceptional Human Longevity. *Experimental Gerontology,* 41**,** 98-102.

Fontana, L. & Partridge, L. 2015. Promoting Health And Longevity Through Diet: From Model Organisms To Humans. *Cell,* 161**,** 106-118.

Fontana, L., Cummings, N. E., Arriola Apelo, S. I., Neuman, J. C., Kasza, I., Schmidt, B. A., Cava, E., Spelta, F., Tosti, V., Syed, F. A., Baar, E. L., Veronese, N., Cottrell, S. E., Fenske, R. J., Bertozzi, B., Brar, H. K., Pietka, T., Bullock, A. D., Figenshau, R. S., Andriole, G. L., Merrins, M. J., Alexander, C. M., Kimple, M. E. & Lamming, D. W. 2016. Decreased Consumption Of Branched-Chain Amino Acids Improves Metabolic Health. *Cell Reports,* 16**,** 520-530.

Fontana, L., Villareal, D. T., Das, S. K., Smith, S. R., Meydani, S. N., Pittas, A. G., Klein, S., Bhapkar, M., Rochon, J., Ravussin, E., Holloszy, J. O. & Group, T. C. S. 2015. Effects Of 2-Year Calorie Restriction On Circulating Levels Of Igf-1, Igf-Binding Proteins And Cortisol In Nonobese Men And Women: A Randomised Clinical Trial. *Aging Cell,* 15**,** 22-27.

Fontana, L., Partridge, L. & Longo, V. D. 2010. Extending Healthy Life Span--From Yeast To Humans. *Science* 328**,** 321-326.

Foretz, M., Guigas, B., Bertrand, L., Pollak, M. & Viollet, B. 2014. Metformin: From Mechanisms Of Action To Therapies. *Cell Metabolism,* 20**,** 953-966.

Franceschi, C. & Campisi, J. 2014. Chronic Inflammation (Inflammaging) And Its Potential Contribution To Age-Associated Diseases. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences,* 69**,** S4-S9.

Franceschi, C., Capri, M., Monti, D., Giunta, S., Olivieri, F., Sevini, F., Panourgia, M. P., Invidia, L., Celani, L., Scurti, M., Cevenini, E., Castellani, G. C. & Salvioli, S. 2007. Inflammaging And Anti-Inflammaging: A Systemic Perspective On Aging And Longevity Emerged From Studies In Humans. *Mechanisms Of Ageing And Development,* 128**,** 92-105.

Freda, P. U. 2002. Somatostatin Analogs In Acromegaly. *The Journal Of Clinical Endocrinology & Metabolism,* 87**,** 3013-3018.

Freitas-Simoes, T.-M., Ros, E. & Sala-Vila, A. 2016. Nutrients, Foods, Dietary Patterns And Telomere Length: Update Of Epidemiological Studies And Randomised Trials. *Metabolism,* 65**,** 406-415.

Friedman, D. B. & Johnson, T. E. 1988. A Mutation In The Age-1 Gene In Caenorhabditis Elegans Lengthens Life And Reduces Hermaphrodite Fertility. *Genetics,* 118**,** 75-86.

Fries, J.F., 1980. Aging, Natural Death, and the Compression of Morbidity. *New England Journal of Medicine,* 303:130-135.

Fulop, T., Dupuis, G., Baehl, S., Page, A., Bourgade, K., Frost, E., Witkowski, J. M., Pawelec, G., Larbi, A. & Cunnane, S. 2015. From Inflamm-Aging To Immune-Paralysis: A Slippery Slope During Aging For Immune-Adaptation. *Biogerontology***,** 1-11.

Gann, P. H., Hennekens, C. H., Ma, J., Longcope, C. & Stampfer, M. J. 1996. Prospective Study Of Sex Hormone Levels And Risk Of Prostate Cancer. *Jnci Journal Of The National Cancer Institute,* 88**,** 1118-1126.

Garcia Caraballo, S. C., Comhair, T. M., Dejong, C. H. C., Lamers, W. H. & Koehler, S. E. 2017. Dietary Treatment Of Fatty Liver: High Dietary Protein Content Has An Antisteatotic And Antiobesogenic Effect In Mice. *Biochimica Et Biophysica Acta (Bba) - Molecular Basis Of Disease,* 1863**,** 1789-1804.

Gardner, J.P. Kimura, M., Chai, W., Durrani, J.F., Tchakmakjian, L., Cao, X., Lu, X., Li, G., Peppas, A.P., Skurnick, J., Wright, W.E., Shay, J.W., Aviv, A.., 2007. Telomere Dynamics in Macaques and Humans. *The Journals of Gerontology: Series A*, 62(4), pp.367–374.

Garratt, M., Nakagawa, S. & Simons, M. J. P. 2016. Comparative Idiosyncrasies In Life Extension By Reduced Mtor Signalling And Its Distinctiveness From Dietary Restriction. *Aging Cell***,** 1-7.

Gibbs, V. K. & Smith Jr, D. L. 2016. Nutrition And Energetics In Rodent Longevity Research. *Experimental Gerontology***,** 1-7.

Giblin, W. & Lombard, D. B. 2016. Sirtuins, Healthspan, And Longevity In Mammals. Elsevier.

Gladyshev, V. N. 2016. Aging: Progressive Decline In Fitness Due To The Rising Deleteriome Adjusted By Genetic, Environmental, And Stochastic Processes. *Aging Cell***,** 1-9.

Gokarn, R., Solon-Biet, S., Youngson, N. A., Wahl, D., Cogger, V. C., Mcmahon, A. C., Cooney, G. J., O Ballard, J. W., Raubenheimer, D., Morris, M. J., Simpson, S. J. & Le Couteur, D. G. 2017. The Relationship Between Dietary Macronutrients And Hepatic Telomere Length In Aging Mice. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences*.

Gokarn, R., Solon-Biet, S. M., Cogger, V. C., Cooney, G. J., Wahl, D., Mcmahon, A. C., Mitchell, J. R., Mitchell, S. J., Hine, C., De Cabo, R., Raubenheimer, D., Simpson, S. J. & Le Couteur, D. G. 2018. Long-Term Dietary Macronutrients And Hepatic Gene Expression In Aging Mice. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences*.

Gomes, A. P., Price, N. L., Ling, A. J. Y., Moslehi, J. J., Montgomery, M. K., Rajman, L., White, J. P., Teodoro, J. S., Wrann, C. D., Hubbard, B. P., Mercken, E. M., Palmeira, C. M., De Cabo, R., Rolo, A. P., Turner, N., Bell, E. L. & Sinclair, D. A. 2013. Declining Nad+ Induces A Pseudohypoxic State Disrupting Nuclear-Mitochondrial Communication During Aging. *Cell,* 155**,** 1624-1638.

Gopalan, S., Carja, O., Fagny, M., Patin, E., Myrick, J. W., Mcewen, L. M., Mah, S. M., Kobor, M. S., Froment, A., Feldman, M. W., Quintana-Murci, L. & Henn, B. M. 2017. Trends In Dna Methylation With Age Replicate Across Diverse Human Populations. *Genetics,* 206**,** 1659-1674.

Gorbunova, V., Seluanov, A., Mao, Z. & Hine, C. 2007. Changes In Dna Repair During Aging. *Nucleic Acids Research,* 35**,** 7466-7474.

Gosby, A. K., Conigrave, A. D., Raubenheimer, D. & Simpson, S. J. 2013. Protein Leverage And Energy Intake. *Obesity Reviews : An Official Journal Of The International Association For The Study Of Obesity,* 15**,** 183-191.

Gowans, G. J., Hawley, S. A., Ross, F. A. & Hardie, D. G. 2013. Amp Is A True Physiological Regulator Of Amp-Activated Protein Kinase By Both Allosteric Activation And Enhancing Net Phosphorylation. *Cell Metabolism,* 18**,** 556-566.

Grandison, R. C., Piper, M. D. W. & Partridge, L. 2009. Amino-Acid Imbalance Explains Extension Of Lifespan By Dietary Restriction In Drosophila. *Nature,* 462**,** 1061-1064.

Gredilla, R., Bohr, V. A. & Stevnsner, T. 2010. Mitochondrial Dna Repair And Association With Aging--An Update. *Experimental Gerontology,* 45**,** 478-488.

Green, D. R., Galluzzi, L. & Kroemer, G. 2011. Mitochondria And The Autophagy-Inflammation-Cell Death Axis In Organismal Aging. *Science,* 333**,** 1109-1112.

Group, U. P. D. S. U. 1998. Effect Of Intensive Blood-Glucose Control With Metformin On Complications In Overweight Patients With Type 2 Diabetes (Ukpds 34). *The Lancet,* 352**,** 854-865.

Guarente, L. 2014. Aging Research-Where Do We Stand And Where Are We Going? *Cell,* 159**,** 15-19.

Guarente, L. 2008. Mitochondria—A Nexus For Aging, Calorie Restriction, And Sirtuins? *Cell,* 132**,** 171-176.

Guarente, L. 2011. Sirtuins, Aging, And Medicine. *New England Journal Of Medicine,* 364**,** 2235-2244.

Haigis, M. C. & Yankner, B. A. 2010. The Aging Stress Response. *Molecular Cell,* 40**,** 333-344.

Han, E.-S. & Hickey, M. 2005. Microarray Evaluation Of Dietary Restriction. *The Journal Of Nutrition,* 135**,** 1343-1346.

Hansen, M. & Kennedy, B. K. 2016. Does Longer Lifespan Mean Longer Healthspan? *Trends In Cell Biology,* 26**,** 565-568.

Harbauer, A. B., Zahedi, R. P., Sickmann, A., Pfanner, N. & Meisinger, C. 2014. The Protein Import Machinery Of Mitochondria-A Regulatory Hub In Metabolism, Stress, And Disease. *Cell Metabolism,* 19**,** 357-372.

Hardie, D. G. 2014. Ampk-Sensing Energy While Talking To Other Signaling Pathways. *Cell Metabolism,* 20**,** 939-952.

Hardie, D. G., Ross, F. A. & Hawley, S. A. 2012. Ampk: A Nutrient And Energy Sensor That Maintains Energy Homeostasis. *Nature Publishing Group,* 13**,** 251-262.

Hardman, S. E., Hall, D. E., Cabrera, A. J., Hancock, C. R. & Thomson, D. M. 2014. The Effects Of Age And Muscle Contraction On Ampk Activity And Heterotrimer Composition. *Experimental Gerontology,* 55**,** 120-128.

Harman, D. 1956. Aging: A Theory Based On Free Radical And Radiation Chemistry. *Journal Of Gerontology,* 11**,** 298-300.

Harrison, D. E., Strong, R., Sharp, Z. D., Nelson, J. F., Astle, C. M., Flurkey, K., Nadon, N. L., Wilkinson, J. E., Frenkel, K., Carter, C. S., Pahor, M., Javors, M. A., Fernandez, E. & Miller, R. A. 2009. Rapamycin Fed Late In Life Extends Lifespan In Genetically Heterogeneous Mice. *Nature,* 310**,** 1193.

Harvey, A. E., Lashinger, L. M., Hays, D., Harrison, L. M., Lewis, K., Fischer, S. M. & Hursting, S. D. 2014. Calorie Restriction Decreases Murine And Human Pancreatic Tumor Cell Growth, Nuclear Factor-Kb Activation, And Inflammation-Related Gene Expression In An Insulin-Like Growth Factor-1-Dependent Manner. *Plos One,* 9**,** E94151.

Harvie, M. & Howell, A. 2012. Energy Restriction And The Prevention Of Breast Cancer. *Proceedings Of The Nutrition Society,* 71**,** 263-275.

Hausenblas, H. A., Schoulda, J. A. & Smoliga, J. M. 2014. Resveratrol Treatment As An Adjunct To Pharmacological Management In Type 2 Diabetes Mellitus-Systematic Review And Meta-Analysis. *Molecular Nutrition & Food Research,* 59**,** 147-159.

Hayflick, L. & Moorhead, P. S. 1961. The Serial Cultivation Of Human Diploid Cell Strains. *Experimental Cell Research,* 25**,** 585-621.

He, X.-Y., Zhao, X.-L., Gu, Q., Shen, J.-P., Hu, Y. & Hu, R.-M. 2012. Calorie Restriction From A Young Age Preserves The Functions Of Pancreatic B Cells In Aging Rats. *The Tohoku Journal Of Experimental Medicine,* 227**,** 245-252.

Heilbronn, L. K. & Ravussin, E. 2003. Calorie Restriction And Aging: Review Of The Literature And Implications For Studies In Humans. *The American Journal Of Clinical Nutrition,* 78**,** 361-369.

Heilbronn, L. K., De Jonge, L., Frisard, M. I., Delany, J. P., Larson-Meyer, D. E., Rood, J., Nguyen, T., Martin, C. K., Volaufova, J., Most, M. M., Greenway, F. L., Smith, S. R., Deutsch, W. A., Williamson, D. A., Ravussin, E. & Pennington Calerie Team, F. T. 2006. Effect Of 6-Month Calorie Restriction On Biomarkers Of Longevity, Metabolic Adaptation, And Oxidative Stress In Overweight Individuals. *Jama,* 295**,** 1539-1548.

Heilbronn, L. K., Civitarese, A. E., Bogacka, I., Smith, S. R., Hulver, M. & Ravussin, E. 2012. Glucose Tolerance And Skeletal Muscle Gene Expression In Response To Alternate Day Fasting. *Obesity Research,* 13**,** 574-581.

Hekimi, S., Lapointe, J. & Wen, Y. 2011. Taking A "Good" Look At Free Radicals In The Aging Process. *Trends In Cell Biology,* 21**,** 569-576.

Hew, J., Solon-Biet, S. M., Mcmahon, A. C., Ruohonen, K., Raubenheimer, D., Ballard, J. W. O., Le Couteur, D. G., Nicholls, C., Li, Z., Maitz, P. K. M., Wang, Y. & Simpson, S. J. 2016. The Effects Of Dietary Macronutrient Balance On Skin Structure In Aging Male And Female Mice. *Plos One,* 11**,** E0166175.

Hill, J. O., Melanson, E. L. & Wyatt, H. T. 2000. Dietary Fat Intake And Regulation Of Energy Balance: Implications For Obesity. *The Journal Of Nutrition,* 130**,** 284s-288s.

Hiona, A., Sanz, A., Kujoth, G. C., Pamplona, R., Seo, A. Y., Hofer, T., Someya, S., Miyakawa, T., Nakayama, C., Samhan-Arias, A. K., Servais, S., Barger, J. L., Portero-Otín, M., Tanokura, M., Prolla, T. A. & Leeuwenburgh, C. 2010. Mitochondrial Dna Mutations Induce Mitochondrial Dysfunction, Apoptosis And Sarcopenia In Skeletal Muscle Of Mitochondrial Dna Mutator Mice. *Plos One,* 5**,** E11468.

Hofmann, J. W., Zhao, X., De Cecco, M., Peterson, A. L., Pagliaroli, L., Manivannan, J., Hubbard, G. B., Ikeno, Y., Zhang, Y., Feng, B., Li, X., Serre, T., Qi, W., Van Remmen, H., Miller, R. A., Bath, K. G., De Cabo, R., Xu, H., Neretti, N. & Sedivy, J. M. 2015. Reduced Expression Of Myc Increases Longevity And Enhances Healthspan. *Cell,* 160**,** 477-488.

Holmes, A. J., Chew, Y. V., Colakoglu, F., Cliff, J. B., Klaassens, E., Read, M. N., Solon-Biet, S. M., Mcmahon, A. C., Cogger, V. C., Ruohonen, K., Raubenheimer, D., Le Couteur, D. G. & Simpson, S. J. 2016. Diet-Microbiome Interactions In Health Are Controlled By Intestinal Nitrogen Source Constraints. *Cell Metabolism***,** 1-13.

Holmes, B. F., Kurth-Kraczek, E. J. & Winder, W. W. 1999. Chronic Activation Of 5′-Amp-Activated Protein Kinase Increases Glut-4, Hexokinase, And Glycogen In Muscle. *Journal Of Applied Physiology,* 87**,** 1990-1995.

Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., Géloën, A., Even, P. C., Cervera, P. & Le Bouc, Y. 2003. Igf-1 Receptor Regulates Lifespan And Resistance To Oxidative Stress In Mice. *Nature,* 421**,** 182-187.

Houtkooper, R. H., Pirinen, E. & Auwerx, J. 2012. Sirtuins As Regulators Of Metabolism And Healthspan.

Hu, Z., Chen, K., Xia, Z., Chavez, M., Pal, S., Seol, J.-H., Chen, C.-C., Li, W. & Tyler, J. K. 2014. Nucleosome Loss Leads To Global Transcriptional Up-Regulation And Genomic Instability During Yeast Aging. *Genes & Development,* 28**,** 396-408.

Hu, S., Wang, L., Yang, D., Li, L., Togo, J., Wu, Y., Liu, Q., Li, B., Li, M., Wang, G., Zhang, X., Niu, C., Li, J., Xu, Y., Couper, E., Whittington-Davies, A., Mazidi ,M., Luo, L., Wang S., Douglas, A., Speakman J.R., 2018. Dietary Fat, but Not Protein or Carbohydrate, Regulates Energy Intake and Causes Adiposity in Mice. *Cell Metabolism*, *28*(3), 415–431.e4.

Hughes, A. L. & Gottschling, D. E. 2012. An Early Age Increase In Vacuolar Ph Limits Mitochondrial Function And Lifespan In Yeast. *Nature*, 1-7.

Humeau, A., Chapeau Blondeau, F. O., Rousseau, D., Rousseau, P., Trzepizur, W. & Abraham, P. 2008. Multifractality, Sample Entropy, And Wavelet Analyses For Age-Related Changes In The Peripheral Cardiovascular System: Preliminary Results. *Medical Physics,* 35**,** 717-723.

Ingram, D. K. & De Cabo, R. 2017. Calorie Restriction In Rodents: Caveats To Consider. *Ageing Research Reviews,* 39**,** 15-28.

Ingram, D. K., Cutler, R. G., Weindruch, R., Renquist, D. M., Knapka, J. J., April, M., Belcher, C. T., Clark, M. A., Hatcherson, C. D. & Marriott, B. M. 1990. Dietary Restriction And Aging: The Initiation Of A Primate Study. *Journal Of Gerontology,* 45**,** B148-63.

Jacobi, D., Liu, S., Burkewitz, K., Kory, N., Knudsen, N. H., Alexander, R. K., Unluturk, U., Li, X., Kong, X., Hyde, A. L., Gangl, M. R., Mair, W. B. & Lee, C.-H. 2015. Hepatic Bmal1 Regulates Rhythmic Mitochondrial Dynamics And Promotes Metabolic Fitness. *Cell Metabolism***,** 1-13.

Jager, S., Handschin, C., St Pierre, J. & Spiegelman, B. M. 2007. Amp-Activated Protein Kinase (Ampk) Action In Skeletal Muscle Via Direct Phosphorylation Of Pgc-1 *Proceedings Of The National Academy Of Sciences,* 104**,** 12017-12022.

Jamieson, H. A., Hilmer, S. N., Cogger, V. C., Warren, A., Cheluvappa, R., Abernethy, D. R., Everitt, A. V., Fraser, R., De Cabo, R. & Le Couteur, D. G. 2007. Caloric Restriction Reduces Age-Related Pseudocapillarization Of The Hepatic Sinusoid. *Experimental Gerontology,* 42**,** 374-378.

Jang, M., Park, S. Y., Kim, Y. W., Jung, S. P. & Kim, J. Y. 2017. Regulating Hypothalamus Gene Expression In Food Intake: Dietary Composition Or Calorie Density? *Diabetes & Metabolism Journal,* 41**,** 121-127.

Janssens, G. E. & Veenhoff, L. M. 2016. Evidence For The Hallmarks Of Human Aging In Replicatively Aging Yeast. *Microbial Cell,* 3**,** 263-274.

Janssens, G. E., Meinema, A. C., González, J., Wolters, J. C., Schmidt, A., Guryev, V., Bischoff, R., Wit, E. C., Veenhoff, L. M. & Heinemann, M. 2015. Author Response. *Elife,* 4**,** 1315.

Jenkins, N. L., Mccoll, G. & Lithgow, G. J. 2004. Fitness Cost Of Extended Lifespan In Caenorhabditis Elegans. *Proceedings Of The Royal Society B: Biological Sciences,* 271**,** 2523-2526.

Jensen, K., Mcclure, C., Priest, N. K. & Hunt, J. 2015. Sex-Specific Effects Of Protein And Carbohydrate Intake On Reproduction But Not Lifespan In Drosophila Melanogaster. *Aging Cell***.**

Jensen, M. B. & Jasper, H. 2014. Mitochondrial Proteostasis In The Control Of Aging And Longevity. *Cell Metabolism,* 20**,** 214-225.

Jewell, J. L., Russell, R. C. & Guan, K.L. 2013. Amino Acid Signalling Upstream Of Mtor. *Nature Reviews Molecular Cell Biology,* 14**,** 133-139.

Jia, K. 2004. The Tor Pathway Interacts With The Insulin Signaling Pathway To Regulate C. Elegans Larval Development, Metabolism And Life Span. *Development,* 131**,** 3897-3906.

Jia, K., Cui, C., Gao, Y., Zhou, Y., & Cui, Q., 2018. An analysis of aging-related genes derived from the Genotype-Tissue Expression project (GTEx). *Cell Death Discovery*, *5*(1), 38.

Jin, C., Li, J., Green, C. D., Yu, X., Tang, X., Han, D., Xian, B., Wang, D., Huang, X., Cao, X., Yan, Z., Hou, L., Liu, J., Shukeir, N., Khaitovich, P., Chen, C. D., Zhang, H., Jenuwein, T. & Han, J.-

D. J. 2011. Histone Demethylase Utx-1 Regulates C. Elegans Life Span By Targeting The Insulin/Igf-1 Signaling Pathway. *Cell Metabolism,* 14**,** 161-172.

Jin, K. 2010. Modern Biological Theories Of Aging. *Aging And Disease,* 1**,** 72-74.

Johnson, F. B., Sinclair, D. A. & Guarente, L. 1999. Molecular Biology Of Aging. *Cell,* 96**,** 291-302.

Johnson, S. C., Rabinovitch, P. S. & Kaeberlein, M. 2013. Mtor Is A Key Modulator Of Ageing And Age-Related Disease. *Nature,* 493**,** 338-345.

Jones, B. 2012. Clonal Mosaicism Linked To Age And Cancer Risk. *Nature Reviews Genetics,* 13**,** 452-452.

Junnila, R. K., List, E. O., Berryman, D. E., Murrey, J. W. & Kopchick, J. J. 2013. The Gh/Igf-1 Axis In Ageing And Longevity. *Nature Reviews Endocrinology,* 9**,** 366-376.

Kaeberlein, M. & Powers Iii, R. W. 2007. Sir2 And Calorie Restriction In Yeast: A Skeptical Perspective. *Ageing Research Reviews,* 6**,** 128-140.

Kapahi, P., Zid, B. M., Harper, T., Koslover, D., Sapin, V. & Benzer, S. 2004. Regulation Of Lifespan In Drosophila By Modulation Of Genes In The Tor Signaling Pathway. *Current Biology,* 14**,** 885-890.

Kark, J. D., Goldberger, N., Kimura, M., Sinnreich, R. & Aviv, A. 2012. Energy Intake And Leukocyte Telomere Length In Young Adults. *The American Journal Of Clinical Nutrition,* 95**,** 479-487.

Katic, M. & Kahn, C. R. 2005. The Role Of Insulin And Igf-1 Signaling In Longevity. *Cellular And Molecular Life Sciences,* 62**,** 320-343.

Kennedy, B. K. & Pennypacker, J. K. 2014. Drugs That Modulate Aging: The Promising Yet Difficult Path Ahead. *Translational Research,* 163**,** 456-465.

Kennedy, B. K., Berger, S. L., Brunet, A., Campisi, J., Cuervo, A. M., Epel, E. S., Franceschi, C., Lithgow, G. J., Morimoto, R. I., Pessin, J. E., Rando, T. A., Richardson, A., Schadt, E. E., Wyss-Coray, T. & Sierra, F. 2014. Geroscience: Linking Aging To Chronic Disease. *Cell,* 159**,** 709-713.

Kennedy, B. K., Austriaco Jr., N. R., Zhang, J. & Guarente, L. 1995. Mutation In The Silencing Gene S/R4 Can Delay Aging In S. Cerevisiae. *Cell,* 80**,** 485-496.

Kennedy, B. K., Austriaco, N. R. & Guarente, L. 1994. Daughter Cells Of Saccharomyces Cerevisiae From Old Mothers Display A Reduced Life Span. *The Journal Of Cell Biology,* 127**,** 1985-1993.

Kenyon, C., Chang, J., Gensch, E., Rudner, A. & Tabtiang, R. 1993. A C. Elegans Mutant That Lives Twice As Long As Wild Type. *Nature,* 366**,** 461-464.

Kiecolt-Glaser, J. K., Preacher, K. J., Maccallum, R. C., Atkinson, C., Malarkey, W. B. & Glaser, R. 2003. Chronic Stress And Age-Related Increases In The Proinflammatory Cytokine Il-6. *Proceedings Of The National Academy Of Sciences,* 100**,** 9090-9095.

Kim, C. H., Lee, E. K., Choi, Y. J., An, H. J., Chung, H. O., Park, D. E., Ghim, B. C., Yu, B. P., Bhak, J. & Chung, H. Y. 2016a. Short-Term Calorie Restriction Ameliorates Genomewide, Age-Related Alterations In Dna Methylation. *Aging Cell***,** 1-8.

Kim, S., Villeponteau, B. & Jazwinski, S. M. 1996. Effect Of Replicative Age On Transcriptional Silencing Near Telomeres Insaccharomyces Cerevisiae. *Biochemical And Biophysical Research Communications,* 219**,** 370-376.

Kirkwood, T. 2010. Why Can't We Live Forever? *Scientific American*, 303, 42-49.

Kirkwood, T. B. L. & Austad, S. N. 2000. Why Do We Age? *Nature Publishing Group,* 408**,** 233-238.

Kirkwood, T. B. L. & Holliday, R. 1979. The Evolution Of Ageing And Longevity. *Proceedings Of The Royal Society B: Biological Sciences,* 205**,** 531-546.

Kirkwood, T. B. L. & Melov, S. 2011. On The Programmed/Non-Programmed Nature Of Ageing Within The Life History. *Current Biology,* 21**,** R701-R707.

Klein, R., Myers, C. E., Cruickshanks, K. J., Gangnon, R. E., Danforth, L. G., Sivakumaran, T. A., Iyengar, S. K., Tsai, M. Y. & Klein, B. E. K. 2014. Markers Of Inflammation, Oxidative Stress, And Endothelial Dysfunction And The 20-Year Cumulative Incidence Of Early Age-Related Macular Degeneration. *Jama Ophthalmology,* 132**,** 446.

Kola, B. 2008. Role Of Amp-Activated Protein Kinase In The Control Of Appetite. *Journal Of Neuroendocrinology,* 20**,** 942-951.

Kopchick, J. J., Parkinson, C., Stevens, E. C. & Trainer, P. J. 2002. Growth Hormone Receptor Antagonists: Discovery, Development, And Use In Patients With Acromegaly. *Endocrine Reviews,* 23**,** 623-646.

Kowald, A. & Kirkwood, T. B. L. 2016. Can Aging Be Programmed? A Critical Literature Review. *Aging Cell***,** 1-13.

Krude, H., Biebermann, H., Luck, W., Horn, R., Brabant, G. & Grüters, A. 1998. Severe Early-Onset Obesity, Adrenal Insufficiency And Red Hair Pigmentation Caused By Pomc; Mutations In Humans. *Nature Genetics,* 19**,** 155-157.

Kujoth, G. C. 2005. Mitochondrial Dna Mutations, Oxidative Stress, And Apoptosis In Mammalian Aging. *Science,* 309**,** 481-484.

Kurth-Kraczek, E. J., Hirshman, M. F., Goodyear, L. J. & Winder, W. W. 1999. 5' Amp-Activated Protein Kinase Activation Causes Glut4 Translocation In Skeletal Muscle. *Diabetes,* 48, 1667-1671.

Kyng, K. J. & Bohr, V. A. 2005. Gene Expression And Dna Repair In Progeroid Syndromes And Human Aging. *Ageing Research Reviews,* 4**,** 579-602.

Labbadia, J. & Morimoto, R. I. 2015. The Biology Of Proteostasis In Aging And Disease. *Annual Review Of Biochemistry,* 84**,** 435-464.

Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., Messadeq, N., Milne, J., Lambert, P., Elliott, P., Geny, B., Laakso, M., Puigserver, P. & Auwerx, J. 2006. Resveratrol Improves Mitochondrial Function And Protects Against Metabolic Disease By Activating Sirt1 And Pgc-1α. *Cell,* 127**,** 1109-1122.

Lamming, D. W., Ye, L., Sabatini, D. M. & Baur, J. A. 2013. Rapalogs And Mtor Inhibitors As Anti-Aging Therapeutics. *Journal Of Clinical Investigation,* 123**,** 980-989.

Lamming, D. W., Ye, L., Katajisto, P., Goncalves, M. D., Saitoh, M., Stevens, D. M., Davis, J. G., Salmon, A. B., Richardson, A., Ahima, R. S., Guertin, D. A., Sabatini, D. M. & Baur, J. A. 2012. Rapamycin-Induced Insulin Resistance Is Mediated By Mtorc2 Loss And Uncoupled From Longevity. *Science,* 335**,** 1638-1643.

Lane, M. A., Ingram, D. K., Cutler, R. G., Knapka, J. J., Barnard, D. E. & Roth, G. S. 1992. Dietary Restriction In Nonhuman Primates: Progress Report On The Nia Study. *Annals Of The New York Academy Of Sciences,* 673**,** 36-45.

Laplante, M. & Sabatini, D. M. 2009. Mtor Signaling At A Glance. *Journal Of Cell Science,* 122**,** 3589-3594.

Larsen, S., Nielsen, J., Hansen, C. N., Nielsen, L. B., Wibrand, F., Stride, N., Schroder, H. D., Boushel, R., Helge, J. W., Dela, F. & Hey-Mogensen, M. 2012. Biomarkers Of Mitochondrial Content In Skeletal Muscle Of Healthy Young Human Subjects. *The Journal Of Physiology,* 590**,** 3349-3360.

Laun, P., Pichova, A., Madeo, F., Fuchs, J., Ellinger, A., Kohlwein, S., Dawes, I., Frohlich, K.-U. & Breitenbach, M. 2001. Aged Mother Cells Of Saccharomyces Cerevisiae Show Markers Of Oxidative Stress And Apoptosis. *Molecular Microbiology,* 39**,** 1166-1173.

Laun, P., Ramachandran, L., Jarolim, S., Herker, E., Liang, P., Wang, J., Weinberger, M., Burhans, D., Suter, B., Madeo, F., Burhans, W. & Breitenbach, M. 2005. A Comparison Of The Aging And Apoptotic Transcriptome Of Saccharomyces Cerevisiae. *Fems Yeast Research,* 5**,** 1261-1272.

Laurie, C. C., Laurie, C. A., Rice, K., Doheny, K. F., Zelnick, L. R., Mchugh, C. P., Ling, H., Hetrick, K. N., Pugh, E. W., Amos, C., Wei, Q., Wang, L.-E., Lee, J. E., Barnes, K. C., Hansel, N. N., Mathias, R., Daley, D., Beaty, T. H., Scott, A. F., Ruczinski, I., Scharpf, R. B., Bierut, L. J., Hartz, S. M., Landi, M. T., Freedman, N. D., Goldin, L. R., Ginsburg, D., Li, J., Desch, K. C., Strom, S. S., Blot, W. J., Signorello, L. B., Ingles, S. A., Chanock, S. J., Berndt, S. I., Le Marchand, L., Henderson, B. E., Monroe, K. R., Heit, J. A., De Andrade, M., Armasu, S. M., Regnier, C., Lowe, W. L., Hayes, M. G., Marazita, M. L., Feingold, E., Murray, J. C., Melbye, M., Feenstra, B., Kang, J. H., Wiggs, J. L., Jarvik, G. P., Mcdavid, A. N., Seshan, V. E., Mirel, D. B., Crenshaw, A., Sharopova, N., Wise, A., Shen, J., Crosslin, D. R., Levine, D. M., Zheng, X., Udren, J. I., Bennett, S., Nelson, S. C., Gogarten, S. M., Conomos, M. P., Heagerty, P., Manolio, T., Pasquale, L. R., Haiman, C. A., Caporaso, N. & Weir, B. S. 2012. Detectable Clonal Mosaicism From Birth To Old Age And Its Relationship To Cancer. *Nature Genetics,* 44**,** 642-650.

Le Couteur, D. G., De Cabo, R., Longo, D. L., Fauci, A. S., Kasper, D. L. & Hauser, S. L. 2012a. Biology Of Aging: Harrison's Principles Of Internal Medicine 18e Vol 2.

Le Couteur, D. G., Solon-Biet, S., Wahl, D., Cogger, V. C., Willcox, B. J., Willcox, D. C., Raubenheimer, D. & Simpson, S. J. 2016. New Horizons: Dietary Protein, Ageing And The Okinawan Ratio. 45**,** 443-447.

Le Couteur, D. G., Warren, A., Cogger, V. C., Smedsrød, B., Sørensen, K. K., De Cabo, R., Fraser, R. & Mccuskey, R. S. 2008. Old Age And The Hepatic Sinusoid. *The Anatomical Record: Advances In Integrative Anatomy And Evolutionary Biology,* 291**,** 672-683.

Le Couteur, D. G., Sinclair, D. A., Cogger, V. C., Mcmahon, A. C., Warren, A., Everitt, A. V., Lebel, M. & De Cabo, R. 2010. The Aging Liver And The Effects Of Long Term Caloric Restriction *Springer Science***,** 1-28.

Le Couteur, D. G., Solon-Biet, S., Cogger, V. C., Mitchell, S. J., Senior, A., De Cabo, R., Raubenheimer, D. & Simpson, S. J. 2015. The Impact Of Low-Protein High-Carbohydrate Diets On Aging And Lifespan. 73**,** 1237-1252.

Le Couteur, D. G., Mclachlan, A. J., Quinn, R. J., Simpson, S. J. & De Cabo, R. 2012b. Aging Biology And Novel Targets For Drug Discovery. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences,* 67**,** 168-174.

Le Couteur, D. G., Naganathan, V., Cogger, V. C., Simpson, S. J. & De Cabo, R. 2014. Biology Of Ageing.

Le Couteur, D. G., Tay, S. S., Solon-Biet, S., Bertolino, P., Mcmahon, A. C., Cogger, V. C., Colakoglu, F., Warren, A., Holmes, A. J., Pichaud, N., Horan, M., Correa, C., Melvin, R. G., Turner, N., Ballard, J. W. O., Ruohonen, K., Raubenheimer, D. & Simpson, S. J. 2014b. The Influence Of Macronutrients On Splanchnic And Hepatic Lymphocytes In Aging Mice. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences*, 1-9.

Lecoultre, V., Ravussin, E. & Redman, L. M. 2011. The Fall In Leptin Concentration Is A Major Determinant Of The Metabolic Adaptation Induced By Caloric Restriction Independently Of The Changes In Leptin Circadian Rhythms. *The Journal Of Clinical Endocrinology & Metabolism,* 96**,** E1512-E1516.

Lee, C. & Longo, V. D. 2011. Fasting Vs Dietary Restriction In Cellular Protection And Cancer Treatment: From Model Organisms To Patients. *Oncogene,* 30**,** 3305-3316.

Lee, K. P., Simpson, S. J., Clissold, F. J., Brooks, R., Ballard, J. W. O., Taylor, P. W., Soran, N. & Raubenheimer, D. 2008. Lifespan And Reproduction In Drosophila: New Insights From Nutritional Geometry. *Proceedings Of The National Academy Of Sciences Of The United States Of America,* 105**,** 2498-2503.

Levine, M. E., Suarez, J. A., Brandhorst, S., Balasubramanian, P., Cheng, C.-W., Madia, F., Fontana, L., Mirisola, M. G., Guevara-Aguirre, J., Wan, J., Passarino, G., Kennedy, B. K., Wei, M., Cohen, P., Crimmins, E. M. & Longo, V. D. 2014. Low Protein Intake Is Associated With A Major Reduction In Igf-1, Cancer, And Overall Mortality In The 65 And Younger But Not Older Population. *Cell Metabolism,* 19**,** 407-417.

Liao, C. Y., Rikke, B. A., Johnson, T. E., Diaz, V. & Nelson, J. F. 2010. Genetic Variation In The Murine Lifespan Response To Dietary Restriction: From Life Extension To Life Shortening. *Aging Cell,* 9**,** 92-95.

Liesa, M. & Shirihai, O. S. 2013. Mitochondrial Dynamics In The Regulation Of Nutrient Utilization And Energy Expenditure. *Cell Metabolism,* 17**,** 491-506.

Lin, S. S., Manchester, J. K. & Gordon, J. I. 2001. Enhanced Gluconeogenesis And Increased Energy Storage As Hallmarks Of Aging In Saccharomyces Cerevisiae. *The Journal Of Biological Chemistry,* 276**,** 36000-36007.

Liu, K., Zhou, R., Wang, B. & Mi, M. T. 2014a. Effect Of Resveratrol On Glucose Control And Insulin Sensitivity: A Meta-Analysis Of 11 Randomised Controlled Trials. *The American Journal Of Clinical Nutrition,* 99**,** 1510-1519.

Longo, V. D., Ellerby, L. M., Bredesen, D. E., Valentine, J. S. & Gralla, E. B. 1997. Human Bcl-2 Reverses Survival Defects In Yeast Lacking Superoxide Dismutase And Delays Death Of Wild-Type Yeast. *The Journal Of Cell Biology,* 137**,** 1581-1588.

Longo, V. D., Antebi, A., Bartke, A., Barzilai, N., Brown-Borg, H. M., Caruso, C., Curiel, T. J., De Cabo, R., Franceschi, C., Gems, D., Ingram, D. K., Johnson, T. E., Kennedy, B. K., Kenyon, C., Klein, S., Kopchick, J. J., Lepperdinger, G., Madeo, F., Mirisola, M. G., Mitchell, J. R., Passarino, G., Rudolph, K. L., Sedivy, J. M., Shadel, G. S., Sinclair, D. A., Spindler, S. R., Suh, Y., Vijg, J., Vinciguerra, M. & Fontana, L. 2015. Interventions To Slow Aging In Humans: Are We Ready? *Aging Cell*

Longo, V. D., Mitteldorf, J. & Skulachev, V. P. 2005. Programmed And Altruistic Ageing. *Nature Reviews Genetics,* 6**,** 866-872.

Lord, C. J. & Ashworth, A. 2012. The Dna Damage Response And Cancer Therapy. *Nature,* 481**,** 287-294.

Lord, C. L., Timney, B. L., Rout, M. P. & Wente, S. R. 2015. Altering Nuclear Pore Complex Function Impacts Longevity And Mitochondrial Function In S. Cerevisiae. *The Journal Of Cell Biology,* 208**,** 729-744.

López-Otín, C., Galluzzi, L., Freije, J. M. P., Madeo, F. & Kroemer, G. 2016. Metabolic Control Of Longevity. 166**,** 802-821.

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. 2013. The Hallmarks Of Aging. *Cell,* 153**,** 1194-1217.

Ludlow, A. T., Witkowski, S., Marshall, M. R., Wang, J., Lima, L. C. J., Guth, L. M., Spangenburg, E. E. & Roth, S. M. 2012. Chronic Exercise Modifies Age-Related Telomere Dynamics In A Tissue-Specific Fashion. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences,* 67**,** 911-926.

Maegawa, S., Hinkal, G., Kim, H. S., Shen, L., Zhang, L., Zhang, J., Zhang, N., Liang, S., Donehower, L. A. & Issa, J. P. J. 2010. Widespread And Tissue Specific Age-Related Dna Methylation Changes In Mice. *Genome Research,* 20**,** 332-340.

Maggio, M., Guralnik, J. M., Longo, D. L. & Ferrucci, L. 2006. Interleukin-6 In Aging And Chronic Disease: A Magnificent Pathway. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences,* 61**,** 575-584.

Magyar, K., Halmosi, R., Palfi, A., Feher, G., Czopf, L., Fulop, A., Battyany, I., Sumegi, B., Toth, K. & Szabados, E. 2012. Cardioprotection By Resveratrol: A Human Clinical Trial In Patients With Stable Coronary Artery Disease. *Clinical Hemorheology And Microcirculation,* 50**,** 179-187.

Mair, W., Piper, M. D. W. & Partridge, L. 2005. Calories Do Not Explain Extension Of Life Span By Dietary Restriction In Drosophila. *Plos Biology,* 3**,** E223.

Margolick, J. B. & Ferrucci, L. 2015. Accelerating Aging Research: How Can We Measure The Rate Of Biologic Aging? *Experimental Gerontology,* 64**,** 78-80.

Mariño, G., Pietrocola, F., Madeo, F. & Kroemer, G. 2014. Caloric Restriction Mimetics: Natural/Physiological Pharmacological Autophagy Inducers. *Autophagy,* 10**,** 1879-1882.

Marra, G. & Wood, S. N. 2012. Coverage Properties Of Confidence Intervals For Generalised Additive Model Components. *Scandinavian Journal Of Statistics,* 39**,** 53-74.

Martinez, D. E. 1998. Mortality Patterns Suggest Lack Of Senescence In Hydra. *Experimental Gerontology,* 33**,** 217-225.

Mattison, J. A., Colman, R. J., Beasley, T. M., Allison, D. B., Kemnitz, J. W., Roth, G. S., Ingram, D. K., Weindruch, R., De Cabo, R. & Anderson, R. M. 2017. Caloric Restriction Improves Health And Survival Of Rhesus Monkeys. *Nature Communications,* 8**,** 14063.

Mattison, J. A., Roth, G. S., Beasley, T. M., Tilmont, E. M., Handy, A. M., Herbert, R. L., Longo, D. L., Allison, D. B., Young, J. E., Bryant, M., Barnard, D., Ward, W. F., Qi, W., Ingram, D. K. & De Cabo, R. 2012. Impact Of Caloric Restriction On Health And Survival In Rhesus Monkeys From The Nia Study. *Nature***,** 1-5.

Mccay, C.M., Crowell, M.F. & Maynard, L.A., 1935. The Effect Of Retarded Growth Upon The Length Of Life Span And Upon The Ultimate Body Size. *The Journal Of Nutrition* 10(1) Pp63-79

Mcfaline-Figueroa, J. R., Vevea, J., Swayne, T. C., Zhou, C., Liu, C., Leung, G., Boldogh, I. R. & Pon, L. A. 2011. Mitochondrial Quality Control During Inheritance Is Associated With Lifespan And Mother-Daughter Age Asymmetry In Budding Yeast. *Aging Cell,* 10**,** 885-895.

Mclean, A. J. & Le Couteur, D. G. 2004. Aging Biology And Geriatric Clinical Pharmacology. *Pharmacological Reviews,* 56**,** 163-184.

Medawar, P. B. 1952. *An Unsolved Problem Of Biology*.

Medvedev, Z. A. 2008. An Attempt At A Rational Classification Of Theories Of Ageing. *Biological Reviews,* 65**,** 375-398.

Mei, S.-C. & Brenner, C. 2015. Calorie Restriction-Mediated Replicative Lifespan Extension In Yeast Is Non-Cell Autonomous. *Plos Biology,* 13**,** E1002048.

Melendez, A. 2003. Autophagy Genes Are Essential For Dauer Development And Life-Span Extension In C. Elegans. *Science,* 301**,** 1387-1391.

Melouane, A., Ghanemi, A., Aubé, S., Yoshioka, M. & St-Amand, J. 2018. Differential Gene Expression Analysis In Ageing Muscle And Drug Discovery Perspectives. *Ageing Research Reviews,* 41**,** 53-63.

Mercken, E. M., Carboneau, B. A., Krzysik-Walker, S. M. & De Cabo, R. 2012. Of Mice And Men: The Benefits Of Caloric Restriction, Exercise, And Mimetics. *Ageing Research Reviews,* 11**,** 390-398.

Merkwirth, C., Jovaisaite, V., Durieux, J., Matilainen, O., Jordan, S. D., Quirós, P. M., Steffen, K. K., Williams, E. G., Mouchiroud, L., Tronnes, S. U., Murillo, V., Wolff, S. C., Shaw, R. J., Auwerx, J. & Dillin, A. 2016. Two Conserved Histone Demethylases Regulate Mitochondrial Stress-Induced Longevity. *Cell,* 165**,** 1209-1223.

Merrill, G. F., Kurth, E. J., Hardie, D. G. & Winder, W. W. 1997. Aica Riboside Increases Amp-Activated Protein Kinase, Fatty Acid Oxidation, And Glucose Uptake In Rat Muscle. *American Journal Of Physiology-Endocrinology And Metabolism,* 273**,** E1107-E1112.

Michán, S. & Sinclair, D. 2007. Sirtuins In Mammals: Insights Into Their Biological Function. *The Biochemical Journal,* 404**,** 1-13.

Michán, S., Li, Y., Chou, M. M.-H., Parrella, E., Ge, H., Long, J. M., Allard, J. S., Lewis, K., Miller, M., Xu, W., Mervis, R. F., Chen, J., Guerin, K. I., Smith, L. E. H., Mcburney, M. W., Sinclair, D. A., Baudry, M., De Cabo, R. & Longo, V. D. 2010. Sirt1 Is Essential For Normal Cognitive Function And Synaptic Plasticity. *The Journal Of Neuroscience,* 30**,** 9695-9707.

Miller, R. A., Buehner, G., Chang, Y., Harper, J. M., Sigler, R. & Smith-Wheelock, M. 2005. Methionine-Deficient Diet Extends Mouse Lifespan, Slows Immune And Lens Aging, Alters Glucose, T4, Igf-I And Insulin Levels, And Increases Hepatocyte Mif Levels And Stress Resistance. *Aging Cell,* 4**,** 119-125.

Milman, S., Atzmon, G., Huffman, D. M., Wan, J., Crandall, J. P., Cohen, P. & Barzilai, N. 2014. Low Insulin-Like Growth Factor-1 Level Predicts Survival In Humans With Exceptional Longevity. *Aging Cell,* 13**,** 769-771.

Milman, S., Huffman, D. M. & Barzilai, N. 2016. The Somatotropic Axis In Human Aging: Framework For The Current State Of Knowledge And Future Research. *Cell Metabolism,* 23**,** 980-989.

Minamino, T., Orimo, M., Shimizu, I., Kunieda, T., Yokoyama, M., Ito, T., Nojima, A., Nabetani, A., Oike, Y., Matsubara, H., Ishikawa, F. & Komuro, I. 2009. A Crucial Role For Adipose Tissue P53 In The Regulation Of Insulin Resistance. *Nature Medicine,* 15**,** 1082-1087.

Minokoshi, Y., Alquier, T., Furukawa, N., Kim, Y.-B., Lee, A., Xue, B., Mu, J., Foufelle, F., Ferré, P., Birnbaum, M. J., Stuck, B. J. & Kahn, B. B. 2004. Amp-Kinase Regulates Food Intake By Responding To Hormonal And Nutrient Signals In The Hypothalamus. *Nature,* 428**,** 569-574.

Mirzaei, H., Suarez, J. A. & Longo, V. D. 2014. Protein And Amino Acid Restriction, Aging And Disease: From Yeast To Humans. *Trends In Endocrinology & Metabolism,* 25**,** 558-566.

Missov, T. I. & Lenart, A. 2013. Gompertz-Makeham Life Expectancies: Expressions And Applications. *Theoretical Population Biology,* 90**,** 29-35.

Mitchell, S. J., Madrigal-Matute, J., Scheibye-Knudsen, M., Fang, E., Aon, M., González-Reyes, J. A., Cortassa, S., Kaushik, S., Gonzalez-Freire, M., Patel, B., Wahl, D., Ali, A., Calvo-Rubio, M., Burón, M. I., Guiterrez, V., Ward, T. M., Palacios, H. H., Cai, H., Frederick, D. W., Hine, C., Broeskamp, F., Habering, L., Dawson, J., Beasley, T. M., Wan, J., Ikeno, Y., Hubbard, G., Becker, K. G., Zhang, Y., Bohr, V. A., Longo, D. L., Navas, P., Ferrucci, L., Sinclair, D. A., Cohen, P., Egan, J. M., Mitchell, J. R., Baur, J. A., Allison, D. B., Anson, R. M., Villalba, J. M., Madeo, F., Cuervo, A. M., Pearson, K. J., Ingram, D. K., Bernier, M. & De Cabo, R. 2016. Effects Of Sex, Strain, And Energy Intake On Hallmarks Of Aging In Mice. *Cell Metabolism,* 23**,** 1093-1112.

Mizushima, S., Moriguchi, E. H., Ishikawa, P., Hekman, P., Nara, Y., Mimura, G., Moriguchi, Y. & Yamori, Y. 1997. Fish Intake And Cardiovascular Risk Among Middle-Aged Japanese In Japan And Brazil. *European Journal of Preventive Cardiology* 4**,** 191-199.

Mons, U., Müezzinler, A., Schöttker, B., Dieffenbach, A. K., Butterbach, K., Schick, M., Peasey, A., De Vivo, I., Trichopoulou, A., Boffetta, P. & Brenner, H. 2017. Leukocyte Telomere Length And All-Cause, Cardiovascular Disease, And Cancer Mortality: Results From Individual-Participant-Data Meta-Analysis Of 2 Large Prospective Cohort Studies. *American Journal Of Epidemiology,* 185**,** 1317-1326.

Moretto, T. L., Benfato, I. D., De Carvalho, F. P., Barthichoto, M., Le Sueur-Maluf, L. & De Oliveira, C. A. M. 2017. The Effects Of Calorie-Matched High-Fat Diet Consumption On Spontaneous Physical Activity And Development Of Obesity. *Life Sciences,* 179**,** 30-36.

Moskalev, A. A., Shaposhnikov, M. V., Plyusnina, E. N., Zhavoronkov, A., Budovsky, A., Yanai, H. & Fraifeld, V. E. 2013. The Role Of Dna Damage And Repair In Aging Through The Prism Of Koch-Like Criteria. *Ageing Research Reviews,* 12**,** 661-684.

Most, J., Tosti, V., Redman, L. M. & Fontana, L. 2017. Calorie Restriction In Humans: An Update. *Ageing Research Reviews,* 39**,** 36-45.

Munkácsy, E. & Rea, S. L. 2014. The Paradox Of Mitochondrial Dysfunction And Extended Longevity. *Experimental Gerontology***,** 1-32.

Muoio, D. M. 2014. Metabolic Inflexibility: When Mitochondrial Indecision Leads To Metabolic Gridlock. *Cell,* 159**,** 1253-1262.

Müller, U. C. & Deller, T. 2017. *The Physiological Functions Of The Amyloid Precursor Protein Gene Family*, Frontiers In Molecular Neuroscience.

Nakagawa, S., Lagisz, M., Hector, K. L. & Spencer, H. G. 2012. Comparative And Meta-Analytic Insights Into Life Extension Via Dietary Restriction. *Aging Cell,* 11**,** 401-409.

Nehlin, J. O. 2016. *Biomarkers Of Replicative Senescence Revisited*. Springer, Chambers.

Nemoto, S. 2004. Nutrient Availability Regulates Sirt1 Through A Forkhead-Dependent Pathway. *Science,* 306**,** 2105-2108.

Newgard, C. B., & Pessin, J. E. 2014. Recent Progress in Metabolic Signaling Pathways Regulating Aging and Life Span. *The Journals of Gerontology Series a: Biological Sciences and Medical Sciences*, *69*, S21–S27.

Ng, T. P., Feng, L., Yap, K. B., Lee, T. S., Tan, C. H. & Winblad, B. 2014. Long-Term Metformin Usage And Cognitive Function Among Older Adults With Diabetes. *Journal Of Alzheimer's Disease*, 41, 61-68.

Nisoli, E., Tonello, C., Cardile, A., Cozzi, V., Bracale, R., Tedesco, L., Falcone, S., Valerio, A., Cantoni, O., Clementi, E., Moncada, S. & Carruba, M. O. 2005. Calorie Restriction Promotes Mitochondrial Biogenesis By Inducing The Expression Of Enos. *Science,* 310**,** 314-317.

Novelle, M. G., Wahl, D., Diéguez, C., Bernier, M. & De Cabo, R. 2015. Resveratrol Supplementation: Where Are We Now And Where Should We Go? *Ageing Research Reviews,* 21**,** 1-15.

Oberdoerffer, P. & Sinclair, D. A. 2007. The Role Of Nuclear Architecture In Genomic Instability And Ageing. *Nature Reviews Molecular Cell Biology,* 8**,** 692-702.

Okauchi, N., Mizuno, A., Yoshimoto, S., Zhu, M., Sano, T. & Shima, K. 1995. Is Caloric Restriction Effective In Preventing Diabetes Mellitus In The Otsuka Long Evans Tokushima Fatty Rat, A Model Of Spontaneous Non-Insulin-Dependent Diabetes Mellitus? *Diabetes Research And Clinical Practice,* 27**,** 97-106.

Osada, J. 2013. The Use Of Transcriptomics To Unveil The Role Of Nutrients In Mammalian Liver. *Isrn Nutrition,* 2013**,** 1-19.

Ou, H.-L. & Schumacher, B. 2017. Dna Damage Responses And P53 In The Aging Process. *Blood,* 131**,** 488-495.

Ozsarlak-Sozer, G., Kerry, Z., Gokce, G., Oran, I. & Topcu, Z. 2010. Oxidative Stress In Relation To Telomere Length Maintenance In Vascular Smooth Muscle Cells Following Balloon Angioplasty. *Journal Of Physiology And Biochemistry,* 67**,** 35-42.

Pacholec, M., Bleasdale, J. E., Chrunyk, B., Cunningham, D., Flynn, D., Garofalo, R. S., Griffith, D., Griffor, M., Loulakis, P., Pabst, B., Qiu, X., Stockman, B., Thanabal, V., Varghese, A., Ward, J., Withka, J. & Ahn, K. 2010. Srt1720, Srt2183, Srt1460, And Resveratrol Are Not Direct Activators Of Sirt1. *The Journal Of Biological Chemistry,* 285**,** 8340-8351.

Palacio, F., Lacoretz, M. & Ordano, M. 2014. Bird-Mediated Selection On Fruit Display Traits In Celtis Ehrenbergiana (Cannabaceae) *Ecology And Evolution***,** 51-62.

Palikaras, K., Lionaki, E. & Tavernarakis, N. 2015. Coordination Of Mitophagy And Mitochondrial Biogenesis During Ageing In C. Elegans. *Nature,* 521**,** 525-528.

Pani, G. 2010. P66shc And Ageing: Ros And Tor? *Aging,* 2**,** 514-518.

Paolisso, G. 2001. Low Insulin Resistance And Preserved Β-Cell Function Contribute To Human Longevity But Are Not Associated With Th–Ins Genes. *Experimental Gerontology,* 37**,** 149-156.

Park, S.-K. & Prolla, T. A. 2005. Lessons Learned From Gene Expression Profile Studies Of Aging And Caloric Restriction. *Ageing Research Reviews,* 4**,** 55-65.

Partridge, L. 2012. Nutrient Sensing Pathways And Ageing. *Free Radical Biology And Medicine,* 53**,** S3.

Passtoors, W. M., van den Akker, E. B., Deelen, J., Maier, A. B., van der Breggen, R., Jansen, R., Trompet, S., van Heemst, D., Derhovanessian, E., Pawelec, G., van Ommen, G.J., Slagboom,

P.E., Beekman, M., 2015. IL7R gene expression network associates with human healthy ageing. *Immunity & Ageing*, *12*(1), 21.

Payne, B. A. I. & Chinnery, P. F. 2015. Mitochondrial Dysfunction In Aging: Much Progress But Many Unresolved Questions. *Biochimica Et Biophysica Acta,* 1847**,** 1347-1353.

Pazoki-Toroudi, H., Amani, H., Ajami, M., Nabavi, S. F., Braidy, N., Kasi, P. D. & Nabavi, S. M. 2016. Targeting Mtor Signaling By Polyphenols: A New Therapeutic Target For Ageing. *Ageing Research Reviews***,** 1-12.

Picca, A., Pesce, V., & Lezza, A. M. S., 2017. Does eating less make you live longer and better? An update on calorie restriction. *Clinical Interventions in Aging*, *12*, 1887–1902.

Piper, M. D. W., Partridge, L., Raubenheimer, D. & Simpson, S. J. 2011. Dietary Restriction And Aging: A Unifying Perspective. *Cell Metabolism,* 14**,** 154-160.

Plank, M., Wuttke, D., Van Dam, S., Clarke, S. A. & De Magalhães, J. P. 2012. A Meta-Analysis Of Caloric Restriction Gene Expression Profiles To Infer Common Signatures And Regulatory Mechanisms. *Molecular Biosystems,* 8**,** 1339.

Popper, H. 1986. Aging And The Liver. *Progress In Liver Diseases,* 8**,** 659-683.

Potter, W. B., O'riordan, K. J., Barnett, D., Osting, S. M. K., Wagoner, M., Burger, C. & Roopra, A. 2010. Metabolic Regulation Of Neuronal Plasticity By The Energy Sensor Ampk. *Plos One,* 5**,** E8996.

Prolla, T. A. & Denu, J. M. 2014. Nad+ Deficiency In Age-Related Mitochondrial Dysfunction. *Cell Metabolism,* 19**,** 178-180.

Pyo, J. O., Yoo, S. M., Ahn, H. H., Nah, J., Hong, S. H., Kam, T. I., Jung, S. & Jung, Y. K. 2013. Overexpression Of Atg5 In Mice Activates Autophagy And Extends Lifespan. *Nature Communications,* 4**,** 604.

Raefsky, S. M. & Mattson, M. P. 2017. Adaptive Responses Of Neuronal Mitochondria To Bioenergetic Challenges: Roles In Neuroplasticity And Disease Resistance. *Free Radical Biology And Medicine,* 102**,** 203-216.

Rafie, N., Golpour Hamedani, S., Barak, F., Safavi, S. M. & Miraghajani, M. 2016. Dietary Patterns, Food Groups And Telomere Length: A Systematic Review Of Current Studies. *European Journal Of Clinical Nutrition,* 71**,** 151-158.

Ramsey, J. J., Colman, R. J., Binkley, N. C., Christensen, J. D., Gresl, T. A., Kemnitz, J. W. & Weindruch, R. 2000. Dietary Restriction And Aging In Rhesus Monkeys: The University Of Wisconsin Study. *Experimental Gerontology,* 35**,** 1131-1149.

Rattan, S. I. S. 2006. Theories Of Biological Aging: Genes, Proteins, And Free Radicals. *Free Radical Research,* 40**,** 1230-1238.

Raubenheimer, D. & Simpson, S. J. 1997. Integrative Models Of Nutrient Balancing: Application To Insects And Vertebrates. *Nutrition Research Reviews,* 10**,** 151-179.

Raubenheimer, D. & Simpson, S. J. 2016. Nutritional Ecology And Human Health. *Annual Review Of Nutrition,* 36**,** 603-626.

Ravussin, E., Redman, L. M., Rochon, J., Das, S. K., Fontana, L., Kraus, W. E., Romashkan, S., Williamson, D. A., Meydani, S. N., Villareal, D. T., Smith, S. R., Stein, R. I., Scott, T. M., Stewart, T. M., Saltzman, E., Klein, S., Bhapkar, M., Martin, C. K., Gilhooly, C. H., Holloszy, J. O., Hadley, E. C., Roberts, S. B. & Kritchevsky, S. 2015. A 2-Year Randomised Controlled Trial

Of Human Caloric Restriction: Feasibility And Effects On Predictors Of Health Span And Longevity. *The Journals Of Gerontology: Series A,* 70**,** 1097-1104.

Razi, S., Cogger, V. C., Kennerson, M., Benson, V. L., Mcmahon, A. C., Blyth, F. M., Handelsman, D. J., Seibel, M. J., Hirani, V., Naganathan, V., Waite, L., De Cabo, R., Cumming, R. G. & Le Couteur, D. G. 2017. Sirt1 Polymorphisms And Serum-Induced Sirt1 Protein Expression In Aging And Frailty: The Champ Study. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences,* 72**,** 870-876.

Rea, S. L., Ventura, N. & Johnson, T. E. 2007. Relationship Between Mitochondrial Electron Transport Chain Dysfunction, Development, And Life Extension In Caenorhabditis Elegans. *Plos Biology,* 5**,** E259.

Reaven, E. P. & Reaven, G. M. 1981. Structure And Function Changes In The Endocrine Pancreas Of Aging Rats With Reference To The Modulating Effects Of Exercise And Caloric Restriction. *Journal Of Clinical Investigation,* 68**,** 75-84.

Redman, L.M. et al., 2018. Metabolic Slowing and Reduced Oxidative Damage with Sustained Caloric Restriction Support the Rate of Living and Oxidative Damage Theories of Aging. *Cell Metabolism*, 27(4), pp.805–815.e4.

Renka, R. J. 1996. Algorithm 751: Tripack: A Constrained Two-Dimensional Delaunay Triangulation Package. *Acm Transactions On Mathematical Software (Toms),* 22**,** 1-8.

Reverter-Branchat, G., Cabiscol, E., Tamarit, J. & Ros, J. 2004. Oxidative Damage To Specific Proteins In Replicative And Chronological-Aged Saccharomyces Cerevisiae: Common Targets And Prevention By Calorie Restriction. *The Journal Of Biological Chemistry,* 279**,** 31983-31989.

Reznick, R. M., Zong, H., Li, J., Morino, K., Moore, I. K., Yu, H. J., Liu, Z.-X., Dong, J., Mustard, K. J., Hawley, S. A., Befroy, D., Pypaert, M., Hardie, D. G., Young, L. H. & Shulman, G. I. 2007. Aging-Associated Reductions In Amp-Activated Protein Kinase Activity And Mitochondrial Biogenesis. *Cell Metabolism,* 5**,** 151-156.

Riley, J. C. 2005a. Estimates Of Regional And Global Life Expectancy, 1800-2001. *Population And Development Review,* 31**,** 537-543.

Ristow, M., Zarse, K., Oberbach, A., Klöting, N., Birringer, M., Kiehntopf, M., Stumvoll, M., Kahn, C. R. & Blüher, M. 2009. Antioxidants Prevent Health-Promoting Effects Of Physical Exercise In Humans. *Proceedings Of The National Academy Of Sciences Of The United States Of America,* 106**,** 8665-8670.

Rizza, W., Veronese, N. & Fontana, L. 2014. What Are The Roles Of Calorie Restriction And Diet Quality In Promoting Healthy Longevity? *Ageing Research Reviews,* 13**,** 38-45.

Rizzi, F., Trougakos, I. P., Pintus, G. & Sykiotis, G. P. 2016. Redox Status And Proteostasis In Ageing And Disease. *Oxidative Medicine And Cellular Longevity,* 2016**,** 1-2.

Ruderman, N. B., Carling, D., Prentki, M. & Cacicedo, J. M. 2013. Ampk, Insulin Resistance, And The Metabolic Syndrome. *Journal Of Clinical Investigation,* 123**,** 2764-2772.

Rudolph, K. L., Chang, S., Lee, H.-W., Blasco, M., Gottlieb, G. J., Greider, C. & Depinho, R. A. 1999. Longevity, Stress Response, And Cancer In Aging Telomerase-Deficient Mice. *Cell,* 96**,** 701-712.

Sahin, E. & Depinho, R. A. 2012. Axis Of Ageing: Telomeres, P53 And Mitochondria. *Nature Reviews* 13**,** 397-404.

Sahin, E. & Depinho, R. A. 2010. Linking Functional Decline Of Telomeres, Mitochondria And Stem Cells During Ageing. *Nature,* 464**,** 520-528.

Salminen, A. & Kaarniranta, K. 2009. Regulation Of The Aging Process By Autophagy. *Trends In Molecular Medicine,* 15**,** 217-224.

Salminen, A., Kaarniranta, K. & Kauppinen, A., 2016. Age-Related Changes In Ampk Activation: Role For Ampk Phosphatases And Inhibitory Phosphorylation By Upstream Signaling Pathways. *Ageing Research Reviews*, 28, Pp.15–26.

Sands, W. A., Page, M. M. & Selman, C. 2017. Proteostasis And Ageing: Insights From Long-Lived Mutant Mice. *The Journal Of Physiology,* 595**,** 6383-6390.

Satoh, A., Imai, S.-I. & Guarente, L. 2017. The Brain, Sirtuins, And Ageing. *Nature Publishing Group,* 18**,** Nrn.2017.42-374.

Scheibye-Knudsen, M., Mitchell, S. J., Fang, E. F., Iyama, T., Ward, T., Wang, J., Dunn, C. A., Singh, N., Veith, S., Hasan-Olive, M. M., Mangerich, A., Wilson, M. A., Mattson, M. P., Bergersen, L. H., Cogger, V. C., Warren, A., Le Couteur, D. G., Moaddel, R., Wilson, D. M., Croteau, D. L., De Cabo, R. & Bohr, V. A. 2014. A High-Fat Diet And Nad(+) Activate Sirt1 To Rescue Premature Aging In Cockayne Syndrome. *Cell Metabolism,* 20**,** 840-855.

Schmucker, D. L. 2005. Age-Related Changes In Liver Structure And Function: Implications For Disease ? *Experimental Gerontology,* 40**,** 650-659.

Schmucker, D. L. 1998. Aging And The Liver: An Update. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences,* 53a**,** B315-B321.

Schotta, G. 2004. A Silencing Pathway To Induce H3-K9 And H4-K20 Trimethylation At Constitutive Heterochromatin. *Genes & Development,* 18**,** 1251-1262.

Schwarz, J., Tomé, D., Baars, A., Hooiveld, G. J. E. J. & Müller, M. 2012. Dietary Protein Affects Gene Expression And Prevents Lipid Accumulation In The Liver In Mice. *Plos One,* 7**,** E47303.

Schwörer, S., Becker, F., Feller, C., Baig, A. H., Köber, U., Henze, H., Kraus, J. M., Xin, B., Lechel, A., Lipka, D. B., Varghese, C. S., Schmidt, M., Rohs, R., Aebersold, R., Medina, K. L., Kestler, H. A., Neri, F., Von Maltzahn, J., Tümpel, S. & Rudolph, K. L. 2016a. Epigenetic Stress Responses Induce Muscle Stem-Cell Ageing By Hoxa9 Developmental Signals. *Nature,* 540**,** 428 Ep --432.

Scialò, F., Sriram, A., Fernández-Ayala, D., Gubina, N., Lõhmus, M., Nelson, G., Logan, A., Cooper, H. M., Navas, P., Enríquez, J. A., Murphy, M. P. & Sanz, A. 2016. Mitochondrial Ros Produced Via Reverse Electron Transport Extend Animal Lifespan. *Cell Metabolism,* 23**,** 725-734.

Seals, D. R. & Melov, S. 2014. Translational Geroscience: Emphasizing Function To Achieve Optimal Longevity. *Aging,* 6**,** 718-730.

Seo, K., Choi, E., Lee, D., Jeong, D.-E., Jang, S. K. & Lee, S.-J. 2013. Heat Shock Factor 1 Mediates The Longevity Conferred By Inhibition Of Tor And Insulin/Igf-1 Signaling Pathways In C. Elegans. *Aging Cell,* 12**,** 1073-1081.

Sevini, F., Giuliani, C., Vianello, D., Giampieri, E., Santoro, A., Biondi, F., Garagnani, P., Passarino, G., Luiselli, D., Capri, M., Franceschi, C. & Salvioli, S. 2014. Mtdna Mutations In Human Aging And Longevity: Controversies And New Perspectives Opened By High-Throughput Technologies. *Experimental Gerontology***,** 1-33.

Sgro, C. M. & Partridge, L. 1999. A Delayed Wave Of Death From Reproduction In Drosophila. *Science,* 286**,** 2521-2524.

Shanahan, T. 1998. The Troubled Past And Uncertain Future Of Group Selectionism. *Endeavour,* 22**,** 57-60.

Sharma, P. K., Agrawal, V. & Roy, N. 2010. Mitochondria-Mediated Hormetic Response In Life Span Extension Of Calorie-Restricted Saccharomyces Cerevisiae. *Age,* 33**,** 143-154.

Simpson, S. J. & Raubenheimer, D. 1993. A Multi-Level Analysis Of Feeding Behaviour: The Geometry Of Nutritional Decisions. *Philosophical Transactions Of The Royal Society Of London B: Biological Sciences,* 342**,** 381-402.

Simpson, S. J. & Raubenheimer, D. 1999. Assuaging Nutritional Complexity: A Geometrical Approach. *Proceedings Of The Nutrition Society,* 58**,** 779-789.

Simpson, S. J. & Raubenheimer, D. 2007. Caloric Restriction And Aging Revisited: The Need For A Geometric Analysis Of The Nutritional Bases Of Aging. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences,* 62**,** 707-713.

Simpson, S. J. & Raubenheimer, D. 2005. Obesity: The Protein Leverage Hypothesis. *Obesity Reviews : An Official Journal Of The International Association For The Study Of Obesity,* 6**,** 133-142.

Simpson, S. J. & Raubenheimer, D. 1995. The Geometric Analysis Of Feeding And Nutrition: A User's Guide. Journal Of Insect Physiology, 41, 545-553.

Simpson, S. J., Le Couteur, D. G., James, D. E., George, J., Gunton, J. E., Solon-Biet, S. M. & Raubenheimer, D. 2017a. The Geometric Framework For Nutrition As A Tool In Precision Medicine. *Nutrition And Healthy Aging,* 4**,** 217-226.

Simpson, S. J., Le Couteur, D. G., Raubenheimer, D., Solon-Biet, S. M., Cooney, G. J., Cogger, V. C., & Fontana, L., 2017b. Dietary protein, aging and nutritional geometry. *Ageing Research Reviews*, *39*, 78–86.

Simpson, S. J., Batley, R. & Raubenheimer, D. 2003. Geometric Analysis Of Macronutrient Intake In Humans: The Power Of Protein? *Appetite,* 41**,** 123-140.

Simpson, S. J., Le Couteur, D. G. & Raubenheimer, D. 2015. Putting The Balance Back In Diet. *Cell,* 161**,** 18-23.

Sinclair, D. A. & Guarente, L. 1997. Extrachromosomal Rdna Circles— A Cause Of Aging In Yeast. *Cell,* 91**,** 1033-1042.

Smeal, T., Claus, J., Kennedy, B., Cole, F. & Guarente, L. 1996. Loss Of Transcriptional Silencing Causes Sterility In Old Mother Cells Of S. Cerevisiae. *Cell,* 84**,** 633-642.

Smith-Vikos, T. & Slack, F. J. 2012. Micrornas And Their Roles In Aging. *Journal Of Cell Science,* 125**,** 7-17.

Smoliga, J. M., Colombo, E. S. & Campen, M. J. 2013. A Healthier Approach To Clinical Trials Evaluating Resveratrol For Primary Prevention Of Age-Related Diseases In Healthy Populations. *Aging,* 5**,** 495-506.

Smoliga, J. M., Vang, O. & Baur, J. A. 2012. Challenges Of Translating Basic Research Into Therapeutics: Resveratrol As An Example. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences,* 67a**,** 158-167.

Soefje, S. A., Karnad, A. & Brenner, A. J. 2011. Common Toxicities Of Mammalian Target Of Rapamycin Inhibitors. *Targeted Oncology,* 6**,** 125-129.

Solon-Biet, S. M., Cogger, V. C., Pulpitel, T., Heblinski, M., Wahl, D., Mcmahon, A. C., Warren, A., Durrant-Whyte, J., Walters, K. A., Krycer, J. R., Ponton, F., Gokarn, R., Wali, J. A., Ruohonen,

K., Conigrave, A. D., James, D. E., Raubenheimer, D., Morrison, C. D., Le Couteur, D. G. & Simpson, S. J. 2016. Defining The Nutritional And Metabolic Context Of Fgf21 Using The Geometric Framework. *Cell Metabolism,* 24**,** 555-565.

Solon-Biet, S. M., Mcmahon, A. C., Ballard, J. W. O., Ruohonen, K., Wu, L. E., Cogger, V. C., Warren, A., Huang, X., Pichaud, N., Melvin, R. G., Gokarn, R., Khalil, M., Turner, N., Cooney, G. J., Sinclair, D. A., Raubenheimer, D., Le Couteur, D. G. & Simpson, S. J. 2014. The Ratio Of Macronutrients, Not Caloric Intake, Dictates Cardiometabolic Health, Aging, And Longevity In Ad Libitum-Fed Mice. *Cell Metabolism,* 19**,** 418-430.

Solon-Biet, S. M., Mitchell, S. J., Coogan, S. C. P., Cogger, V. C., Gokarn, R., Mcmahon, A. C., Raubenheimer, D., De Cabo, R., Simpson, S. J. & Le Couteur, D. G. 2015a. Dietary Protein To Carbohydrate Ratio And Caloric Restriction: Comparing Metabolic Outcomes In Mice. *Cellreports,* 11**,** 1529-1534.

Solon-Biet, S. M., Mitchell, S. J., De Cabo, R., Raubenheimer, D., Le Couteur, D. G. & Simpson, S. J. 2015b. Macronutrients And Caloric Intake In Health And Longevity. *The Journal Of Endocrinology,* 226**,** R17-28.

Solon-Biet, S. M., Walters, K. A., Simanainen, U. K., Mcmahon, A. C., Ruohonen, K., Ballard, J. W. O., Raubenheimer, D., Handelsman, D. J., Le Couteur, D. G. & Simpson, S. J. 2015c. Macronutrient Balance, Reproductive Function, And Lifespan In Aging Mice. *Proceedings Of The National Academy Of Sciences Of The United States Of America,* 112**,** 3481-3486.

Someya, S., Yu, W., Hallows, W. C., Xu, J., Vann, J. M., Leeuwenburgh, C., Tanokura, M., Denu, J. M. & Prolla, T. A. 2010. Sirt3 Mediates Reduction Of Oxidative Damage And Prevention Of Age-Related Hearing Loss Under Caloric Restriction. *Cell,* 143**,** 802-812.

Sommer, M., Poliak, N., Upadhyay, S., Ratovitski, E. A., Nelkin, B. D., Donehower, L. A. & Sidransky, D. 2014. Δnp63&Alpha Overexpression Induces Downregulation Of Sirt1 And An Accelerated Aging Phenotype In The Mouse. *Cell Cycle,* 5**,** 2005-2011.

Song, S., Hooiveld, G. J. E. J., Li, M., Zhao, F., Zhang, W., Xu, X., Müller, M., Li, C. & Zhou, G. 2016. Distinct Physiological, Plasma Amino Acid, And Liver Transcriptome Responses To Purified Dietary Beef, Chicken, Fish, And Pork Proteins In Young Rats. *Molecular Nutrition & Food Research,* 60**,** 1199-1205.

Soultoukis, G. A. & Partridge, L. 2016. Dietary Protein, Metabolism, And Aging. *Annual Review Of Biochemistry,* 85**,** 5-34.

Speakman, J. R., Mitchell, S. E. & Mazidi, M. 2016. Calories Or Protein? The Effect Of Dietary Restriction On Lifespan In Rodents Is Explained By Calories Alone. *Experimental Gerontology***,** 1-11.

Speliotes, E. K., Willer, C. J., Berndt, S. I., Monda, K. L., Thorleifsson, G., Jackson, A. U., Lango Allen, H., Lindgren, C. M., Luan, J. A. A., Mägi, R., Randall, J. C., Vedantam, S., Winkler, T. W., Qi, L., Workalemahu, T., Heid, I. M., Steinthorsdottir, V., Stringham, H. M., Weedon, M. N., Wheeler, E., Wood, A. R., Ferreira, T., Weyant, R. J., Segrè, A. V., Estrada, K., Liang, L., Nemesh, J., Park, J. H., Gustafsson, S., Kilpeläinen, T. O., Yang, J., Bouatia-Naji, N., Esko, T., Feitosa, M. F., Kutalik, Z., Mangino, M., Raychaudhuri, S., Scherag, A., Smith, A. V., Welch, R., Zhao, J. H., Aben, K. K., Absher, D. M., Amin, N., Dixon, A. L., Fisher, E., Glazer, N. L., Goddard, M. E., Heard-Costa, N. L., Hoesel, V., Hottenga, J. J., Johansson, A., Johnson, T., Ketkar, S., Lamina, C., Li, S., Moffatt, M. F., Myers, R. H., Narisu, N., Perry, J. R. B., Peters, M. J., Preuss, M., Ripatti, S., Rivadeneira, F., Sandholt, C., Scott, L. J., Timpson, N. J., Tyrer, J. P., Van Wingerden, S., Watanabe, R. M., White, C. C., Wiklund, F., Barlassina, C., Chasman, D. I.,

Cooper, M. N., Jansson, J. O., Lawrence, R. W., Pellikka, N., Prokopenko, I., Shi, J., Thiering, E., Alavere, H., Alibrandi, M. T. S., Almgren, P., Arnold, A. M., Aspelund, T., Atwood, L. D., Balkau, B., Balmforth, A. J., Bennett, A. J., Ben-Shlomo, Y., Bergman, R. N., Bergmann, S., Biebermann, H., Blakemore, A. I. F., Boes, T., Bonnycastle, L. L., Bornstein, S. R., Brown, M. J., Buchanan, T. A., Et Al. 2010. Association Analyses Of 249,796 Individuals Reveal 18 New Loci Associated With Body Mass Index. *Nature Genetics,* 42**,** 937-948.

Stenesen, D., Suh, J. M., Seo, J., Yu, K., Lee, K.-S., Kim, J.-S., Min, K.-J. & Graff, J. M. 2013. Adenosine Nucleotide Biosynthesis And Ampk Regulate Adult Life Span And Mediate The Longevity Benefit Of Caloric Restriction In Flies. *Cell Metabolism,* 17**,** 101-112.

Stumpferl, S. W., Brand, S. E., Jiang, J. C., Korona, B., Tiwari, A., Dai, J., Seo, J.-G. & Jazwinski, S. M. 2012. Natural Genetic Variation In Yeast Longevity. *Genome Research,* 22**,** 1963-1973.

Suh, Y., Atzmon, G., Cho, M.-O., Hwang, D., Liu, B., Leahy, D. J., Barzilai, N. & Cohen, P. 2008. Functionally Significant Insulin-Like Growth Factor I Receptor Mutations In Centenarians. *Proceedings Of The National Academy Of Sciences Of The United States Of America,* 105**,** 3438-3442.

Sun, A. Y., Wang, Q., Simonyi, A. & Sun, G. Y. 2010. Resveratrol As A Therapeutic Agent For Neurodegenerative Diseases. *Molecular Neurobiology,* 41**,** 375-383.

Sun, L., Sadighi Akha, A. A., Miller, R. A. & Harper, J. M. 2009. Life-Span Extension In Mice By Preweaning Food Restriction And By Methionine Restriction In Middle Age. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences,* 64a**,** 711-722.

Svensson, J., Sjögren, K., Fäldt, J., Andersson, N., Isaksson, O., Jansson, J.-O. & Ohlsson, C. 2011. Liver-Derived Igf-I Regulates Mean Life Span In Mice. *Plos One,* 6**,** E22640.

Swindell, W. R. 2008. Comparative Analysis Of Microarray Data Identifies Common Responses To Caloric Restriction Among Mouse Tissues. *Mechanisms Of Ageing And Development,* 129**,** 138-153.

Swindell, W. R. 2009. Genes And Gene Expression Modules Associated With Caloric Restriction And Aging In The Laboratory Mouse. *Bmc Genomics,* 10**,** 585.

Tacutu, R., Budovsky, A., Yanai, H. & Fraifeld, V. E. 2011. Molecular Links Between Cellular Senescence, Longevity And Age-Related Diseases – A Systems Biology Perspective. *Aging,* 3**,** 1178-1191.

Takahashi, A. C. M., Porta, A., Melo, R. C., Quitério, R. J., Da Silva, E., Borghi-Silva, A., Tobaldini, E., Montano, N. & Catai, A. M. 2012. Aging Reduces Complexity Of Heart Rate Variability Assessed By Conditional Entropy And Symbolic Analysis. *Internal And Emergency Medicine,* 7**,** 229-235.

Takubo, K., Nakamura, K. I., Izumiyama, N., Furugori, E., Sawabe, M., Arai, T., Esaki, Y., Mafune, K. I., Kammori, M., Fujiwara, M., Kato, M., Oshimura, M. & Sasajima, K. 2000. Telomere Shortening With Aging In Human Liver. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences* 55**,** B533-B536.

Tanrikulu-Kucuk, S. & Ademoglu, E. 2012. Dietary Restriction Of Amino Acids Other Than Methionine Prevents Oxidative Damage During Aging: Involvement Of Telomerase Activity And Telomere Length. *Life Sciences,* 90**,** 924-928.

Tarry-Adkins, J. L. & Ozanne, S. E. 2016. Nutrition In Early Life And Age-Associated Diseases. *Ageing Research Reviews***,** 1-10.

Tatar, M. 2001. A Mutant Drosophila Insulin Receptor Homolog That Extends Life-Span And Impairs Neuroendocrine Function. *Science,* 292**,** 107-110.

Timper, K. & Brüning, J. C. 2017. Hypothalamic Circuits Regulating Appetite And Energy Homeostasis: Pathways To Obesity. *Disease Models & Mechanisms,* 10**,** 679-689.

Tomás-Loba, A., Flores, I., Fernandez-Marcos, P. J., Cayuela, M. L., Maraver, A., Tejera, A., Borrás, C., Matheu, A., Klatt, P., Flores, J. M., Vina, J., Serrano, M. & Blasco, M. A. 2008. Telomerase Reverse Transcriptase Delays Aging In Cancer-Resistant Mice. *Cell,* 135**,** 609-622.

Trifunovic, A., Wredenberg, A., Falkenberg, M., Spelbrink, J. N., Rovio, A. T., Bruder, C. E., Bohlooly-Y, M., Gidlöf, S., Oldfors, A., Wibom, R., Törnell, J., Jacobs, H. T. & Larsson, N. G. 2004. Premature Ageing In Mice Expressing Defective Mitochondrial Dna Polymerase. *Nature,* 429**,** 417-423.

Trzeciak, A. R., Mohanty, J. G., Jacob, K. D., Barnes, J., Ejiogu, N., Lohani, A., Zonderman, A. B., Rifkind, J. M. & Evans, M. K. 2012. Oxidative Damage To Dna And Single Strand Break Repair Capacity: Relationship To Other Measures Of Oxidative Stress In A Population Cohort. *Mutation Research/Fundamental And Molecular Mechanisms Of Mutagenesis,* 736**,** 93-103.

Uddin, G. M., Youngson, N. A., Sinclair, D. A. & Morris, M. J. 2016. Head To Head Comparison Of Short-Term Treatment With The Nad(+) Precursor Nicotinamide Mononucleotide (Nmn) And 6 Weeks Of Exercise In Obese Female Mice. *Frontiers In Pharmacology,* 7**,** 258.

Umemura, A., Park, E. J., Taniguchi, K., Lee, J. H., Shalapour, S., Valasek, M. A., Aghajan, M., Nakagawa, H., Seki, E., Hall, M. N. & Karin, M. 2014. Liver Damage, Inflammation, And Enhanced Tumorigenesis After Persistent Mtorc1 Inhibition. *Cell Metabolism,* 20**,** 133-144.

Unal, E., Kinde, B. & Amon, A. 2011. Gametogenesis Eliminates Age-Induced Cellular Damage And Resets Life Span In Yeast. *Science,* 332**,** 1554-1557.

Vaiserman, A. M., Lushchak, O. V. & Koliada, A. K. 2016. Anti-Aging Pharmacology: Promises And Pitfalls. *Ageing Research Reviews***,** 1-27.

Valencia, W. M., Palacio, A., Tamariz, L. & Florez, H. 2017. Metformin And Ageing: Improving Ageing Outcomes Beyond Glycaemic Control. *Diabetologia,* 60**,** 1630-1638.

Valle, A., Silvestri, E., Moreno, M., Chambery, A., Oliver, J., Roca, P. & Goglia, F. 2008. Combined Effect Of Gender And Caloric Restriction On Liver Proteomic Expression Profile. *Journal Of Proteome Research,* 7**,** 2872-2881.

Van De Rest, O., Berendsen, A. A., Haveman-Nies, A. & De Groot, L. C. 2015. Dietary Patterns, Cognitive Decline, And Dementia: A Systematic Review. *Advances In Nutrition: An International Review Journal,* 6**,** 154-168.

Van Heemst, D., Beekman, M., Mooijaart, S. P., Heijmans, B. T., Brandt, B. W., Zwaan, B. J., Slagboom, P. E. & Westendorp, R. G. J. 2005. Reduced Insulin/Igf-1 Signalling And Human Longevity. *Aging Cell,* 4**,** 79-85.

Velázquez-Villegas, L. A., Charabati, T., Contreras, A. V., Alemán, G., Torres, N. & Tovar, A. R. 2016. Pparα Downregulates Hepatic Glutaminase Expression In Mice Fed Diets With Different Protein:Carbohydrate Ratios. *The Journal Of Nutrition,* 146**,** 1634-1640.

Ventura, N., Rea, S. L., Schiavi, A., Torgovnick, A., Testi, R. & Johnson, T. E. 2009. P53/Cep-1 Increases Or Decreases Lifespan, Depending On Level Of Mitochondrial Bioenergetic Stress. *Aging Cell,* 8**,** 380-393.

Vera, E., Bernardes De Jesus, B., Foronda, M., Flores, J. M. & Blasco, M. A. 2013. Telomerase Reverse Transcriptase Synergizes With Calorie Restriction To Increase Health Span And Extend Mouse Longevity. *Plos One,* 8**,** E53760.

Vera, E., Bernardes De Jesus, B., Foronda, M., Flores, J. M. & Blasco, M. A. 2012. The Rate Of Increase Of Short Telomeres Predicts Longevity In Mammals. *Cell Reports,* 2**,** 732-737.

Vermeij, W. P., Hoeijmakers, J. H. J. & Pothof, J. 2016. Genome Integrity In Aging: Human Syndromes, Mouse Models, And Therapeutic Options. *Annual Review Of Pharmacology And Toxicology,* 56**,** 427-445.

Vidaček, N. Š., Nanić, L., Ravlić, S., Sopta, M., Gerić, M., Gajski, G., Garaj-Vrhovac, V. & Rubelj, I. 2017. Telomeres, Nutrition, And Longevity: Can We Really Navigate Our Aging? *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences,* 73**,** 39-47.

Vijg, J. & Campisi, J. 2008. Puzzles, Promises And A Cure For Ageing. *Nature,* 454**,** 1065-1071.

Vilchez, D., Saez, I. & Dillin, A. 2014. The Role Of Protein Clearance Mechanisms In Organismal Ageing And Age-Related Diseases. *Nature Communications,* 5**,** 5659.

Wahl, D., Cogger, V. C., Solon-Biet, S. M., Waern, R. V. R., Gokarn, R., Pulpitel, T., Cabo, R. D., Mattson, M. P., Raubenheimer, D., Simpson, S. J. & Le Couteur, D. G. 2016. Nutritional Strategies To Optimise Cognitive Function In The Aging Brain. *Ageing Research Reviews,* 31**,** 80-92.

Walker, D. W., Mccoll, G., Jenkins, N. L., Harris, J. & Lithgow, G. J. 2000. Evolution Of Lifespan In C. Elegans. *Nature,* 405**,** 296-297.

Wanders, D., Ghosh, S., Stone, K. P., Van, N. T. & Gettys, T. W. 2014. Transcriptional Impact Of Dietary Methionine Restriction On Systemic Inflammation: Relevance To Biomarkers Of Metabolic Disease During Aging. *Biofactors (Oxford, England),* 40**,** 13-26.

Wang, C. P., Lorenzo, C., Habib, S. L., Jo, B. & Espinoza, S. E. 2017. Differential Effects Of Metformin On Age Related Comorbidities In Older Men With Type 2 Diabetes. *Journal Of Diabetes And Its Complications,* 31**,** 679-686.

Warnat, P., Eils, R. & Brors, B. 2005. Cross-Platform Analysis Of Cancer Microarray Data Improves Gene Expression Based Classification Of Phenotypes. *Bmc Bioinformatics,* 6**,** 265.

Warshamana-Greene, G. S., Litz, J., Buchdunger, E., García-Echeverría, C., Hofmann, F. & Krystal, G. W. 2005. The Insulin-Like Growth Factor-I Receptor Kinase Inhibitor, Nvp-Adw742, Sensitizes Small Cell Lung Cancer Cell Lines To The Effects Of Chemotherapy. *Clinical Cancer Research,* 11**,** 1563-1571.

Waterson, M. J. & Horvath, T. L. 2015. Neuronal Regulation Of Energy Homeostasis: Beyond The Hypothalamus And Feeding. *Cell Metabolism,* 22**,** 962-970.

Wei, G. Z., Wang, F., Zhao, Y. G., Li, S. S., Shi, M. L., Gao, K., Luo, Y. & Tang, W. R. 2016. Association Of Longevity With Tnf-A G308a And Il-6 G174c Polymorphic Inflammatory Biomarkers In Caucasians: A Meta-Analysis. *Zeitschrift Für Gerontologie Und Geriatrie,* 49**,** 706-713.

Wei, M., Fabrizio, P., Madia, F., Hu, J., Ge, H., Li, L. M. & Longo, V. D. 2009. Tor1/Sch9-Regulated Carbon Source Substitution Is As Effective As Calorie Restriction In Life Span Extension. *Plos Genetics,* 5**,** E1000467.

Wei, Y., Zhang, Y. J., Cai, Y. & Xu, M. H. 2015. The Role Of Mitochondria In Mtor-Regulated Longevity. 90**,** 167-181.

Weimer, S., Priebs, J., Kuhlow, D., Groth, M., Priebe, S., Mansfeld, J., Merry, T. L., Dubuis, S., Laube, B., Pfeiffer, A. F., Schulz, T. J., Guthke, R., Platzer, M., Zamboni, N., Zarse, K. & Ristow, M. 2014. D-Glucosamine Supplementation Extends Life Span Of Nematodes And Of Ageing Mice. *Nature Communications,* 5**,** 1469.

Weismann, A. 1882. *Über Die Dauer Des Lebens,* Jena, Germany, Verlag Von Gustav Fisher.

Willcox, B. J., Willcox, D. C. & Ferrucci, L. 2008. Secrets Of Healthy Aging And Longevity From Exceptional Survivors Around The Globe: Lessons From Octogenarians To Supercentenarians. *The Journals Of Gerontology Series A: Biological Sciences And Medical Sciences,* 63**,** 1181-1185.

Willcox, D. C., Willcox, B. J., Todoriki, H., Curb, J. D. & Suzuki, M. 2006. Caloric Restriction And Human Longevity: What Can We Learn From The Okinawans? *Biogerontology,* 7**,** 173-177.

Williams, G. C. 1957. Pleiotropy, Natural Selection, And The Evolution Of Senescence. *Evolution,* 11**,** 398.

Williams, L. D., Burdock, G. A., Edwards, J. A., Beck, M. & Bausch, J. 2009. Safety Studies Conducted On High-Purity Trans-Resveratrol In Experimental Animals. *Food And Chemical Toxicology,* 47**,** 2170-2182.

Wood, S. 2006. *Generalised Additive Models*, Crc Press.

Wood, S. N. 2013. A Simple Test For Random Effects In Regression Models. *Biometrika,* 100**,** 1005-1010.

Wood, S. N. 2011. Fast Stable Restricted Maximum Likelihood And Marginal Likelihood Estimation Of Semiparametric Generalised Linear Models - Wood - 2010 - Journal Of The Royal Statistical Society: Series B (Statistical Methodology) - Wiley Online Library. … *Of The Royal Statistical Society: Series B*

Wood, S. N. 2003. Thin Plate Regression Splines. *Journal Of The Royal Statistical Society: Series B (Statistical Methodology),* 65**,** 95-114.

Woods, S. C. & Begg, D. P. 2015. Food For Thought: Revisiting The Complexity Of Food Intake. *Cell Metabolism,* 22**,** 348-351.

Xiao, F., Huang, Z., Li, H., Yu, J., Wang, C., Chen, S., Meng, Q., Cheng, Y., Gao, X., Li, J., Liu, Y. & Guo, F. 2011. Leucine Deprivation Increases Hepatic Insulin Sensitivity Via Gcn2/Mtor/S6k1 And Ampk Pathways. *Diabetes,* 60**,** 746-756.

Xie, Z., Zhang, Y., Zou, K., Brandman, O., Luo, C., Ouyang, Q. & Li, H. 2012. Molecular Phenotyping Of Aging In Single Yeast Cells Using A Novel Microfluidic Device. *Aging Cell,* 11**,** 599-606.

Xu, B. & Xie, X. 2016. Neurotrophic Factor Control Of Satiety And Body Weight. *Nature Reviews Neuroscience,* 17**,** 282-292.

Yamamoto, Y., Tanahashi, T., Kawai, T., Chikahisa, S., Katsuura, S., Nishida, K., Teshima-Kondo, S., Sei, H. & Rokutan, K. 2009. Changes In Behavior And Gene Expression Induced By Caloric Restriction In C57bl/6 Mice. *Physiological Genomics,* 39**,** 227-235.

Yang, J. L., Weissman, L., Bohr, V. A. & Mattson, M. P. 2008. Mitochondrial Dna Damage And Repair In Neurodegenerative Disorders. *Dna Repair,* 7**,** 1110-1120.

Yao, Y., Tsuchiyama, S., Yang, C., Bulteau, A. L., He, C., Robison, B., Tsuchiya, M., Miller, D., Briones, V., Tar, K., Potrero, A., Friguet, B., Kennedy, B. K. & Schmidt, M. 2015. Proteasomes, Sir2, And Hxk2 Form An Interconnected Aging Network That Impinges On The Ampk/Snf1-Regulated Transcriptional Repressor Mig1. *Plos Genetics,* 11**,** E1004968.

Yashin, A. I., Wu, D., Arbeeva, L. S., Kovtun, M., Kulminski, A. & Ukraintseva, S. V. 2015b. Genetic Heterogeneity And Its Role In Gwas Of Human Aging And Longevity. *The Gerontologist,* 55**,** 859-859.

Yee, C., Yang, W. & Hekimi, S. 2014. The Intrinsic Apoptosis Pathway Mediates The Pro-Longevity Response To Mitochondrial Ros In C. Elegans. *Cell,* 157**,** 897-909.

Yeo, G. S. H. & Heisler, L. K. 2012. Unraveling The Brain Regulation Of Appetite: Lessons From Genetics. *Nature Neuroscience,* 15**,** 1343-1349.

Yoshino, J., Conte, C., Fontana, L., Mittendorfer, B., Imai, S.-I., Schechtman, K. B., Gu, C., Kunz, I., Fanelli, F. R., Patterson, B. W. & Klein, S. 2012. Resveratrol Supplementation Does Not Improve Metabolic Function In Nonobese Women With Normal Glucose Tolerance. *Cell Metabolism,* 16**,** 658-664.

Yui, J., Chiu, C.P. & Lansdorp, P.M., 1998. Telomerase activity in candidate stem cells from fetal liver and adult bone marrow. *Blood*, 91(9), pp.3255–3262.

Zarse, K., Schmeisser, S., Groth, M., Priebe, S., Beuster, G., Kuhlow, D., Guthke, R., Platzer, M., Kahn, C. R. & Ristow, M. 2012. Impaired Insulin/Igf1 Signaling Extends Life Span By Promoting Mitochondrial L-Proline Catabolism To Induce A Transient Ros Signal. *Cell Metabolism,* 15**,** 451-465.

Zhang, C. & Cuervo, A. M. 2008. Restoration Of Chaperone-Mediated Autophagy In Aging Liver Improves Cellular Maintenance And Hepatic Function. *Nature Medicine,* 14**,** 959-965.

Zhang, G., Li, J., Purkayastha, S., Tang, Y., Zhang, H., Yin, Y., Li, B., Liu, G. & Cai, D. 2013. Hypothalamic Programming Of Systemic Ageing Involving Ikk-**B**, Nf-**K**b And Gnrh. *Nature* Vol 548 1-8.

Zhong, L. & Mostoslavsky, R. 2011. Fine Tuning Our Cellular Factories: Sirtuins In Mitochondrial Biology. *Cell Metabolism,* 13**,** 621-626.

Zhong, L., D'urso, A., Toiber, D., Sebastian, C., Henry, R. E., Vadysirisack, D. D., Guimaraes, A., Marinelli, B., Wikstrom, J. D., Nir, T., Clish, C. B., Vaitheesvaran, B., Iliopoulos, O., Kurland, I., Dor, Y., Weissleder, R., Shirihai, O. S., Ellisen, L. W., Espinosa, J. M. & Mostoslavsky, R. 2010. The Histone Deacetylase Sirt6 Regulates Glucose Homeostasis Via Hif1α. *Cell*, 140, 280-293.

Zhang, Q., Nogales-Cadenas, R., Lin, J.-R., Zhang, W., Cai, Y., Vijg, J., & Zhang, Z. D., 2016. Systems-level analysis of human aging genes shed new light on mechanisms of aging. *Human Molecular Genetics*, *25*(14), 2934–2947.

Zid, B. M., Rogers, A. N., Katewa, S. D., Vargas, M. A., Kolipinski, M. C., Lu, T. A., Benzer, S. & Kapahi, P. 2009. 4e-Bp Extends Lifespan Upon Dietary Restriction By Enhancing Mitochondrial Activity In Drosophila. *Cell* 139**,** 149-160.

Zoncu, R., Efeyan, A. & Sabatini, D. M. 2010. Mtor: From Growth Signal Integration To Cancer, Diabetes And Ageing. *Nature Publishing Group***,** 1-15.