A Metabolomic Approach to Assessing Life–History Traits in *Caenorhabditis elegans*

Sarah Katherine Davies

July 2011
The work contained within this thesis is my own, except where otherwise stated. All else is appropriately referenced.
Abstract

The proximate causes of ageing and the biological processes that determine lifespan are still unclear. However, many studies using model organisms have led to the identification of genes associated with longevity. While there is a clear link between changes in metabolism and changes in longevity, there has been relatively little ageing-related research that has measured metabolites directly. Metabolic profiling of low molecular weight metabolites (metabolomics) has an advantage over other ‘omics’ techniques, in that it directly samples the metabolic changes in an organism, and integrates information from changes at the gene, transcript and protein levels, as well as post-translational modification.

This thesis demonstrates that metabolic profiling provides a new and useful phenotyping tool for studying ageing in the nematode *Caenorhabditis elegans*. Using both nuclear magnetic resonance (NMR) spectroscopy and gas chromatography–mass spectrometry (GC–MS), I have identified metabolites that are linked with long life. I have carried out the first characterisation of the *C. elegans* metabolome throughout both development and ageing. Comparing these metabolic changes in wild type worms with those seen in a long-lived mutant aid the understanding of when and how mutant worms acquire their long-lived phenotype. In addition to this, I have examined the effects on metabolism of a commonly used technique in *C. elegans* ageing research: the inhibition of DNA synthesis to maintain synchronous ageing populations. This provided a way to control for the effects of this technique when used in my work, but also demonstrated that its use may result in artefacts in data. I have also investigated the effect of mutation accumulation on the *C. elegans* metabolic profile. I have shown that metabolomics provides a way to obtain new phenotypes in this type of study, and novel information about the variation that occurs as a result of spontaneous mutation.
Acknowledgements

Firstly, I’d like to thank my supervisors, Austin Burt, Armand Leroi and Jake Bundy, for their insight and discussion throughout my PhD. Jake, in particular, has been amazingly patient, putting up with an endless stream of silly questions on a day–to–day basis, and arranging lab days out with picnics and punting. I’d also like to thank the Bundy lab; Volker, Florian, Manuel and Greg have all been a huge help, with everything from GC–MS problems to filling tiny balloons with helium.

Huge thanks to Amy, who has managed to keep me (relatively) sane, with drinks and coffee breaks, and being generally lovely and very rational when things have been stressful. And helping me move flat, multiple times...

My Mum and Dad have been, as always, brilliant and I can’t really say thanks enough – they’ve helped me so, so much. I’m sure they know far more about worms than they ever wanted to know. And Jess and Bella – thanks so much for the letters of encouragement, and winning lottery tickets (!) – they made everything a bit better.

Finally, Jake Pearce has been more amazing than I can ever say, helping me with everything from data analysis to \LaTeX. He has proof–read multiple drafts, explained PLS model validation over, and over, and over again, and has generally helped me through nearly every thesis–based panic. He has also managed to put up with me at home, being incredibly supportive, always making sure I stuck at it when I just wanted to give up, and happily spending days taking photos of pigeons to cheer me up. Thank you!
# Contents

## Abbreviations

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Introduction</td>
</tr>
<tr>
<td>1.1</td>
<td>Aims and objectives</td>
</tr>
<tr>
<td>1.2</td>
<td><em>Caenorhabditis elegans</em></td>
</tr>
<tr>
<td>1.3</td>
<td>Ageing</td>
</tr>
<tr>
<td>1.4</td>
<td>Metabolomics</td>
</tr>
<tr>
<td>1.5</td>
<td>A metabolomic approach for the study of ageing</td>
</tr>
<tr>
<td>1.6</td>
<td>Thesis outline</td>
</tr>
</tbody>
</table>

## Materials and Methods

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Materials and Methods</td>
</tr>
<tr>
<td>2.1</td>
<td><em>C. elegans</em> strains</td>
</tr>
<tr>
<td>2.2</td>
<td>Maintenance of <em>C. elegans</em></td>
</tr>
<tr>
<td>2.2.1</td>
<td>Nematode Growth Medium</td>
</tr>
<tr>
<td>2.2.2</td>
<td>OP50 food source</td>
</tr>
<tr>
<td>2.2.3</td>
<td>Egg preparation</td>
</tr>
<tr>
<td>2.3</td>
<td><em>C. elegans</em> longevity assays</td>
</tr>
<tr>
<td>2.4</td>
<td>Preparation of <em>C. elegans</em> for metabolomic analysis</td>
</tr>
<tr>
<td>2.4.1</td>
<td>Harvesting <em>C. elegans</em></td>
</tr>
<tr>
<td>2.4.2</td>
<td>Extraction of metabolites</td>
</tr>
<tr>
<td>2.5</td>
<td>NMR spectroscopy</td>
</tr>
<tr>
<td>2.5.1</td>
<td>Sample preparation</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Data acquisition</td>
</tr>
<tr>
<td>2.5.3</td>
<td>Data processing</td>
</tr>
<tr>
<td>2.6</td>
<td>Gas chromatography mass spectrometry</td>
</tr>
<tr>
<td>2.6.1</td>
<td>Sample preparation</td>
</tr>
<tr>
<td>2.6.2</td>
<td>Data acquisition</td>
</tr>
<tr>
<td>2.6.3</td>
<td>Data processing</td>
</tr>
<tr>
<td>2.7</td>
<td>Data analysis</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>2.7.1 Survival analysis</td>
<td>50</td>
</tr>
<tr>
<td>2.7.2 Data normalisation</td>
<td>50</td>
</tr>
<tr>
<td>2.7.3 General statistical analyses</td>
<td>50</td>
</tr>
<tr>
<td>2.7.4 Multivariate analysis</td>
<td>51</td>
</tr>
<tr>
<td>3 Method Development and Validation</td>
<td>55</td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>55</td>
</tr>
<tr>
<td>3.2 Results</td>
<td>56</td>
</tr>
<tr>
<td>3.2.1 Metabolomic analysis of a long–lived mutant</td>
<td>56</td>
</tr>
<tr>
<td>3.2.2 Metabolomic analysis of an epistatic interaction</td>
<td>60</td>
</tr>
<tr>
<td>3.3 Discussion</td>
<td>67</td>
</tr>
<tr>
<td>4 The Effect of 5-fluoro-2-deoxyuridine on the <em>C. elegans</em> Metabolome</td>
<td>75</td>
</tr>
<tr>
<td>4.1 Introduction</td>
<td>75</td>
</tr>
<tr>
<td>4.2 Results</td>
<td>81</td>
</tr>
<tr>
<td>4.3 Discussion</td>
<td>101</td>
</tr>
<tr>
<td>5 Metabolomic analysis of the <em>C. elegans</em> life cycle</td>
<td>107</td>
</tr>
<tr>
<td>5.1 Introduction</td>
<td>107</td>
</tr>
<tr>
<td>5.2 Results</td>
<td>111</td>
</tr>
<tr>
<td>5.3 Discussion</td>
<td>134</td>
</tr>
<tr>
<td>6 Metabolomic analysis of mutation accumulation in <em>C. elegans</em></td>
<td>143</td>
</tr>
<tr>
<td>6.1 Introduction</td>
<td>143</td>
</tr>
<tr>
<td>6.2 Methods</td>
<td>148</td>
</tr>
<tr>
<td>6.3 Results</td>
<td>149</td>
</tr>
<tr>
<td>6.4 Discussion</td>
<td>167</td>
</tr>
<tr>
<td>7 Discussion</td>
<td>175</td>
</tr>
<tr>
<td><strong>Bibliography</strong></td>
<td>193</td>
</tr>
</tbody>
</table>
List of Tables

2.1 *C. elegans* strains ................................................. 43

4.1 FUdR and genotype interaction: Two-way ANOVA ............ 96

5.1 *C. elegans* growth parameters ................................. 108

6.1 Mutation Accumulation: number of changed metabolites in a
given line ............................................................ 157

6.2 Mutation Accumulation: number of lines in which a given
metabolite has changed ............................................. 161
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>The <em>C. elegans</em> life cycle</td>
<td>21</td>
</tr>
<tr>
<td>1.2</td>
<td>The <em>daf-2, daf-16</em> pathway</td>
<td>23</td>
</tr>
<tr>
<td>1.3</td>
<td>Example NMR spectrum</td>
<td>37</td>
</tr>
<tr>
<td>2.1</td>
<td>PLS modelling, cross validation</td>
<td>53</td>
</tr>
<tr>
<td>3.1</td>
<td>Method development and validation: survival analysis</td>
<td>57</td>
</tr>
<tr>
<td>3.2</td>
<td>Metabolic profiling of 10–day–old <em>daf-2(m41)</em> and wild type worms</td>
<td>58</td>
</tr>
<tr>
<td>3.3</td>
<td>Metabolite levels in 10–day–old wild type and <em>daf-2(m41)</em> worms</td>
<td>59</td>
</tr>
<tr>
<td>3.4</td>
<td>NMR analysis of an epistatic interaction: PCA scores</td>
<td>61</td>
</tr>
<tr>
<td>3.5</td>
<td>NMR analysis of an epistatic interaction: relative concentrations of metabolites</td>
<td>63</td>
</tr>
<tr>
<td>3.6</td>
<td>GC–MS analysis of an epistatic interaction: PLS–DA scores</td>
<td>64</td>
</tr>
<tr>
<td>3.7</td>
<td>GC–MS analysis of an epistatic interaction: PLS–DA loadings</td>
<td>65</td>
</tr>
<tr>
<td>3.8</td>
<td>GC–MS analysis of an epistatic interaction: DAF-16 dependence</td>
<td>66</td>
</tr>
<tr>
<td>4.1</td>
<td>The effect of FUdR on the <em>C. elegans</em> metabolome: survival analysis</td>
<td>80</td>
</tr>
<tr>
<td>4.2</td>
<td>The effect of FUdR on the <em>C. elegans</em> metabolome: relative concentrations of metabolites</td>
<td>84</td>
</tr>
<tr>
<td>4.3</td>
<td>The effect of FUdR on the <em>C. elegans</em> metabolome: All data, PCA scores</td>
<td>85</td>
</tr>
<tr>
<td>4.4</td>
<td>The effect of FUdR on the <em>C. elegans</em> metabolome: PCA loadings</td>
<td>86</td>
</tr>
<tr>
<td>4.5</td>
<td>The effect of FUdR on the <em>C. elegans</em> metabolome: Treatment–separated PCA scores and loadings</td>
<td>88</td>
</tr>
<tr>
<td>4.6</td>
<td>The effect of FUdR on the <em>C. elegans</em> metabolome: Treatment–separated, PCA loadings scatter plots</td>
<td>90</td>
</tr>
</tbody>
</table>
6.4 Mutation Accumulation analysis: PCA scores, PC2 vs PC3 . 152
6.5 Mutation Accumulation analysis: PCA scores, PC2 vs PC4 . 153
6.6 Mutation Accumulation analysis: grouping on PC2 . . . . . . 155
6.7 Mutation Accumulation analysis: Grouping observed on PC2
relates to two groups of MA lines . . . . . . . . . . . . . . . . 156
6.8 Mutation Accumulation analysis: PC2 loadings . . . . . . . 158
6.9 Mutation Accumulation analysis: Distribution of the number
of changed metabolites per line . . . . . . . . . . . . . . . . 159
6.10 Mutation Accumulation analysis: Correlation between the
Number of changed metabolites in a given line and mean score
on PC2 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 160
6.11 Mutation Accumulation analysis: Metabolites most likely to
change with mutation accumulation . . . . . . . . . . . . . . 162
6.12 Mutation Accumulation analysis: Metabolite centrality . . . 164
6.13 Mutation Accumulation analysis: Correlation between metabo-
type and fitness . . . . . . . . . . . . . . . . . . . . . . . . . 166
**Abbreviations**

Common abbreviations used throughout this thesis. Where relevant, additional terms are defined in the text.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BCAA</td>
<td>Branched-chain amino acid</td>
</tr>
<tr>
<td>DR</td>
<td>Dietary restriction</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FID</td>
<td>Free induction decay</td>
</tr>
<tr>
<td>FUdR</td>
<td>Fluorodeoxyuridine</td>
</tr>
<tr>
<td>GC–MS</td>
<td>Gas–chromatography mass–spectrometry</td>
</tr>
<tr>
<td>GPC</td>
<td>Glycerophosphocholine</td>
</tr>
<tr>
<td>HCA</td>
<td>Hierarchical cluster analysis</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IIS</td>
<td>Insulin/IGF–like signalling</td>
</tr>
<tr>
<td>MA</td>
<td>Mutation accumulation</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OPLS</td>
<td>Orthogonal partial least squares</td>
</tr>
<tr>
<td>OPLS–DA</td>
<td>Orthogonal partial least squares discriminant analysis</td>
</tr>
<tr>
<td>PC</td>
<td>Principal component</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-OH kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PLS</td>
<td>Partial least squares</td>
</tr>
<tr>
<td>PLS–DA</td>
<td>Partial least squares discriminant analysis</td>
</tr>
<tr>
<td>RMA</td>
<td>Reduced major axis</td>
</tr>
<tr>
<td>RNAi</td>
<td>Ribonucleic acid interference</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SGK</td>
<td>Serum– and glucocorticoid–inducible kinase</td>
</tr>
<tr>
<td>TOR</td>
<td>Target of rapamycin</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>NGM</td>
<td>Nematode growth–medium</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Aims and objectives

The proximate causes of ageing, and the biological processes that determine lifespan are still unclear. However, many studies using model organisms have led to the identification of genes that can be associated with longevity. Metabolomics, or metabolic profiling of low molecular weight metabolites (Fiehn, 2002), is an effective way to break down the complex longevity phenotype into smaller elements to provide intermediate phenotypes of ageing. This work will examine the changes in metabolism that occur with the ageing process in the nematode *Caenorhabditis elegans*. It will investigate the metabolic features that are associated with long–lived animals, during both development and the ageing process, and the features associated with altered fecundity, a trait often associated with ageing. It will also consider the effect of the accumulation of mutations on the *C. elegans* metabolic profile.
1 Introduction

1.2 Caenorhabditis elegans

\textit{Caenorhabditis elegans} is a free–living, soil dwelling nematode, around 1 mM in length at adulthood, and comprising only 959 cells. Fed on bacteria, worms can be easily and cheaply cultured in large numbers under laboratory conditions, and frozen for long–term storage. As a multicellular eukaryote with a short generation time, \textit{C. elegans} has been used as a model organism since 1974 (Brenner, 1974). The lineage of all 959 cells of a hermaphrodite is known and therefore \textit{C. elegans} is a common choice for the study of development. The \textit{C. elegans} genome has been fully sequenced (\textit{C. elegans} Sequencing Consortium, 1998) – the first multicellular organism genome sequenced – and it is relatively easy to disrupt specific gene function with RNA interference (RNAi), by injection, soaking or by culturing worms on genetically transformed \textit{E. coli} (Murphy et al., 2003). The \textit{C. elegans} genome is freely available via www.wormbase.org (Harris, 2004), a central data repository for information on \textit{C. elegans} as well as other nematodes. This makes \textit{C. elegans} ideal for use in genomic studies, as well as for use in proteomics, transcriptomics and metabolomics.

Most \textit{C. elegans} individuals are hermaphrodites (around 99.5% of the population on average), with only a small number of males, which are a result of chromosome non–disjunction (Kadandale & Singson, 2004). Males contain exactly 72 cells more than hermaphrodite worms, and these form the male reproductive organs. Hermaphrodites are able to self–fertilise, however if mated with a male, eggs are preferentially fertilised by sperm from the male rather than sperm from the hermaphrodite, and this greatly increases the number of progeny produced (Wood, 1988).
1.2 Caenorhabditis elegans

Figure 1.1: The life cycle of wild type *C. elegans* consists of an embryonic stage, four larval stages (L1–L4) and an adult stage. Each stage ends with a cuticle molt followed by the formation of a new cuticle. Depending on culture temperature, the development of *C. elegans* from egg to adult takes 3–5 days. Under unfavourable conditions, worms enter an alternate L3 stage, the dauer larva. (Diagram adapted from Epstein & Shakes (1995), photographs adapted from Fielenbach & Antebi (2008)).

The *C. elegans* life cycle is shown in Figure 1.1, and consists of embryogenesis and four larval stages (L1–L4) before adulthood. At 20°C, gastrulation takes place over approximately three hours before the egg is laid via the vulva (Wood, 1988). After 7–9 hours, the L1 worm emerges from the egg and then continues through four molts, which occur at the transition from L1 to L2, from L2 to L3, from L3 to L4 and finally from L4 to adult. Worms have a flexible exoskeleton, the cuticle, consisting mainly of cross-linked collagens forming an extracellular matrix. Molting involves the shedding of old cuticle, with a new cuticle generated at each larval stage, allowing for growth of the worm. The cuticle changes in composition and relative thickness at each stage, along with the expression of surface proteins (Cox et al., 1981a,b). At 20°C, the four molts take place over the course of around forty hours. At the final molt, both gonadogenesis and spermatogenesis are complete and
Introduction

Oogenesis begins (Wood, 1988). The progression from embryo to adult takes around three days in total and egg production begins at around 65 hours after worms have hatched (Byerly et al., 1976). Between 200 and 300 viable eggs are produced by self fertilisation.

In unfavourable conditions, such as overcrowding, low food availability and high temperatures, *C. elegans*, at the L2 stage, is able to enter into facultative diapause and develop into an alternative, specialised, third larval stage called the dauer larva. Dauer larvae are non-feeding, have arrested development, and are able to survive for several months, with development continuing as normal when worms are returned to favourable conditions (Wood, 1988). Adult worms that have undergone this dauer diapause have the same fecundity and lifespan as worms that have progressed from embryo to adult without this stage (Wood, 1988).

1.3 Ageing

The genetic control of ageing

Its relatively short average lifespan of 2–3 weeks depending on temperature and food availability (Vanfleteren & Braeckman, 1999), makes *C. elegans* an ideal model organism for studying ageing. In addition to this, many single gene mutations have been discovered to increase *C. elegans* lifespan. The first was the *age-1* mutation, found to increase maximum hermaphrodite lifespan by up to 110%, depending on temperature (Friedman & Johnson, 1988). *Age-1* encodes the homolog of mammalian phosphoinositide-3-OH kinase (PI3K). The next longevity-associated gene to be discovered was *daf-2* (Kenyon et al., 1993), which more than doubled wild type lifespan.
This increase in longevity was found to depend on another gene, \textit{daf-16}. Both \textit{daf-2} and \textit{daf-16} also regulate the formation of long-lived dauer larvae. These three genes are all components of the insulin/IGF-1 signalling (IIS) pathway (Morris \textit{et al.}, 1996; Ogg \textit{et al.}, 1997; Lin, 1997; Kimura, 1997). A simplified version of this pathway is shown in Figure 1.2.

\textbf{Figure 1.2:} \textit{Daf-2} encodes a receptor tyrosine kinase, which binds an insulin–like substrate. This initiates a cascade that involves the activation of the \textit{age-1} encoded kinase, which then activates protein kinase B (PKB), a serine–threonine kinase, encoded by Akt as well as serum– and glucocorticoid–inducible kinase (SGK, not shown). PKB and SGK then phosphorylate the FOXO–related forkhead transcription factor DAF-16 and as a result of this phosphorylation, DAF-16 is retained in the cytoplasm and so is not transcriptionally active. Reduced insulin / IGF-2 signalling results in unphosphorylated DAF-16 entering the nucleus, resulting in the activation of many genes and an increase in longevity.
1 Introduction

The *daf-2* gene encodes a receptor tyrosine kinase, which binds an insulin–like substrate. This initiates a cascade that involves the activation of the *age-1* encoded kinase, which then activates protein kinase B (PKB), a serine–threonine kinase, encoded by *Akt*, as well as serum– and glucocorticoid–inducible kinase (SGK). PKB and SGK then phosphorylate the FOXO–related forkhead transcription factor DAF-16 and as a result of this phosphorylation, DAF-16 is retained in the cytoplasm and so is not transcriptionally active (Paradis & Ruvkun, 1998; Paradis et al., 1999; Hertweck et al., 2004). However, when insulin/IGF-1 signalling is reduced, for example, by mutation of *daf-2* or *age-1*, unphosphorylated DAF-16 is able to enter the nucleus, resulting in the activation of many genes, including those associated with protection against oxidative stress and heat shock, those linked with autophagy and innate immunity as well as other genes associated with survival (Murphy et al., 2003; Lee et al., 2003b; McElwee, 2004; Honda & Honda, 1999; Melendez, 2003). Null *daf-16* (*daf-16*(0)) mutations fully repress the longevity effects of IIS mutations (Kenyon, 2005). Downregulation of IIS has also been found to promote longevity and stress resistance in both *Drosophila* (Clancy, 2001; Tatar, 2001) and in mice (Blüher et al., 2003; Holzenberger et al., 2003).

Much of the work contained within this thesis focusses on mutations in *daf-2*, however there is considerable variation in the phenotype resulting from *daf-2* mutation, depending on the allele studied. The *daf-2* alleles studied here – *daf-2*(m41) and *daf-2(e1370) – represent hypomorphic mutations (Gems et al., 1998), i.e., mutations that cause a partial loss of gene function, reducing expression without the complete loss of function observed as a result of a null mutation. These alleles are known to show differences in longevity, fecundity, and thermotolerance, with *daf-2(e1370) generally longer–lived,
1.3 Ageing

less fecund, and with increased intrinsic thermotolerance compared with
*daf-2(m41)* (Larsen *et al.*, 1995; Gems *et al.*, 1998).

**Reactive oxygen species**

The ‘rate–of–living’ hypothesis states that lifespan is linked with the metabolic
rate of an organism. This hypothesis was generated based on the observation
that species with high metabolic rates generally have shorter lifespans than
those with lower metabolic rates. A mechanism to support this idea was
put forward by Harman (1956), who suggested that reactive oxygen species
(ROS), created within cells as part of normal metabolism, resulted in the ac-
cumulation of cellular damage. The term ROS can be used to describe many
different molecules and free radicals (chemical species with one unpaired
electron and therefore powerful oxidising agents). These species include
superoxide anions, hydrogen peroxide and hydroxide radicals, and may cause
oxidation and damage of cell components. This idea of free radicals pro-
vided evidence to support the rate–of–living theory; a higher metabolic rate
produces a greater number of ROS in a given period of time, leading to
increased cellular damage and therefore a shorter lifespan. Conversely, a
slower metabolic rate results in relatively fewer ROS per unit time, less
cellular damage and increased longevity. However, this free–radical theory of
ageing appears to be more accurate than the closely–related rate–of–living
hypothesis. Some species, for example, birds, have relatively high metabolic
rates and relatively long lifespans and it has been found that this is due
to decreased intracellular production of ROS in these species (Ku *et al.*, 1993).
1 Introduction

The ageing process leads to increased numbers of malfunctioning mitochondria, decreased biogenesis of mitochondria and increased oxidative damage (Guarente, 2008; López-Lluch et al., 2008). Most of the ROS produced within cells originate in mitochondria, and in particular at complexes I and III (NADH dehydrogenase and ubiquinone-cytochrome c reductase respectively) (Turrens, 1997). Both nuclear and mitochondrial DNA accumulate damage as a result of oxidation, but mitochondrial DNA (Beckman & Ames, 1998), possibly due to the close proximity to the most important source of ROS production, is more sensitive to oxidative damage than nuclear DNA. Damaged mitochondrial DNA is likely to lead to impaired mitochondrial function, and these damaged mitochondria produce more ROS, resulting in additional DNA damage and a continuing downward spiral (Finkel & Holbrook, 2000).

A system of antioxidants helps to reduce the net amount of ROS. This defence system includes superoxide dismutase (SOD), converting superoxide to hydrogen peroxide (McCord & Fridovich, 1969), and catalase and glutathione peroxidase, both converting hydrogen peroxide to water. Overexpression of catalase and SOD has long been known to increase longevity in Drosophila (Orr & Sohal, 1994) and treatment with SOD and catalase mimetics increase lifespan in C. elegans (Melov et al., 2000). The amount of oxidative stress a cell experiences is a result of the balance between the production of ROS, and the production and effectiveness of the antioxidant system (Finkel & Holbrook, 2000). External factors such as ultraviolet radiation and hyperthermia may result in the production of high levels of ROS and oxidative stress. This leads to the activation of pathways including p53 activation and the heat shock response (reviewed by Finkel & Holbrook, 2000). Heat shock proteins are involved in ensuring the correct folding, transport and
1.3 Ageing

degradation of other proteins within the cell and the activity of the heat shock response has been shown to decrease with age (Finkel & Holbrook, 2000).

In addition to causing damage to cellular components directly, ROS also result in oxidative glycation reactions between reducing sugars and protein amino groups, giving Advanced Glycation End products (AGEs). These end products are highly reactive, causing protein aggregation through crosslinking, and contributing to the ageing phenotype (reviewed by Singh et al., 2001). Additionally, AGE formation induces the production of free radicals, and decreases the activity of SOD. Together, these processes further increase the level of oxidative damage, and, in turn, the production of AGEs (Singh et al., 2001).

Mitochondria and ageing

In nematodes, mutations in many nuclear genes that encode mitochondrial proteins increase longevity, via a decrease in the rate of metabolism or a decrease in oxidative phosphorylation rate, which leads to reduced levels of reactive oxygen species. The first was discovered by Felkai et al. (1999), who found that worms with mutations in the gene clk-1, which codes for a mitochondrial hydroxylase required for the synthesis of coenzyme Q, were long–lived. The next longevity–associated mitochondrial mutation to be identified was that of the isp-1 gene, which encodes the Rieske iron–sulfur subunit of complex III of the Electron Transport Chain (ETC) (Feng et al., 2001). In addition to these genetic mutations, the reduced synthesis of many mitochondrial components increase longevity, the genes coding for these proteins identified by genome–wide RNAi screens (Dillin, 2002; Lee et al.,
1 Introduction

As well as increasing lifespan, these mutations result in lowered fecundity, increases in developmental times and slowing of movement. The mechanisms by which these mutations influence longevity are unclear. It may be the case that mitochondrial mutations result in a reduced metabolic rate, decreasing ROS production (Feng et al., 2001). Alternatively, mutations in the ETC may lead to an increase in electron leakage, increasing the production of ROS, and activating an adaptive hormesis response (Ventura et al., 2006). Hormesis is the process by which low levels of stress lead to the activation of defence mechanisms, here resulting in an increase in the resistance to oxidative stress and therefore an increase in longevity. While many of these mitochondrial mutations appear to show a synergistic effect with daf-2 mutations, and extend lifespan independently of DAF-16 (Feng et al., 2001; Wong et al., 1995; Dillin, 2002), some have been found to require DAF-16 for lifespan extension (Lee et al., 2003a; Curran & Ruvkun, 2007).

Dietary restriction

Dietary restriction (DR) extends the life span of C. elegans as it does in many other animals (Heilbronn & Ravussin, 2003). The Sir2 gene, shown to be involved in lifespan extension by DR, was first identified in the budding yeast Saccharomyces cerevisiae (Lin et al., 2000). In C. elegans, the longevity-increasing effects of DR can be seen in worms fed a restricted diet and also in various mutants where feeding is affected. For example, eat-2 mutants are unable to feed properly due to a larynx defect, and show increased longevity (Lakowski & Hekimi, 1998). Life span extension in this way is not dependent on functional DAF-16, but instead requires Foxa transcription factor PHA-4
1.3 Ageing

(Panowski et al., 2007). PHA-4 is not required for life span extension that occurs as a result of other regulating pathways, but is essential for all forms of DR–induced increases in longevity – in mutants such as eat-2 and also in worms fed bacteria at a limited concentration (Walker et al., 2005).

Dietary restriction alleviates the decrease in both mitochondrial activity and biogenesis that occur with age. In mammals, it leads to increased expression of PGC-1α, a master regulator of mitochondrial biogenesis and the ROS defence system. It also increases SIRT1 expression. SIRT1 is the closest homologue of yeast SIR2, and its expression can be linked with an increase in mitochondrial biogenesis, decrease in ROS production and lifespan extension (Bishop & Guarente, 2007). Increased mitochondrial biogenesis is beneficial as it leads to more functional mitochondria and therefore less mitochondrial ROS production (Bishop & Guarente, 2007). Overall, these changes lead to a decrease in oxidative damage in metabolically active tissues. This has been shown to be the case in both mice and in humans (Civitarese et al., 2007; Nisoli et al., 2005). CR appears to affect mitochondrial biogenesis and ROS defence genes via endothelial nitric oxide synthase (eNOS) expression (Nisoli et al., 2004, 2005). eNOS–derived nitric oxide directly increases mitochondrial biogenesis (Nisoli et al., 2004) and eNOS Null mutant mice have age–related diseases, a reduced lifespan, reduced mitochondrial biogenesis and reduced SIRT1 expression (Li et al., 2004). The C. elegans homologue of SIRT has yet to be convincingly connected with DR (Bishop & Guarente, 2007), but DR in C. elegans does induce mitochondrial respiration and increases oxidative stress to extend lifespan by hormesis (Schulz et al., 2007).
Introduction

Reproduction and Ageing

When internal resources are limited and insufficient to pay all construction and maintenance costs for two life history traits that share a common resource pool, the result is a trade–off. If there is no variation in resource input, then two traits linked in such a trade–off will be negatively correlated (Zera & Harshman, 2001). In this case, if internal reserves allocated to reproduction in *C. elegans* limit resources available for somatic maintenance, the result will be a trade–off between reproduction and longevity.

This given, it is unsurprising that the laser ablation of germ–line precursors results in a 60% increase in life span, and germ–line mutants (which have a phenotype much like worms with laser–ablated germ–lines) are also long–lived (Mukhopadhyay & Tissenbaum, 2007). However, while the specific ablation of the germ–line extends life span, ablation of reproduction altogether does not affect longevity. Removal of the entire gonad (that is, both the somatic gonad and the germ–line), has little or no effect on life span, despite the complete removal of reproduction. For this reason, it has been suggested that the idea of a trade–off between reproduction and longevity is too simplistic, and that the germ–line sends a specific molecular signal that represses longevity and that the somatic gonad produces a signal opposing the germ–line signal, so controlling lifespan (Hsin & Kenyon, 1999). Germ–line stem-cells, rather than the germ cells themselves appear to be the important factor in wild type longevity (Gems & Riddle, 1996).

Where manipulations are made to attempt to alter the allocation of resources in some way, the strength of a signal may inadvertently be changed, giving the impression of a trade–off or cost of reproduction, where this may not be the case. The fact that elimination of reproduction by removing the
entire gonad has no (or even a detrimental) effect on life span is contrary to the resource allocation trade–off theory. However, it is possible that life span extension following removal of the germ–line requires a signal of some sort from the somatic gonad. It appears that the positive effect of possessing a somatic gonad balances the deleterious effect of possessing a germ–line, so that eliminating the entire gonad leaves longevity unchanged (Leroi, 2001). To properly test this, ideally the effects on lifespan of germ–line in the absence of somatic gonad would be examined, however the presence of germ cells relies upon the somatic gonad.

The germ–line signal, with its negative effect on survival, depends on the presence of a functioning insulin signalling cascade (Patel et al., 2002). Life span extension as a result of an absent germ–line (either through mutation or laser ablation) is dependent on the presence of functional DAF-16, and DAF-16 activity is needed specifically during adulthood for life span extension by germ–line ablation. Daf-16 mutations suppress the longevity–enhancing effects of germ–line ablation, and it seems that in wild type worms, daf-16 responds to a signal from germ cells much as it responds to a signal mediated by daf-2.

Reduced fecundity is observed to varying degrees in daf-2 mutants (alleles e1370, m596, m41, etc.) (Gems et al., 1998), but germ–line ablation in these mutants further increases life span, indicating separate pathways and that life span extension in daf-2 mutants is not a result of a trade–off and the allocation of resources away from reproduction and towards somatic maintenance. Daf-2 mutation and germ–line ablation have synergistic effects on life span, so that when the activity of both germ–line signals and daf-2 decreases, daf-16 activity is permitted to increase even further (Hsin
Introduction

Germ–line signals and wild type DAF-2 activity act in parallel to shorten life span by down regulating daf-16, i.e. germ–line signalling may converge into the IIS pathway downstream of daf-2 to affect activation of DAF-16.

Wild type DAF-2 inhibits life span extension in a similar way to the germ–line signal, and so daf-2 mutants lacking both the germ–line and somatic gonad still have an increased life span relative to wild type, implying that the presence of the somatic gonad is not necessary for life span extension (Barnes & Partridge, 2003).

Metabolism and ageing

Longevity pathways can be associated with specific enzymes involved in metabolism, with many metabolism–linked genes activated by DAF-16 (Lee et al., 2003b; Murphy et al., 2003; Halaschek-Wiener, 2005). In addition to this, proteomic studies have been used to investigate the link between metabolism and longevity in C. elegans. For example, it has been found that wild type worms have a greater abundance of proteins involved with lipid transport and translation elongation than daf-2 worms, while these mutants have a comparatively high abundance of proteins involved in the biosynthesis of amino acids and the metabolism of ROS, as well as those involved with carbohydrate metabolism (Dong et al., 2007).

Longevity–associated pathways have also been linked with key regulators of metabolism in C. elegans. The ratio of AMP to ATP is a measure of energy availability, and this ratio increases with age and changes in response to environmental stress. In worms, the AMP–activated protein kinase subunit AAK-2 acts as a sensor, being activated by AMP. Both environmental stress
1.3 Ageing

and lowered insulin signalling have been found to increase longevity via
*aak-2*, which acts in parallel to *daf-16* to control lifespan (Apfeld et al.,
2004). Wang et al. (2008) used laser ablation of germ line precursor cells to
investigate fat metabolism in these worms. It was found that germline stem
cells modulate lipid metabolism by inducing a specific fat lipase throughout
the worm, which in turn affects longevity. Lipid hydrolysis was found to be
activated via the *daf-16* pathway.

TOR (target–of–rapamycin) protein kinases have been shown to respond to
nutrients and growth factors to control the growth of cells. They are involved
in signalling to downstream translation machinery in response to environmen-
tal nutrient availability (Kim et al., 2002; Hara et al., 2002). In *C. elegans*, the
TOR homologue regulates dauer diapause, altering metabolism in response
to changes in environmental nutrient abundance to cause the accumulation of
lipids. The transcription of raptor, which is encoded by *daf-15* and is a regu-
latory associated protein of TOR, is regulated by DAF-16. Together, raptor
and TOR regulate metabolism and increase lifespan (Jia et al., 2004).

In addition to this, long–lived dauer larvae are known to have altered
metabolism, as reviewed by Braeckman et al. (2000), with a decreased
metabolic rate and a reliance on lipids as a primary energy reserve rather
than the combination of lipid and carbohydrate metabolism observed in non–
dauer worms. Dauers also excrete dauer inducing pheromone in response
to changes in food–availability. This pheromone consists of ascaroside
derivatives with short, fatty acid–like side chains (Butcher et al., 2009).
Changes in metabolism associated with dauer formation have been directly
measured by Fuchs et al. (2010), with some metabolites – phosphoserine
and hydroxyproline – found to be unique to the dauer stage. It is possible
that these modified amino acids are a result of the use of the extracellular matrix, as well as other proteins, as another energy store.

1.4 Metabolomics

Metabolomics is an untargeted approach that allows the quantification of many metabolites – the intermediates and products of metabolism – in a given tissue or organism, at a given time, simultaneously (Goodacre et al., 2004). Changes in environment, disease and mutation may all cause changes in the levels of metabolites in a system, and metabolomics allows an insight into these responses (Nicholson et al., 1999; Fiehn, 2002; Want et al., 2005). Raamsdonk et al. (2001) found that metabolic pathway mutations in yeast that showed no visual phenotype did, however, have a metabolic phenotype, demonstrating the usefulness of metabolomics as a phenotyping tool. These ‘silent’ phenotypes have also been identified in C. elegans (Blaise et al., 2007).

Metabolomic sample preparation (reviewed in Dunn & Ellis, 2005) depends on the platform used, but when profiling intracellular metabolites, first involves the rapid stopping or ‘quenching’ of enzymatic activity by quick-freezing or treatment with acid. This is followed by the extraction of metabolites from frozen, homogenised samples using combinations of cold solvents, for example, methanol/water, methanol/chloroform/water, or acetonitrile/water (Lin et al., 2007). An alternative to the analysis of intracellular metabolites, ‘metabolic footprinting’ involves the analysis of metabolites excreted by a system, or the ‘exometabolome’ (Kell et al., 2005). This may involve the analysis of urine, or of microbial growth medium, and
1.4 Metabolomics

has been used in metabolomic analysis of C. elegans (Butler et al., 2010; Martin et al., 2011).

The choice of platform for metabolomics will depend on the level of sensitivity required, the metabolites to be quantified and factors such as ease of sample preparation. The two main platforms for metabolic profiling are Nuclear Magnetic Resonance (NMR) Spectroscopy, and Mass Spectrometry (MS). Proton NMR–based profiling allows the identification and quantification of a large number of metabolites across many metabolite classes and gives a good overview of the metabolome of an organism. NMR also has the advantage of being non-destructive, and requires only minimal sample preparation. MS is more sensitive than NMR, able to detect small molecules in the nanomolar range rather than the millimolar and micromolar range that can be detected with NMR (Want et al., 2005). However, it is destructive to samples. Here, both NMR and Gas Chromatography coupled to Mass Spectrometry (GC–MS) have been used, and are described in more detail below.

Nuclear Magnetic Resonance

NMR is based on the spin of atomic nuclei, which is determined by the number of protons and neutrons within the nucleus. An odd number of protons or neutrons result in an intrinsic magnetic moment and a non-zero spin (Keeler, 2010). The most common isotope used in NMR spectroscopy is $^1$H. In $^1$H (as well as in $^{13}$C and $^{19}$F), nuclei have a spin of one half, and may exist in one of two states, ‘spin–up’ or ‘spin–down’. These states have the same energy levels and under normal conditions, the number of atoms in each state will be approximately equal. However, when a magnetic field
is applied, the spins that are orientated with the direction of the magnetic field will now be at a lower energy level than those spins orientated against the direction of the magnetic field. At any given time, more nuclei will populate the lower energy state than the higher energy state (Bothwell & Griffin, 2010). The proportion of nuclei that exist at each state is determined by the difference in energy between the two states, and this energy gap is proportional to the strength of the magnetic field applied. The sensitivity of NMR spectroscopy depends on the difference in energy states, with higher magnetic field strengths resulting in greater sensitivity.

Following the polarisation of nuclei in a magnetic field, a pulse of electromagnetic radiation at a frequency corresponding to the difference in energy states is applied. This causes the excitation of nuclei from the lower energy state to the higher energy state. Following this excitation, nuclei at the higher energy state begin to return to their lower energy state, in a process termed ‘relaxation’ (Keeler, 2010). NMR spectra are a result of the energy released as the nuclei return to their lower energy states. This signal, the ‘Free Induction Decay’ (FID), is made up of signals from the combination of nuclei in a given sample, each resonating at its own frequency. The signal decays as the population of nuclei return to their relaxed state. This signal of intensity vs. time can be Fourier–transformed to give a frequency–domain NMR spectrum, i.e. intensity vs NMR frequency (Bothwell & Griffin, 2010). The chemical shift of a nucleus is compared to that of a reference substance (in hertz) and then divided by the frequency at which the spectrometer operates (in megahertz). This ratio means that the dimensionless chemical shift is given in ppm.
The electrons that orbit each nucleus in a sample shield the nucleus from the applied magnetic field, so that the overall field experienced by a nucleus depends on how shielded the nucleus is. This nuclear shielding, and therefore the resonant frequency of a nucleus, depends on neighbouring nuclei and their electronic environment. Adjacent nuclei cause peaks to be split into doublets or triplets and therefore provides information on chemical structure. The position on the ppm scale, as well as peak splitting, allows the identification of metabolites. The concentration of a given metabolite is directly proportional to the total of the integrals of each peak associated with that metabolite (Keeler, 2010).

An example *C. elegans* extract NMR spectrum is given in Figure 1.3.

**Figure 1.3:** Example NMR spectrum (*C. elegans* extract).

**Gas–Chromatography Mass–Spectrometry**

Here, mass–spectrometry coupled to gas–chromatography has been used. Gas–chromatography provides a way to separate components of a mixed
sample so that they enter the mass spectrometer sequentially. The GC–MS is made up of two components: the gas–chromatograph and the mass–spectrometer (Roessner et al., 2000). The gas–chromatography stage involves inserting a sample into a capillary column. To improve their volatility, samples are derivatised prior to injection, in order to decrease the boiling point of polar molecules. Derivatization accentuates the differences in the compounds contained within the sample, to aid the chromatographic separation.

Different molecules within the sample travel at different speeds down this column, based on their chemical properties, separating them in the process. The time taken for a given molecule to pass down the capillary is called the ‘retention time’.

Downstream of the gas–chromatograph, the separated molecules enter the mass spectrometer. Here, compounds undergo ionisation to form ionised fragments (Want et al., 2007). The ionisation technique used here is Electron Impact (EI) ionisation, which involves the molecules being bombarded with free electrons, which causes each molecule to fragment in a characteristic pattern. This is followed by the separation of ions based on their mass to charge ratio \( m/z \). Ions are then detected, in this case by a single quadrupole detector, and processed to generate mass spectra. Data evaluation involves the use of both retention times (generated as a result of the gas–chromatography step), and fragmentation patterns (from the mass–spectrometry component).

Gas chromatography is able to provide high resolution chromatograms, and can differentiate between molecules that have the same retention time. Different molecules may result in similar mass spectra, and so combining
these two processes increases the chance that a molecule is identified correctly (Roessner et al., 2000).

**Metabolomic data analysis**

The data obtained from metabolomic analysis is multivariate. In order to examine the relationships between all samples and all variables, it is necessary to look at all of the data obtained simultaneously by reducing the data to a few independent summary variables. The thousands of data points produced by metabolomic analysis can be reduced using multivariate techniques such as principal component analysis (PCA) (Wold et al., 1987). This allows us to reduce a large number of possibly correlated variables to a smaller number of uncorrelated variables or ‘Principal Components’ in order to obtain an overview of a dataset, and to identify any patterns that may exist within the data. The first principal component accounts for as much data variability as possible, the second for as much of the remaining variability as possible, and so on. Multivariate analysis may be supervised, where structure in the data is taken into account and separation between groups is forced, or unsupervised, where no structure in the data is taken into account. PCA is an example of unsupervised analysis.

In addition to multivariate analysis, it is possible to determine the relative concentration of individual metabolites within a given sample so that univariate analysis techniques can be applied. All data analysis techniques used are described in more detail in Chapter 2.
1 Introduction

1.5 A metabolomic approach for the study of ageing

It is clear that there is an important link between longevity and ageing, and metabolism. Additionally, there are many genes that, when mutated, produce superficially similar effects on lifespan. Using an untargeted / unselective method for the profiling of low molecular weight metabolites should provide intermediate phenotypes of ageing and longevity. This may then be used to determine the functional relationships of target genes as well as determining those metabolites influencing longevity. An advantage of metabolomics, is that it allows us to look at changes in metabolites, not just changes in the genes that control metabolism. This is useful, as a significant proportion of regulation may occur at the post–transcriptional or post–translational level. Metabolomics has already been used to study the effects of ageing. Wang et al. (2007) looked at the effects of ageing and dietary restriction (DR) on the metabolome of Labradors throughout their lifetime using the urinary metabolic profiles of both control–fed and diet–restricted dogs. The results suggested that ageing has a greater effect on the metabolic profile than does DR. Age related changes have also been studied in rats (Williams et al., 2005). 1H NMR and HPLC–TOF MS were used to look at endogenous metabolites excreted in urine.

While some C. elegans metabolic profiling experiments have been carried out (Blaise et al., 2007; Atherton et al., 2008; Swire et al., 2009; Fuchs et al., 2010; Martin et al., 2011; Butler et al., 2010; Szeto et al., 2011), at the outset of this work, no literature existed in this area.
1.6 Thesis outline

This thesis will examine the usefulness of metabolomics as a new phenotyping tool for studying life history traits in *C. elegans*. It begins with the analysis of an epistatic interaction, using NMR spectroscopy and GC–MS to identify metabolic phenotypes that are related to longevity. It then investigates the effects on metabolism of a commonly–used technique in *C. elegans* ageing research: the inhibition of DNA synthesis to maintain synchronous populations. These results will then help to control for the use of this technique in the first characterisation of the *C. elegans* metabolome throughout both development and ageing. Comparing the metabolic changes in wild type worms with those observed in a long–lived mutant should aid the understanding of when and how mutant worms acquire their long–lived phenotype. Finally, it will examine the effect of mutation on the *C. elegans* metabolic profile, with the first metabolomic study of mutation accumulation.
1 Introduction
2 Materials and Methods

2.1 C. elegans strains

Table 2.1 shows the mutant C. elegans strains used. All mutant strains were obtained from the Caenorhabditis Genetics Centre (http://www.cbs.umn.edu/CGC/).

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Gene</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>wild type</td>
<td>-</td>
</tr>
<tr>
<td>DR1565</td>
<td>daf-2</td>
<td>m41</td>
</tr>
<tr>
<td>CB1370</td>
<td>daf-2</td>
<td>e1370</td>
</tr>
<tr>
<td>DR26</td>
<td>daf-16</td>
<td>m26</td>
</tr>
<tr>
<td>DR1309</td>
<td>daf-2; daf-16</td>
<td>e1370; m26</td>
</tr>
</tbody>
</table>

Table 2.1: C. elegans strains

2.2 Maintenance of C. elegans

2.2.1 Nematode Growth Medium

Nematodes were maintained on Nematode Growth Medium (NGM), consisting of 17 g\textsuperscript{l}\textsuperscript{-1} agar, 3 g NaCl, 2.5 g peptone, 1 mM CaCl\textsubscript{2}, 1 mM MgSO\textsubscript{4}, 25 mM K\textsubscript{2}HPO\textsubscript{4} (pH 6), and 0.1% cholesterol. Cholesterol was dissolved in ethanol, filter-sterilised and added after sterilising the remaining mixture in
an autoclave. Approximately 20 ml of the liquid NGM was poured into each 90 mM diameter Petri dish and once set, plates were seeded with \textit{E. coli} strain OP50 as a food source.

For fluorodeoxyuridine (FUdR) plates, FUdR was dissolved in water, filter-sterilised and added to the liquid NGM after autoclaving to give a final concentration of 100 mg l$^{-1}$.

Where required, worms were transferred to plates containing FUdR at young adulthood. Worms were washed from plates using M9 buffer (22 mM KH$_2$PO$_4$, 42 mM Na$_2$HPO$_4$, 85.5 mM NaCl, 1 mM MgSO$_4$) into 15 ml centrifuge tubes and allowed to settle naturally for 3 minutes before the majority of the supernatant was removed. The remaining suspension of worms was then pipetted onto seeded FUdR plates.

\subsection*{2.2.2 OP50 food source}

\textit{E. coli} OP50 is a uracil auxotroph which prevents overgrowth of the bacterial lawn. The OP50 was cultured overnight in 10 ml of Luria broth and then stored at 4 $^\circ$C until use. To seed plates with bacteria, 200 ml of the OP50 culture was added to each plate and spread with a sterile, L-shaped spreader. Plates were incubated overnight at 37 $^\circ$C before use.

As FUdR prevents all DNA synthesis, \textit{E. coli} is not able to proliferate on NGM containing FUdR. For this reason, an overnight \textit{E. coli} culture was concentrated by a factor of 50 before aliquots were spread on NGM containing FUdR, as described by Mitchell \textit{et al.} (1979). These seeded plates were then incubated overnight at 37$^\circ$C and this resulted in a lawn of \textit{E. coli}. 

44
2.2.3 Egg preparation

To obtain age–synchronised populations, gravid adults were washed from plates with M9 buffer into 15 ml centrifuge tubes and centrifuged at 1300 g for 1 minute before the supernatant was removed. Bleaching solution (20% NaOCl (4%), 5% 10 M NaOH) was added to the pellet and the tubes shaken well at regular intervals for a total of 10 minutes. The tubes were then centrifuged at 1300 g for 1 minute and the supernatant removed. M9 buffer was used to wash the pellet (centrifugation followed by removal of the supernatant). The pellet was then resuspended in 0.1 ml M9 buffer, and transferred to a clean NGM plate seeded with E. coli OP50. This method leaves only those worms protected by the egg shell.

2.3 C. elegans longevity assays

Synchronised populations of L4 larvae were grown at a density of ten worms per plate. During the egg–laying period, worms were transferred to new plates daily, so that the original population could be separated from their progeny. When all strains had ceased to lay eggs, worms were transferred to new plates every two days. Worms were scored as dead with the absence of movement and the failure to respond to several light pokes with a platinum wire, as described by Gems et al. (1998). The number of surviving worms, cumulative number of dead worms, and missing worms per plate were recorded at each transfer point. Missing worms, and deaths occurring as a result of internal hatching of progeny, were recorded as censored data.
2 Materials and Methods

2.4 Preparation of *C. elegans* for metabolomic analysis

2.4.1 Harvesting *C. elegans*

Worms were washed into sterile, 15 ml screw–capped tubes from plates with M9 buffer, allowed to settle at the bottom of the tube for 1 minute, and the supernatant removed. The resulting pellets were transferred to 2 ml microcentrifuge tubes and immediately snap–frozen in liquid nitrogen to quench metabolism. Samples were then stored at -80°C until use.

Where older adult worms were required (except where the use of FUdR prevented the production of progeny) their offspring were removed by repeated filtering using 50 µm Nitex membranes (Sefar Ltd., Bury, UK). Worms were washed from agar plates using M9 buffer, and the resulting suspension filtered using the 50 µm membrane. The larger, older worms were retained by the membrane, and were transferred to new agar plates by gently pressing the membrane onto fresh plates. This process was then repeated to ensure all small, young worms were removed.

2.4.2 Extraction of metabolites

In Chapters 3 and 4, each 0.5 ml frozen pellet was ground using a liquid nitrogen–cooled pestle and mortar before the addition of 2 ml 100% methanol, resulting in an overall methanol concentration of approximately 80%. After further grinding, the sample was pipetted into a microcentrifuge tube. The pestle and mortar were washed with 2 ml 80% methanol, which was transferred to a second tube. All extraction solvents used were ice–cold. The
tubes were incubated on ice for 10 minutes and then centrifuged for 5 minutes at 13000g. The supernatants were then combined and vacuum–dried overnight.

In Chapters 5 and 6, worms were transferred to in 2 ml tubes containing a 0.3 ml volume of 0.1 mM diameter SiO₂ beads before being snap–frozen. For extraction, 1.2 ml 100% methanol was added to each tube containing a 0.3 ml worm pellet, and a Precellys 24–Dual bead beater (Bertin Technologies) was used to homogenise the samples, operated at 6500 rpm for 60 seconds. This process was repeated with an 80% methanol wash. Again, all extraction solvents used were ice–cold. After centrifugation for for 5 minutes at 13000 g, supernatants from the two steps were combined and transferred to new tubes and vacuum–dried overnight.

2.5 NMR spectroscopy

2.5.1 Sample preparation

650 μl NMR buffer (95% \(^7\)H₂O, 0.1 M phosphate buffer, pH 7.0, 0.97 mM trimethylsilyl propanoic acid (TSP)) was added to each dried–down sample, sonicated for 1 minute and then centrifuged for 10 minutes at 16000 g. 600 μl of the supernatant was transferred into an NMR tube.

2.5.2 Data acquisition

NMR spectra were acquired essentially as described by Beckonert et al. (2007) using a Bruker Avance DRX600 spectrometer with a 14 T magnet, equipped with a 5 mm cryogenically cooled inverse probe. However, for life
2 Materials and Methods

cycle data (Chapter 5), a Bruker Avance II 800 spectrometer, equipped with a triple–resonance cryoprobe was used.

Typically, spectra were acquired over a 12 kHz spectral width into 32K data points, using a 1D NOESY pulse sequence for improved baseline shape over a simple presaturation experiment. Eight dummy scans and from 256 to 512 scans were acquired per free induction decay (FID). An additional 3.5 s relaxation recovery delay was included per scan, giving a total recycle time of 5 s.

2.5.3 Data processing

The spectra were processed in iNMR 2 (http://www.inmr.net), using 1.5 fold zero filling and an exponential apodization factor of 0.5 to 1 Hz. Following Fourier transformation, the spectra were corrected for phase, the TSP peak referenced to 0 ppm, and a first–order polynomial fitted to the baseline, using the software proprietary algorithms. Individual spectra were corrected manually if necessary. The integrate function was used to set manual bins such that all resonances were, as far as possible, assigned to a single bin, and no bins contained only noise across all spectra.

Individual metabolites were identified and quantified using Chenomx NMR Suite 5.0 (Chenomx, Edmonton, Canada). This software aids the identification of compounds based on a database of standards, and allows the concentrations of these metabolites to be calculated, using an internal standard of known concentration (here, TSP).
2.6 Gas chromatography mass spectrometry

2.6.1 Sample preparation

20 µl quantitation standard mixture (1.5 mM 2,2,3-d3-leucine, 1.3 mM 13C-[U]-glucose) was added to each extracted sample before vacuum–drying in Agilent 2 ml autosampler vials. Samples were derivatized using silylation, to produce silyl derivatives (active hydrogens are replaced with a TMS trimethylsilyl group) which are more volatile, less stable, and more thermally stable. Derivatization was carried out as described by Lisec et al. (2006): 40 µl Methoxyamine hydrochloride (20 mg ml⁻¹ in pyridine) was added to each tube and the samples were incubated at 37°C for 2 hours with shaking. Following incubation, 70 µl N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and 10 µl injection standard (2-fluorobiphenyl) was added to each tube. This was followed by a further incubation period of 30 minutes at 37°C with shaking.

2.6.2 Data acquisition

Samples were analysed on an Agilent 5975c quadrupole mass spectrometer with a 7890 gas chromatograph, equipped with a 30 m x 0.25 mm HP–5ms column, and splitless injector. The method was based on that described by Lisec et al. (2006). The injector port was heated to 230 °C and the source to 200 °C. Carrier gas was helium (1 ml min⁻¹). 1 µl of sample was injected, using the following temperature program: isothermal at 70 °C for 5 minutes, followed by a 5 °C/min ramp to 230 °C, followed by a 10 °C/min ramp to 330 °C, and isothermal for 5 minutes. The mass spectrometer was set to scan from 50–600 Da at 5,000 Da/s.
2 Materials and Methods

2.6.3 Data processing

Metabolites were identified using the Fiehn Library (Kind et al., 2009) and AMDIS, a deconvolution programme (Stein, 1999). Validation of this identification and peak integration for quantification was then carried out using the GAVIN script for Matlab (Behrends et al., 2011).

2.7 Data analysis

2.7.1 Survival analysis

Kaplan–Meier analysis (Bland & Altman, 1998) was performed on survival data to generate survival curves using Aabel v3 (Gigawiz Ltd. Co.). This method allows for censored data, and therefore takes into account data associated with missing worms, or with death due to internal hatching of progeny.

2.7.2 Data normalisation

Processed data were normalised following the method of Dieterle et al. (2006), in which all spectra are compared to a reference spectrum, as before, using the median fold change across all variables. Normalisation was carried out using Microsoft Excel for Mac v12.2.9.

2.7.3 General statistical analyses

Linear regressions were carried out using Aabel v3 (Gigawiz Ltd. Co.). Linear regressions are OLS (ordinary least squares) regressions unless otherwise
2.7 Data analysis

stated. In some cases a Reduced Major Axis (RMA) regression has been used. An RMA regression may be of use when the direction of causality between variables (i.e. which variable is independent and which is dependent) is not known (Smith, 2009).

T-tests were carried out using Microsoft Excel for Mac v12.2.9, and correlation matrices (Spearman’s rank correlation coefficient and associated p-values) were generated in MATLAB (R2010b, The Mathwork Inc.).

2.7.4 Multivariate analysis

All multivariate analyses were carried out using SIMCA-P+ v12.0.1 (Umetrics), with figures generated using Aabel v3 (Gigawiz Ltd. Co.).

Unsupervised analysis

The unsupervised multivariate analysis used was Principal Component Analysis (PCA), as described by Wold et al. (1987). PCA reduces the dimensionality of a dataset by generating a set of uncorrelated variables (principal components) from a set of correlated variables. Each principal component explains less of the total variance in the data than the previous component. PCA results in a matrix of scores, summarising the original variables, and a matrix of loadings, showing the relative influence of each variable. Together, the scores and loadings for a given component show which variables are responsible for any patterns observed in the data. The number of components fitted in a model is determined by cross validation to test the significance of each principal component.
2 Materials and Methods

For each analysis, the total amount of variance explained by the model is given by $R^2$ and is quoted either for individual components or as a cumulative value, taking into account all fitted principal components. The cumulative cross validated goodness of fit statistic across all components, $Q^2$ (cumulative), is also given for each model, and provides an estimate of how well the model would be able to describe additional data. This makes it possible to judge whether the model is specific to the current data, or could adequately describe new samples, i.e., whether the model is over–fitted.

Supervised analysis

The supervised analysis methods used were Partial Least–Squares Regression (PLS) and PLS–Discriminant Analysis (PLS–DA) as well as Orthogonal–PLS (OPLS) and Orthogonal PLS–Discriminant Analysis (PLS–DA).

PLS regression, described by Wold et al. (2001), is used to find the relationship between two matrices, $X$ and $Y$, where the $X$ matrix contains predictive variables and the $Y$ matrix contains dependent variables. PLS modelling aims to maximise the correlation between these matrices by projecting the predictor variables and the observable variables to a new space and generating latent variables that are maximally associated with the $Y$ variable. PLS regression is well suited to datasets where the $X$ matrix contains more variables than observations. PLS–DA is used when the $Y$ variable groups the data into one of two groups (i.e. is binary). OPLS (Trygg, 2002) is a modification of PLS, removing variation in the $X$ matrix that is orthogonal to $Y$, and therefore unrelated to $Y$. This results
2.7 Data analysis

in a predictive component (variation that is linearly related to Y) and an orthogonal component (orthogonal to Y).

Model statistics are given for each analysis. The total amount of variance explained by the model is given by $R^2$(cumulative), with $R^2_X$ and $R^2_Y$ representing the X and Y matrices respectively. For PLS models, $Q^2$ estimates the ability of the model to predict an unknown Y, given the corresponding X variables. As with PCA, a large $Q^2$ value indicates the model is not over–fitted.

Cross validation determines the number of significant PLS components fitted in a model. A subset of observations are removed from the model, so that Y values for this subset are predicted by the model. These predicted values are compared with the true values. This is repeated so that each observation has been included in the removed subset once.

![Figure 2.1: Validation of an OPLS regression model, from section 5.2.](image)

All PLS models were validated using permutation analysis (Lindgren et al., 1996). Permutation analysis randomises the pairings of the X and Y matrices, generating a model in which there is no true relationship between X and Y. For each permutation of the Y vector, a model is generated with the same number of components as the model to be validated. $Q^2$ statistics are generated for each of these models, resulting in a distribution of $Q^2$. 

53
values, for models where X and Y are unrelated. The cumulative $Q^2$ value associated with the model to be validated should be clearly distinct from the randomised distribution of $Q^2$ values. Figure 2.1 shows the result of this analysis for the OPLS model generated in section 5.2. In this case, and in all other PLS or OPLS models presented, the actual $Q^2$ value is clearly distinct from and greater than the overall randomised $Q^2$ distribution.
3 Method Development and Validation

3.1 Introduction

While some literature on the subject now exists (Blaise et al., 2007; Atherston et al., 2008; Swire et al., 2009), the use of metabolomics to study *C. elegans* is still relatively untested and at the outset of this analysis, no metabolomic analysis of *C. elegans* had been published. To demonstrate that a metabolomic approach may be used to analyse *C. elegans* longevity mutants, \(^1\)H NMR spectroscopy was used to obtain metabolic profiles for wild type and *daf-2*(m41) worms. The m41 mutation is a hypomorph mutation that disrupts *daf-2*, which encodes a tyrosine kinase expressed throughout the worm that acts as a receptor for many of the 37 insulin–like ligands present in the *C. elegans* genome (Li et al., 2003; Pierce et al., 2001). Its mutation results in the nuclear localisation of DAF-16, which encodes a FOXO transcription factor, leading to changes in expression of many longevity–linked genes (Lee et al., 2003b; Libina et al., 2003; Lin et al., 2001).

The mutation of one gene that masks the phenotypic effects of a mutation of another gene is referred to as an epistatic interaction. The long–lived
phenotype of *C. elegans* IIS mutants (such as *daf-2* mutants) is repressed by a null mutation in *daf-16*. It may be the case that *daf-2* mutants show phenotypes that are unrelated to their increased longevity. Phenotypes at the metabolite level that are linked with an increase in life span should not be seen when DAF-16 activity is inhibited. This idea has previously been applied to both transcriptomic and proteomic data (Murphy *et al.*, 2003; Dong *et al.*, 2007). In order to establish whether the observed metabolic changes are also DAF-16 repressible, the metabolic profiles of a different hypomorph *daf-2* mutant (*daf-2(e1370)) a *daf-16* mutant (*daf-16(m26)), the associated double mutant (*daf-2(e1370);daf-16(m26)) and a wild type control (N2) were compared using both $^1$H NMR spectroscopy and Gas Chromatography–Mass Spectrometry (GC–MS).

### 3.2 Results

#### 3.2.1 Metabolomic analysis of a long-lived mutant

After confirming the increased longevity of *daf-2(m41)* worms relative to wild type worms via a longevity assay of a population of 100 worms of each strain (Figure 3.1 A), NMR spectroscopy was used to obtain the metabolic profiles of 10–day–old wild type and *daf-2(m41)* worms grown at 15°C until the L4 stage, when the incubation temperature was increased to 22.5°C.

To ensure that only old worms were sampled at this point, their offspring were removed regularly by repeated filtering using 50 µm Nitex membranes as described in Chapter 2. At ten days post hatching, worms were washed from plates with M9 buffer and quick–frozen in liquid nitrogen. Samples were stored at -80°C before extraction with 80% methanol and analysis by
3.2 Results

Figure 3.1: Survival analysis of: (A) daf-2(m41) and wild type worms (N2) and (B) mutants used in epistasis experiment: daf-2(e1370), daf-16(m26), double mutant daf-2(e1370); daf-16(m26) and wild type (N2).

NMR spectroscopy. Spectra were manually binned as described in Chapter 2 and the resulting data normalised using median fold change normalisation. As the aim of this study was only to determine whether a clear difference between strains could be seen, at this point, identification of metabolites was not important.

Principal Component Analysis (PCA) of log transformed data resulted in a three component model, where the first three Principal Components (PCs) explain 50, 11 and 7% of the variance respectively. The two strains are clearly separable on PC1 and hierarchical cluster analysis (HCA) confirms the presence of two groups: one containing mostly wild type samples and the other containing mostly mutant worms (Figure 3.2 A). The relative concentrations and loadings for bins with the most substantial loadings (>0.1 and < -0.1, 34 bins of a total of 179) on PC1 are shown in Figure 3.2 B. The positions of these bins are also shown.

The relative concentrations of 26 metabolites were determined in wild type and daf-2(m41) worms as described in Chapter 2. Of these, 15 differed significantly between strains (two-tailed t-test, p < 0.05, Figure 3.3). The daf-2(m41) mutants had elevated levels of alanine, succinate, choline, tre-
Figure 3.2: A. $^1$H NMR spectroscopy followed by principal component analysis demonstrates that daf-2(m41) and wild type worms have distinct metabolic profiles and are clearly separable along PC1 (means and 95% CI are given by ellipses). In the three component model, the first three PCs explain 50, 11 and 7% of the variance respectively and the two strains can be clearly separated on PC1. Hierarchical cluster analysis separates the samples into two groups, one containing mostly wild type worm samples and the other containing mostly mutant worm samples. B. The spectrum shows the median of five N2 samples, with intensities $>5.0$ ppm scaled by a factor of 10. The heat map shows the relative concentration of 34 bins out of a total of 179 with a substantial ($>0.1$ and $<-0.1$) loading on PC 1, their position on the spectrum (above) and their loadings (below).
3.2 Results

Figure 3.3: Mean levels of individual metabolites in wild type and *daf-2* worms. The concentrations of all metabolites shown differed significantly (*p* < 0.5) between strains. Error bars represent the standard error of the mean for each metabolite.

halose, lactate, nicotinate, phenylalanine and tryptophan, and decreased levels of glutamate, formate, phosphocholine, malate, NAD+, aspartate and betaine relative to wild type worms. However, a Bonferroni correction to correct for multiple comparisons gave an adjusted significance level of 0.002.
Using this corrected value, aspartate, lactate, glutamate, betaine, succinate, nicotinate, alanine, and NAD+ were still found to differ significantly between the strains.

3.2.2 Metabolomic analysis of an epistatic interaction

Longevity analysis of all strains gave the expected results, with daf-2 worms having a greater mean life span than all other strains, and both daf-16 and daf-2;daf-16 mutants showing approximately wild type longevity (Figure 3.1 B). These results confirmed that the daf-2–induced lifespan extension was fully DAF-16 repressible under the conditions of this laboratory. For metabolomic analysis, synchronised populations were grown at 15°C on NGM plates until the L4 stage, when the incubation temperature was increased to 22.5°C. At six days post hatching, all strains were washed from plates with M9 buffer and quick–frozen in liquid nitrogen, following filtration using 50 µm Nitex membranes to ensure that only old worms were sampled (as described in Chapter 2). Samples were stored at -80°C before extraction with 80% methanol. From each sample, 20% of the total extraction volume was stored for analysis with GC–MS and the remainder used for analysis by NMR spectroscopy. GC–MS is more sensitive than NMR spectroscopy and therefore requires less biomass (in this case fewer worms) per sample. This further analysis of the samples also gives a greater coverage of the metabolome than NMR alone.
3.2 Results

Figure 3.4: The metabolomes of *daf-2* worms, *daf-16* worms, double mutant (*daf-2;daf-16*) worms and wild type worms were compared using $^1$H NMR spectroscopy. PCA and cluster analysis divide the samples into two main groups. One cluster contains the *daf-2* worms, and another cluster contains the other three strains. The first 3 principal components explain 31, 21 and 13% of the variance respectively.
3 Method Development and Validation

Analysis by $^1$H NMR spectroscopy

All spectra were manually binned and then normalised using median fold change normalisation. Principal component analysis (a three component model where the first three principal components explain 31, 21 and 13% of the total variance respectively) and HCA of log transformed data divide the samples into two main groups; one cluster contains the *daf-2* worms, and another cluster contains the other three strains (Figure 3.4). While this demonstrates that the metabolic phenotype of *daf-2* worms at least partially depends on DAF-16, there is clearly some structure within the cluster containing wild type, *daf-16* and double mutant worms. Double mutants form a sub-cluster clearly distinct from wild type worms, implying that not all of the *daf-2* phenotype is DAF-16 dependent.

For each sample, forty metabolites were identified and quantified. Analysis of the pattern of change of 11 metabolites found to vary in *daf-2*(e1370) mutants compared to wild type showed that only four – isoleucine, valine, leucine and phosphocholine – follow the classic pattern of DAF-16 dependence. That is, the relative concentrations of each of these metabolites were significantly elevated or reduced ($p < 0.05$) in *daf-2*(e1370) mutants but not in *daf-16* mutants or double mutant worms (Figure 3.5). Other metabolites showed more complex patterns of epistasis. For example, relative concentrations of lysine, lactate and glycerol were all significantly different in both *daf-16* worms and double mutants compared with wild type worms ($p < 0.05$; two–tailed t–test). Additionally, while *daf-16* mutants have wild type levels of trehalose, the relative concentration of the sugar in double mutants was no different to that observed in *daf-2* mutant worms.

$^1$Experimental data obtained jointly with Jonathan Viney, Imperial College.
Figure 3.5: Analysis of the levels of 11 metabolites found to vary in daf-2(e1370) mutants compared to wild type showed that only four – isoleucine, valine, leucine and phosphocholine – follow the classic pattern of DAF-16 dependence. That is, the relative concentrations of each of these metabolites were significantly elevated or reduced (p < 0.05) in daf-2(e1370) mutants but not in daf-16 mutants or double mutant worms. Other metabolites showed more complex patterns of epistasis. Error bars indicate one standard deviation from the mean.
3 Method Development and Validation

Analysis by Gas Chromatography Mass Spectrometry

Figure 3.6: Partial least squares discriminant analysis (three component model, $R^2_X$ (cumulative) = 0.433, $R^2_Y$ (cumulative) = 0.719, $Q^2$ (cumulative) = 0.517) divides the samples into the two groups seen in the NMR dataset. One large cluster contains daf-2(e1370) worms, and another cluster contains the other three strains. These two clusters are separated on the second component.

Metabolites were identified based on their retention time and mass spectrum and then quantified (as described in Chapter 2), giving a total of 148 metabolites that were used for subsequent data analysis. These data were normalised using median fold change normalisation and scaled to unit variance. While PCA using NMR data showed clear separation between groups, the larger number of variables identified by GC–MS and possible higher variability resulted in poor discrimination between groups by PCA for GC–MS data. However, a supervised analysis, Partial Least Squares Discriminant Analysis (PLS–DA), of log transformed data, gives good separation between groups (Figure 3.6). PLS–DA (a three component model, $R^2_X$ (cumulative) = 0.433, $R^2_Y$ (cumulative) = 0.719, $Q^2$ (cumulative) = 0.517), where each strain is given a different class, divides the samples into the two
3.2 Results

Figure 3.7: Loadings corresponding to the PLS–DA scores in Figure 3.6. Only the first two components are shown.
Figure 3.8: Again, leucine and valine show DAF-16 dependence, as does dopamine, which was undetected by NMR spectroscopy. As before, trehalose appears to be DAF-16 independent, and succinate shows a more complex pattern of epistasis consistent with the pattern seen in the NMR data. In the notched box and whisker plots, whiskers are extended to the extreme data points, and the box spans the distance between the two quartiles surrounding the median. The width of a notch is proportional to the interquartile range of the sample, and inversely proportional to the square root of the number of samples. Where there is no overlap between the notches of two boxed, the medians of the two groups are significantly different.
groups seen in the NMR dataset. One large cluster contains \textit{daf-2(e1370)} worms, and another cluster contains the other three strains. These two clusters are separated on the second component. As with the NMR dataset, some structure can be seen in the wild type, \textit{daf-16}, double mutant cluster. Figure 3.7 shows the loadings for the first two components.

A two–way ANOVA, where the factors one and two were defined as \textit{daf-2} and \textit{daf-16} respectively, identified those metabolites whose relative concentrations were significantly different (p < 0.05) in \textit{daf-2} worms only. Of the 148 metabolites found to be present in the samples, seven showed classical DAF-16 dependence in this way. These were adenosine, citrate, dopamine, leucine, valine, phenylalanine and putrescine. A selection of these are shown in Figure 3.8 along with trehalose and succinate. As before, trehalose appears to be DAF-16 independent, and succinate shows a more complex pattern of epistasis consistent with the pattern seen in the NMR data. Leucine and valine once again show DAF-16 dependence (although isoleucine does not) and dopamine, putrescine, adenosine, citrate and phenylalanine have been identified as additional potential metabolites that may play a role in the ageing process.

3.3 Discussion

The comparison of the metabolic profile of wild type worms with that of long–lived mutant \textit{daf-2(m41)} was a proof–of–principle experiment that has demonstrated that untargeted metabolic profiling using NMR spectroscopy was sensitive enough to detect differences between these strains. This also provided confirmation that the metabolome of this long–lived strain differed
from that of wild type worms. A wide range of metabolites could be detected in the tissue extracts, including amino acids, aromatic compounds and sugars. Analysis of an epistasis interaction showed that metabolomics is a good tool for identifying metabolic changes associated with long life. These experiments have also shown that both NMR spectroscopy and GC–MS are suitable platforms for *C. elegans* metabolomics studies, with GC–MS supporting many of the findings made using NMR as well as allowing the identification of additional metabolites.

The initial analysis, using *daf-2(m41)* mutants, identified several metabolites that change in this long lived strain relative to wild type. For example, in *daf-2(m41)* worms, trehalose levels were found to be elevated relative to wild type. This disaccharide is important in nematodes as a carbohydrate storage molecule, and is thought to confer stress resistance in many invertebrates, protecting lipids and proteins from desiccation or freezing damage (Behm, 1997; Pellerone *et al.*, 2003). It has been suggested that trehalose is a ‘longevity assurance sugar’ and the genes involved in its synthesis are known to be up–regulated in IIS mutants (Lamitina & Strange, 2005; McElwee *et al.*, 2006). Other metabolites with altered levels in *daf-2(m41)* such as malate and succinate, which are associated with carbohydrate metabolism, and choline, which is involved in lipid metabolism, may be further signatures of longevity.

Unsupervised multivariate analysis of the epistasis NMR data divides the samples into two main groups; one containing the *daf-2* mutants and the other containing all other strains (*daf-16* worms, double mutants and wild type worms). There is, however, some structure within the wild type, *daf-16*, double mutant cluster, with double mutants clearly separable from wild
type worms. While the long–lived phenotype of daf–2 mutants is DAF-16 repressible, other daf–2 related phenotypes at the metabolic level clearly are not. Those metabolites found to show classical DAF-16 dependence may therefore have a causal role in making worms long–lived. Equally, those found not to be DAF-16 dependent may not contribute to long life. Trehalose has been found in increased concentrations in age–1 mutants, daf–2 mutants and dauers (Lamitina & Strange, 2005; McElwee et al., 2006), as well as the two long–lived mutants studied here. However, these results suggest that trehalose levels are not DAF-16 dependent, and this is in contrast to previous studies. Two of the genes upregulated by DAF-16 are tps–1 and tps–2 (McElwee et al., 2003; Murphy et al., 2003). These encode trehalose-6-phosphate synthase, which is responsible for the biosynthesis of trehalose. However, differences in gene expression may not always reflect functional changes, which may explain this contradiction. Trehalose treatment has been found to increase longevity and slow ageing in wild type C. elegans and in daf–2; daf–16 double mutants but not in daf–2 mutants, suggesting that treatment with trehalose and daf–2 mutation extend lifespan via a common mechanism (Honda et al., 2010). The lifespan extension of a daf–16 null mutant, however, suggests that an increase in longevity resulting from trehalose treatment does not require DAF-16, which is consistent with the metabolomic data. While often thought to be an important metabolite for longevity, the DAF-16 independence seen in trehalose levels (in both NMR and GC–MS data) suggests that daf–2 may regulate this metabolite via a transcription factor that acts parallel to DAF-16.

NMR analysis suggests the branched–chain amino acids, isoleucine, leucine and valine are all DAF-16 repressible, with significantly increased levels in daf–2(e1370) mutants compared with all other strains (p<0.5). Martin et al.
(2011) also found high levels of BCAAs in *daf-2(e1370)* worms, along with altered transcript levels of genes involved in BCAA metabolism compared with wild type. Again, these changes were found to be DAF-16 repressible. This result is consistent with the findings that not only do branched–chain amino acids (BCAAs) extend the chronological lifespan of yeast (Alvers et al., 2009), but that mice given a diet supplemented with BCAAs have an increased lifespan relative to control mice (D’antona et al., 2010). Here the authors show that mice fed the BCAA supplement long term had increased mitochondrial biogenesis in cardiac and skeletal muscles, increased SIRT1 expression, and decreased oxidative damage to an increase in expression of ROS defence genes in muscle. BCAA supplements have also been shown to improve age related disorders in rats (Pansarasa et al., 2008) and in humans (Solerte et al., 2008a,b), improving age–related disorders such as sarcopenia (a loss of muscle mass, function and strength with age), insulin resistance and type 2 diabetes. D’antona et al. (2010) also showed that the increase in mitochondrial biogenesis and increased expression of ROS defence genes due to BCAA supplementation was induced by increased expression of endothelial nitric oxide synthase (eNOS), as is the case with increased mitochondrial biogenesis and increased ROS defence due to caloric restriction. Isoleucine, leucine and valine were also all slightly elevated relative to wild type in 10–day old *daf-2(m41)* worms (an increase of 9, 4 and 19% respectively), but this difference was not significant at the 95% significance level (p = 0.35, 0.57 and 0.08 for isoleucine, leucine and valine respectively). It may be the case that the degree to which these metabolite levels change with mutation of *daf-2* may depend on the allele studied, or possibly sampling age.

Another metabolite that appears to be DAF-16 repressible and is therefore potentially associated with longevity is phosphocholine, which is found
in significantly reduced concentration in *daf-2(e1370)* worms compared with the other epistasis strains as well as being found at significantly lower concentration in *daf-2(m41)* worms than wild type worms. Phosphocholine is a precursor of phosphatidylcholine, a phospholipid present in cell membrane bilayers and therefore it is possible that reduced phosphocholine is a result of reduced cell turnover. Phosphocholine is found in high concentration in dauer larvae (Swire *et al.*, 2009), but dauers do not feed and therefore rely on internal energy resources for survival and as a breakdown product of phosphatidylcholine, phosphocholine levels may increase if lipid metabolism is increased. Unlike dauers, *daf-2* worms are able to feed, and therefore do not need to metabolise energy stores, so that phosphocholine levels remain low. Both the BCAAs and phosphocholine have been shown to follow a similar pattern (increases in BCAAs and a decrease in phosphocholine) in other long–lived worms (Fuchs *et al.*, 2010).

Many results from GC–MS analysis are consistent with NMR analysis. For example, once again, the branched–chain amino acids leucine and valine were identified as DAF-16 repressible. One metabolite that could not be identified using NMR but was seen in GC–MS analysis and found to be DAF-16 repressible is dopamine. Dopamine mediates the ‘basal slowing response’ in *C. elegans*. This is the slowing of movement on encountering food, and worms that are unable to produce dopamine fail to slow in response to food (Sawin *et al.*, 2000). The results are consistent with those of Gaglia & Kenyon (2009), who found that the dopamine system is altered in *daf-2* worms and that the changes seen in response to dopamine in the environment are linked with DAF-16 activity in neurons.
Putrescine, phenylalanine, adenosine and citrate were all found at lower concentrations in \textit{daf-2} worms than in the other epistasis strains. Changes in pools of phenylalanine are consistent with the fact that many genes encoding components of the catabolic pathways of phenylalanine are regulated in \textit{daf-2} worms (Dong \textit{et al.}, 2007; Fisher \textit{et al.}, 2008). Putrescine is a product of amino acid breakdown, suggesting that this process may be inhibited in some way in \textit{daf-2} worms.

Some metabolites showed more complex patterns of epistasis. Relative concentrations of lysine, lactate and glycerol were all significantly different in both \textit{daf-16} worms and double mutants compared with wild type worms (p <0.05; two–tailed t–test). The complex patterns of epistasis seen in these metabolites are more difficult to interpret. \textit{Daf-16(0)} null mutants show several subtle phenotypes such as rapid growth, early reproduction and slightly reduced longevity compared to wild type worms. In addition to this, at least some DAF-16 can be detected in the nuclei of wild type worms (Cypser & Johnson, 2003; Weinkove \textit{et al.}, 2006). This suggests that the model of DAF-16 being fully repressed in wild type worms and then activated in \textit{daf-2} mutants is too simple and it appears that some metabolite levels may be sensitive to even very low levels of DAF-16 activity.

\textbf{Conclusion}

It is possible to distinguish between long–lived \textit{daf-2} mutants and wild type \textit{C. elegans} based on metabolic profile. Metabolomics has therefore been shown to provide a new and useful phenotyping tool for studying \textit{C. elegans} ageing and longevity. Both NMR spectroscopy and Gas Chromatography–Mass Spectrometry are good platforms for this analysis, with the more
sensitive GC–MS supporting many of the findings made with NMR as well as allowing the identification of additional metabolites. The analysis of an epistatic interaction using metabolomics is a useful way to help focus future research. While the long–lived phenotype of daf-2 mutants is DAF-16 repressible, it is clear that other daf-2 related phenotypes are not; those metabolites found to show classical DAF-16 dependence may have a causal role in making worms long–lived. Equally, those found not to be DAF-16 dependent may not contribute to long life.
4 The Effect of 5-fluoro-2-deoxyuridine on the *C. elegans* Metabolome

4.1 Introduction

A common problem encountered when analysing ageing in *C. elegans* is that of separating adult worms from their progeny. Under optimal conditions, worms begin to reproduce at around 3 days post–hatching and egg laying continues over a several day period. This means that to obtain a synchronous population of ageing adults, new progeny should be separated from parents before the point at which they can no longer be distinguished from older worms, ensuring that multiple generations are not mixed. If only very small numbers of ageing worms are required, it is possible to regularly transfer individual worms to new plates during the egg–laying period. However, this is not feasible if large numbers of worms are required. For this reason, it is helpful if the worms to be analysed are sterile, so that no progeny are produced and the population remains synchronous for the entire lifespan of the worms. There are two common methods of creating sterile worms.
– the use of fertility mutations (e.g. using fer– mutants) or the use of the chemical 5-fluoro-2-deoxyuridine (FUdR).

**Fertility mutants**

Fer– mutants produce the normal number of eggs and sperm, but are defective in some sperm–specific function required for normal fertilisation – in males, sterility may be due to either defective sperm, or an inability to transfer sperm during copulation – so that fertilised eggs are very rare (Argon & Ward, 1980). Fer– mutants grow and develop like wild–type worms, and egg–laying occurs at the normal time. Fertilisation–defective mutants have been used previously in microarray analysis of ageing in *C. elegans*. Murphy et al. (2003) used RNA interference (RNAi) to reduce *daf-2* and *daf-16* activity, followed by microarray analysis to identify genes with altered expression in these strains. However, instead of using wild type worms, a sterile strain (*fer-15(bn26);fem-1(hc17]*) was subjected to RNAi, so that a population of developmentally synchronous worms was easily obtained. Age–related changes in gene expression were generally no different in *daf-2(RNAi)* and *daf-16(RNAi);daf-2(RNAi)* animals, which is surprising, as the *daf-16* mutation suppresses the longevity–enhancing effects of the *daf-2* mutation. While this unexpected result might be due to an effect of the RNAi itself, the consequences of using sterile worms should also be considered. Similarly, Halaschek-Wiener (2005) used serial analysis of gene expression (SAGE) to analyse the transcriptome of the long–lived mutant *daf-2(m41)*. However, the *daf-2* mutant also carried a temperature–sensitive *fer-15* mutation to prevent the production of offspring, and a *fer-15* mutant was used as a control. While the life span of *fer-15* worms was found to be not significantly
4.1 Introduction

different from N2 (wild type) worms (fer-15 mutants had a maximum life span of 22 days compared with an N2 life span of 20 days), it is possible that the fer-15 mutation may have concealed transcriptional differences between the long-lived daf-2 mutant and wild type worms.

5-fluoro-2-deoxyuridine

Growing C. elegans on 5-fluoro-2-deoxyuridine inhibits DNA synthesis (Mitchell et al., 1979). As the only mitotic activity in adult C. elegans is in the germ line, this, in theory, only affects egg production, killing larvae but not adults. Eliminating egg production in this way has been found not to significantly affect the life span of worms grown under standard conditions (Apfeld & Kenyon, 1998; Hosono et al., 1982), although a slight increase (7%) in lifespan has been reported (Mitchell et al., 1979). It is thought that changes in the ageing process remain qualitatively unchanged (Yasuda et al., 2006; Mitchell et al., 1979).

Like fertilisation mutants, FUdR is often used where populations of synchronous, ageing adults are required (Shaw et al., 2007; Houthoofd et al., 2002; Lee et al., 2003a) and in longevity assays, so that daily transfer of worms to new plates during the reproductive period is not required. While FUdR is regularly used in studies on ageing, FUdR–treated animals survive longer in the presence of a pathogen such as Pseudomonas aeruginosa than do untreated control worms. Miyata et al. (2008) suggest that this is a result of wild type worms prioritising reproduction over immunity (even in the presence of a lethal pathogen) and that embryonic development decreases immunity in adult worms. The energy required for the expression of antimicrobials used in defence may instead be redirected for use in embryonic
4 5-fluoro-2-deoxyuridine

development. When no energy is required for the development of embryos, i.e., when worms are grown on FUdR, energy can be allocated to immunity and therefore worms are able to survive longer. It may also be the case that FUdR prevents the proliferation of the pathogen.

The use of FUdR in the culturing of *C. elegans* is relatively common, and the downstream effects of DNA synthesis inhibition are assumed to be negligible or, at least, have not been properly examined, despite the fact that signals from the reproductive system are believed to affect the lifespan of worms (Hsin & Kenyon, 1999; Lin *et al.*, 2001). The importance of not simply dismissing the possible effects of FUdR has recently been highlighted: the *tub-1* mutation is involved with fat storage modulation and has previously been shown to be long–lived (Mukhopadhyay *et al.*, 2005). However, Aitlhadj & Stürzenbaum (2010) have demonstrated that lifespan extension in *tub-1* worms is seen only when worms are grown in the presence of FUdR, and that the lifespan–extending properties of the mutation are lost when worms are grown on standard NGM plates in the absence of FUdR. This suggests that there may be an interaction between some mutations and the inhibition of DNA synthesis, and draws attention to the need for careful controls to avoid misinterpretation when analysing worms cultured in the presence of FUdR.

The closely related 5-fluorouracil, an anticancer drug, causes defects in both development and reproduction in *C. elegans*, as well as resulting in a smaller body size when worms are hatched onto media containing the chemical (Kumar *et al.*, 2010). High concentrations cause worms to arrest at larval stage. Using as low a concentration of FUdR for effective inhibition of DNA synthesis as possible should help minimise any unwanted effects.
4.2 Results

(Gruber et al., 2009). The timing of transfer of worms to FUdR-containing media is extremely important; transferring worms before growth has ceased can cause abnormalities in body size and morphology (Mitchell et al., 1979; Gandhi et al., 1980). In addition to this, in adults, FUdR has been shown to result in slight changes in the rate of pharyngeal pumping and appears to increase SOD levels (Mitchell et al., 1979; Gandhi et al., 1980; Vanfleteren & Vreese, 1995; Gruber et al., 2009).

Nonetheless, FUdR is extremely useful in metabolomic analysis of synchronous ageing worms where large populations are required for adequate biomass. In addition to this, as FUdR effectively stops reproduction, it may be a good choice as a manipulator for examining life history trade-offs involving reproduction – the possible trade-off between reproduction and longevity, for example. Comparing the metabolic profile of worms grown in the presence of FUdR with those of worms grown without FUdR should help to establish how great an effect the chemical has on the metabolism of C. elegans, and reveal potential interactions with mutations. This should make it possible to adequately control for any effects FUdR may have on a given experiment. Here, I have used proton NMR spectroscopy to examine these effects by comparing wild type worms with long-lived mutant daf-2(m41) grown on both standard NGM agar and on agar containing FUdR.
Figure 4.1: Kaplan–Meier survival analysis for all four treatments: wild type N2 worms grown on standard NGM agar for the entire experiment; wild type worms transferred to plates containing FUdR at adulthood; daf-2(m41) mutant worms grown on standard NGM agar for the entire experiment; daf-2 worms transferred to plates containing FUdR at adulthood. A: survival curves; B: mean and median age at death for all treatments; C: Chi–square test statistics. These results suggest that FUdR does not significantly effect the lifespan of either strain and, irrespective of whether worms have been grown in the presence of FUdR, there is a significant difference in the lifespan of the two strains.
4.2 Results

FUdR does not significantly affect the lifespan of wild type or daf-2(m41) worms

Longevity assays (as described in Chapter 2) were carried out for all four treatments (wild type N2 worms grown on standard NGM agar; wild type worms grown on NGM agar with the addition of FUdR; daf-2(m41) mutant worms grown on standard NGM agar; daf-2 worms grown on NGM agar with the addition of FUdR) at 20°C. Synchronous populations of wild type worms and daf-2(m41) worms were grown at 20°C on standard NGM agar seeded with E. coli OP50. When both strains had reached young adulthood (defined here as the point at which eggs were observed but no hatched progeny were seen, approximately 72 hours post–bleaching), worms were transferred to new agar plates. For each strain, half of the worms were transferred to control plates (standard NGM agar) and half to agar plates containing 100 mg/l FUdR, both seeded with E. coli OP50. As FUdR prevents all DNA synthesis, E. coli is not able to proliferate on agar containing FUdR. For this reason, an overnight E. coli culture was concentrated by a factor of 50 before aliquots were spread on agar containing FUdR, as described by Mitchell et al. (1979). These seeded plates were then incubated overnight at 37°C and this resulted in a lawn of E. coli. E. coli culture for the control, NGM–only plates was prepared in the standard way, as described in chapter two. This was intended to ensure that the two types of plate contained comparable amounts of E. coli. All worms, regardless of whether they were grown on agar containing FUdR, were transferred to new plates every day during the period in which worms on standard NGM continued to lay eggs.
After this period, worms were transferred to new plates approximately every three days.

Figure 4.1 shows the results of a Kaplan–Meier survival analysis on the data. A, B and C show the resulting survival curves, mean and median age at death for each treatment and chi–square test results respectively. FUdR did not significantly affect the lifespan of either strain \((p = 0.19\) for wild type worms transferred to FUdR plates at adulthood compared with wild type worms transferred to control plates, and \(p = 0.11\) for \(daf-2(m41)\) worms transferred to FUdR plates at adulthood compared with \(daf-2(m41)\) worms transferred to control plates) and irrespective of whether worms were grown in the presence of FUdR, there was a significant difference between the lifespan of the two strains \((p = 0.01\) on NGM and \(p < 0.001\) on FUdR).

**FUdR has a greater effect on the *C. elegans* metabolome than does genotype**

Despite no significant differences in longevity for each strain if transferred to FUdR–containing plates at adulthood, FUdR may still have an effect on the metabolome. To investigate this, a large number of synchronised worms were grown under the conditions described above. After transferring worms to either plates containing \(100\ mgL^{-1}\) FUdR or control plates, worms were incubated at \(20^\circ\)C for 24 hours. After this time, all worms were washed from plates using M9 buffer and filtered through nylon mesh to remove eggs and progeny that were present on those plates that did not contain FUdR. After washing with M9 buffer to remove any *E. coli* from the worms, the samples were snap–frozen with liquid nitrogen, and stored at \(-80^\circ\)C before extraction with 80% methanol and analysis by NMR spectroscopy. Samples
4.2 Results

were randomised for harvesting, sample preparation, and analysis by NMR spectroscopy in order to eliminate batch effects. Individual metabolites were identified and quantified for each sample, and the resulting data normalised. The mean relative concentrations of each metabolite identified are given in Figure 4.2.

All data were mean-centred and log transformed before principal component analysis (PCA). In the two-component model, PC1 and PC2 explain 41% and 11.2% of the variance respectively ($R^2$ (cumulative) = 0.522, $Q^2$ (cumulative) = 0.331). Principal component analysis of all data (Figure 4.3) suggests that the presence or absence of FUdR has a greater effect on the metabolome than does the *daf-2(m41)* mutation, with clear separation between worms grown on standard NGM and those grown on NGM containing FUdR along PC1, which explains 41% of the total variance in the data. Though some separation between *daf-2* and wild type worms can be seen along PC2, this principal component explains only 11.2% of the variance.

The addition of FUdR is associated with changes in the levels of specific metabolites in both genotypes

Figure 4.4 shows the loadings associated with each principal component for the model in Figure 4.3. The metabolome of worms transferred to FUdR plates at adulthood is associated with increased levels of lysine, putrescine, GPC, acetate, o-phosphocholine and tyrosine compared with the metabolome of worms transferred to control plates. In contrast, the metabolome of worms allowed to reproduce normally is associated with increased levels of cystathionine, trehalose, glutamate and NAD, and slightly
Figure 4.2: Mean relative concentrations of all metabolites quantified, with magnification of lower concentration metabolites. Error bars indicate the standard error.
4.2 Results

Figure 4.3: Principal Component Analysis of mean-centred, log transformed data. In the two component model, PC1 explains 41% of the total variance and PC2 explains 11.2% ($Q^2$ (cumulative) = 0.331). This analysis suggests that whether worms are grown on agar containing FUdR has a greater effect on metabolome than does genotype, with clear separation between treatments along PC1. There is also some separation between strains along PC2. This can be seen most clearly in B, where each data point is connected to its respective group mean.
Figure 4.4: Principal Component Analysis of mean–centred, log transformed data. Corresponding to the scores shown in Figure 4.3, A and B show the loadings for principal components 1 and 2 respectively.
increased levels of isoleucine and valine relative to the metabolome of worms transferred to FUdR plates.

*Daf-2(m41)* and wild type worms are metabolically distinct, regardless of whether they are allowed to reproduce

To determine whether metabolic differences could be seen clearly on either medium, data were grouped according the presence or absence of FUdR, and analysed in two groups, giving two separate models (Figure 4.5). Again, the data were mean-centred and log transformed before PCA. A and B show worms grown on NGM only ($R^2$X (cumulative) = 0.631, $Q^2$ (cumulative) = -0.112), C and D show strains grown on FUdR only ($R^2$X (cumulative) = 0.384, $Q^2$ (cumulative) = 0.0215). In B and D, each data point is connected to its respective group mean. The strains grown on standard NGM (A and B) are clearly separable along the NGM–only model’s second component, which accounts for 17.7% of the total variance in the model, while in the FUdR–only model (C and D), strains are separable along the first component, which explains 19.6% of the variance in that model.

Where worms have been transferred to FUdR, the greatest source of variance in metabotype is related to genotype (demonstrated by the fact that the strains can be separated along the model’s first component). However, where worms remain on control plates, genotype is not the greatest source of variation. In this model, the second component separates the strains while the first component does not. It appears that the two strains are easier to separate and more distinct from one another when both are transferred to FUdR at adulthood. Despite this, both models clearly separate the two strains within the first two components. *Daf-2(m41)* worms are metabolically...
Figure 4.5: Principal component analysis of mean–centred, log transformed data. A and B show worms grown on NGM only ($R^2_X$ (cumulative) = 0.631, $Q^2$ (cumulative) = -0.112), C and D show strains grown on FUdR only ($R^2_X$ (cumulative) = 0.384, $Q^2$ (cumulative) = 0.0215). In the NGM only model (a four–component model), PC1, PC2, PC3 and PC4 explain 20.7%, 17.7%, 14.1% and 10.6% of the variance respectively, though only the first two components are shown here. In the two–component FUdR only model, PC1 explains 19.6% of the variance and PC2 explains 18.8% of the variance.

A: NGM only, PC1 contributions; B: NGM only, PC2 contributions; C: FUdR only, PC1 contributions; D: FUdR only, PC2 contributions. The strains grown on standard NGM are clearly separable along PC2 in the NGM model, while strains grown on plates containing FUdR are separable along PC1 in the FUdR model. In B and D, each data point is connected to its respective group mean.
distinct from wild type worms whether they are allowed to reproduce normally or are unable to reproduce due to the effect of FUdR.

Metabolites that are most important in separating wild type and \textit{daf-2(m41)} worms vary depending on the presence or absence of FUdR

For each model whose scores are shown in Figure 4.5, the loadings making up each principal component are shown in figures 4.6 and 4.7. Figure 4.6A suggests that when worms are transferred to control plates, relatively high levels of malate, aspartate, threonine and cystathionine are associated with the \textit{daf-2(m41)} metabolome, whereas when worms are transferred to plates containing FUdR, the \textit{daf-2(m41)} metabotype is different, with relatively high levels of o-phosphcholine, glutamine, glutamate and, to a lesser extent, the branched–chain amino acids, isoleucine, leucine and valine compared with wild type worms.

The same strain-related changes in individual metabolites can be seen in worms grown on FUdR plates and those transferred to control plates

Based on principal component analysis, FUdR seems to have a much greater affect on metabolite levels than does genotype, but the \textit{daf-2(m41)} metabolome is clearly distinct from that of wild type worms regardless of the presence of FUdR. However, the loadings shown in figures 4.6 and 4.7 suggest that the most important metabolites for distinguishing between strains change based on whether worms are grown on FUdR-containing plates or control plates, but it is important to know the effects FUdR has
Figure 4.6: Data separated by treatment, loadings scatter plots. Scores shown in Figure 4.5. Principal component analysis of mean-centred, log transformed data. A shows loadings associated with an NGM-only model ($R^2_X$ (cumulative) = 0.631, $Q^2$ (cumulative) = -0.112). B shows loadings associated with an FUdR-only model ($R^2_X$ (cumulative) = 0.384, $Q^2$ (cumulative) = 0.0215).
Figure 4.7: Data separated by treatment, loadings column plots. Scores shown in Figure 4.5. Principal component analysis of mean-centred, log transformed data. A and B show worms grown on NGM only ($R^2X$ (cumulative) = 0.631, $Q^2$ (cumulative) = -0.112), C and D show strains grown on FUdR only ($R^2X$ (cumulative) = 0.384, $Q^2$ (cumulative) = 0.0215).
Figure 4.8: The log transformed fold change of daf-2(m41) relative to wild type for each metabolite versus the log transformed -p-value for each metabolite. A: worms grown on control plates; B: worms transferred to plates containing FUdR. The 0.1, 0.05 and 0.01 significance levels are shown in each plot, and labels are shown for all metabolites that are found at significantly different levels in the two strains on either FUdR or control plates. Tyrosine and alanine are the only metabolites to differ significantly between strains in both treatments at the 5% significance level, but if this is reduced to 10%, 2-Aminoadipate, threonine and glutamine can also be considered significantly different in both treatments. Tyrosine and alanine are the only metabolites to differ significantly between strains in both treatments at the 5% significance level, but if this is reduced to 10%, 2-Aminoadipate, threonine and glutamine can also be considered significantly different in both treatments.
4.2 Results

Figure 4.9: A: log transformed fold change of daf-2(m41) relative to wild type for worms transferred to FUdR–containing plates versus the log transformed fold change for worms transferred to control plates. A Reduced Major Axis (RMA) regression suggests a positive correlation between the fold change values for the two treatments; B: the negative of the log transformed p–value of daf-2(m41) for each metabolite (based on a two–tailed t–test comparing the two strains) for worms transferred to FUdR–containing plates versus the equivalent negative, log transformed p–value for worms transferred to control plates. Again, an RMA regression has been applied and suggests a positive correlation between the p–values for each treatment; C: as B but with the exclusion of tyrosine (whose low p-value may skew the data), which removes the positive correlation seen in B.
on the levels of individual metabolites. I.e., are the differences seen in individual metabolites between wild type and daf-2(m41) worms the same if worms are grown in the presence of FUdR as they are if worms are allowed to reproduce? To investigate this, a two–tailed t–test was carried out for each treatment, testing the differences between each metabolite in the two strains. In addition to this, the fold change for daf-2(m41) relative to wild type for each metabolite was calculated. Figure 4.8 shows the result of the combination of these two analyses. The 0.1, 0.05 and 0.01 significance levels are shown in each plot, so that all metabolites found at significantly different levels (p≤0.1) in the two strains are easily identifiable for both treatments.

Of all metabolites identified, tyrosine and alanine are the only metabolites to differ significantly (p≤0.05) between strains in both treatments. However, if the significance level is reduced to 10%, 2-aminoadipate, threonine and glutamine can also be said to differ significantly in both treatments. Labels are shown for all metabolites that are found at significantly different levels (p≤0.1) in the two strains on either FUdR or control plates. Of the 13 metabolites that are present at significantly different levels (p≤0.1) in the two strains on NGM, nine have the same direction of change on FUdR plates, even if this change is not significant. Of the 12 metabolites that vary significantly between strains on FUdR plates, six show the same direction of change between strains on control plates, even if the change is not significant.

Figure 4.9 B shows the p–value of daf-2(m41) for each metabolite (based on the same two–tailed t–test comparing the two strains) for worms transferred to FUdR–containing plates versus the equivalent p–value for worms transferred to control plates, with a Reduced Major Axis (RMA) regression.
The $R^2$ and $p$-values are shown for each regression. In Figure 4.9 C, tyrosine has been excluded as it has high leverage. This removes the positive correlation seen in B. This suggests that generally, those metabolites with the most significant changes between strains with one treatment will not necessarily have the most significant changes given the alternative treatment. However, Figure 4.9 A shows, for each metabolite, the fold change of $daf-2(m41)$ relative to wild type for worms transferred to FUdR-containing plates versus the equivalent fold change for worms transferred to control plates. Again, an RMA regression suggests a positive correlation between the fold change values for the two treatments. The overall trend seems to be that for each metabolite, the difference between wild type and $daf-2(m41)$ worms is the same whether worms are allowed to reproduce or transferred to plates containing FUdR.

**Some metabolites appear to be affected by an interaction between genotype and FUdR or sterility**

A two-way ANOVA, where factor A = genotype and factor B = treatment (i.e presence or absence of FUdR), was carried out (Table 4.1). Metabolites with a $p$-value of $\leq0.05$ for genotype are found at significantly different levels in the two strains. Metabolites with a $p$-value of $\leq0.05$ for treatment are found at significantly different levels in worms transferred to control plates in worms transferred to plates containing FUdR. Those metabolites with an interaction $p$-value of $\leq0.05$ may be affected by an interaction between genotype and FUdR. For these metabolites, the changes seen between strains may differ depending on whether worms are grown on standard NGM agar or on NGM agar containing FUdR. Conversely, metabolites with an
Table 4.1: A two–way ANOVA, where factor A = genotype and factor B = treatment (i.e presence or absence of FUdR), was carried out. Metabolites with a p–value of ≤0.05 for genotype are found at significantly different levels in the two strains. Metabolites with a p–value of ≤0.05 for treatment are found at significantly different levels in worms transferred to control plates in worms transferred to plates containing FUdR. Those metabolites with an interaction p–value of ≤0.05 may be affected by an interaction between genotype and FUdR. For these metabolites, the changes seen between strains may differ depending on whether worms are grown on standard NGM agar or on NGM agar containing FUdR. Conversely, metabolites with an interaction p–value of >0.05 should show the same changes between strains, regardless of whether worms are transferred to control plates or FUdR plates at adulthood.
4.2 Results

Figure 4.10: Box and whisker plots for those metabolites that have a significant interaction p-value ($p \leq 0.05$). Some metabolites – glycerol and tyrosine – with a significant interaction p-value do not seem to be affected by a genotype / treatment interaction. I.e. the changes between strains appear to occur in the same direction and to a similar degree on both control and FUdR–containing plates. However, with the exception of lactate, it can be seen that most of the metabolites predicted to be affected by an interaction between genotype and treatment do appear to behave differently depending on whether worms are transferred to FUdR or control plates.
interaction p–value of >0.05 should show the same changes between strains, regardless of whether worms are transferred to control plates or FUdR plates at adulthood.

Figure 4.10 shows box and whisker plots for those metabolites that have a significant interaction p–value (p≤0.05). Some metabolites – glycerol and tyrosine – with a significant interaction p–value do not seem to be affected by a genotype / treatment interaction, i.e. the changes between strains appear to occur in the same direction and to a similar degree on both control and FUdR–containing plates. From these plots, however, it can be seen that most of the metabolites predicted to be affected by an interaction between genotype and treatment do behave differently depending on whether worms are transferred to FUdR or control plates. Glutamate, glutamine and 2-aminoadipate are all found at lower levels in *daf-2(m41)* worms than wild type on control plates, but at higher levels in the mutant worms than wild type if they have been transferred to FUdR–containing plates. Lactate and NAD follow the opposite pattern, with higher levels in mutant worms than in wild type on control plates but lower levels in *daf-2(m41)* worms on FUdR plates. In some cases, rather than altering the direction of any difference between strains, FUdR appears to simply introduce or magnify a change. There is very little difference in GPC levels between strains on control media, but on FUdR–containing media, mutants have increased levels of the metabolite. A similar effect is seen in betaine levels, with little genotype–related difference on control plates but a decrease with the *daf-2(m41)* mutation.
Despite a low interaction test statistic, succinate seems to be affected by an interaction between genotype and FUdR.

Of the metabolites that are predicted not to be affected by a genotype / treatment interaction, only succinate appears to behave differently depending on whether or not worms are allowed to reproduce (Figure 4.2). With similar levels in the two strains when transferred to control plates, succinate levels seem to decrease in mutant worms relative to wild type in worms that have been transferred to FUdR–containing plates (Figure 4.11). However, the two–way ANOVA statistics shown in Figure 4.11C suggest that neither genotype or treatment have a significant effect on succinate levels ($p = 0.191$ and $p = 0.134$ respectively).
Figure 4.11: Despite a low interaction test statistic, succinate appears to be affected by an interaction between genotype and treatment. With similar levels in the two strains when transferred to control plates, levels seem to decrease in mutant worms relative to wild type in worms that have been transferred to FUdR–containing plates. A: Box and whisker plot showing relative concentration in all four groups, B: interaction plots, C: Two–way ANOVA statistics imply that neither genotype or treatment have a significant effect on succinate levels.
4.3 Discussion

If the reason for the use of 5-fluoro-2-deoxyuridine is purely to help generate large, synchronised populations of older worms, or to simplify longevity assays, then ideally the effect of the treatment should be negligible. At the very least, if comparing mutants, any effect of the treatment should be significantly less than that of genotype. This is not the case here, where the use of FUdR has a greater effect on the metabolic profile of *C. elegans* than does the *daf-2(m41)* mutation. This is not surprising, however, given that FUdR effectively removes the ability of the worms to reproduce, and the worms sampled were of reproductive age. The second most favourable situation then, would be that despite any effect being larger than desired, it may be easily factored out, with no interaction between the treatment and genotype. Again, this is not the case here, as for a number of metabolites, there is a clear interaction between FUdR and genotype. This novel experiment suggests that for this reason, FUdR is not ideal for use as a tool to simplify the maintenance of synchronised populations when investigating differences between certain strains, despite widespread use of the chemical for this purpose. It also demonstrates that in the study of ageing mutants, longevity assays are insufficient to show that FUdR does not have a significant effect on a given strain, i.e. while it might not change one phenotype, it is possible that it affects others.

The inhibition of reproduction with FUdR results in some changes in metabolome that are seen across strains (Figure 4.4). When reproduction is inhibited, levels of putrescine increase. Putrescine is not only a product of the breakdown of amino acids, but is also important in DNA synthesis (Lee & Maclean, 2011). Inhibiting DNA synthesis would be expected to
affect levels of this metabolite. The addition of FUdR also increases levels of phosphocholine. Phosphocholine may be associated with a reduction in cell turnover, given that it is present in cell membrane bilayers (Swire et al., 2009).

Worms that are unable to reproduce have decreased trehalose levels. High levels of trehalose have been linked with increased longevity, but changes in the levels of trehalose do not appear to be DAF-16 dependent, as described in Chapter 3. Trehalose appears to be important for egg hatching in nematodes (Behm, 1997), and therefore may be expected to be affected by the inhibition of egg production. NAD is also found in lower levels in worms cultured on FUdR than on those allowed to reproduce. Depletion of NAD is associated with delays in gonad development and defects in egg laying behaviour in C. elegans (Vrablik et al., 2009).

The inhibition of reproduction affects which metabolites are most important for distinguishing between wild type and daf-2(m41) worms (Figure 4.6). This clearly demonstrates the importance of taking into account the effects of FUdR when interpreting results. While in some cases, FUdR may magnify or introduce changes in metabolite levels between strains (for example, in GPC, succinate and betaine), in others, an interaction between FUdR and genotype may have more of an impact, with levels of metabolites changing in opposite directions between strains depending on whether worms are able to reproduce. In the mutant, glutamate, glutamine, 2-aminoadipate, lactate and NAD all change in opposite directions relative to wild type depending on the presence or absence of FUdR. This interaction should be taken into account if these metabolites are to be linked with longevity.
The removal of reproduction will clearly affect the metabolic profile of worms in a significant way. However, one factor in addition to this that may have contributed to the substantial differences seen between the two treatments is the way in which the *E. coli* food source was prepared for each type of agar plate. As previously stated, the fact that FUdR prevents all DNA synthesis means that *E. coli* is not able to proliferate on agar containing FUdR. For this reason, an overnight *E. coli* culture was concentrated by a factor of 50 before spreading on agar containing FUdR, the method described by Mitchell *et al.* (1979). These plates were then incubated overnight at 37°C and this resulted in a lawn of *E. coli*. In contrast, *E. coli* culture for the control, NGM–only plates was prepared in the standard way, with overnight bacterial culture spread onto plates (without concentration) and incubated overnight at 37°C. This meant that the two types of plate contained comparable amounts of *E. coli*. One way to eliminate this difference would be to use a concentrated culture of UV–killed bacteria for both types of plate, so that neither type of plate contained proliferating *E. coli*. However, Mitchell *et al.* (1979) reported that the response of *C. elegans* to FUdR was virtually identical on monoxenic and axenic media, implying that FUdR does not need to be ingested by the worm to take effect, and any metabolism of the FUdR by *E. coli* is unlikely to affect the way that the chemical works. Differences in the preparation of *E. coli* for the two types of agar seems unlikely to have a great effect on the metabolome of the worm, if all worms are given a sufficient amount of the food source to prevent any dietary restriction effects, and in both cases the bacteria are metabolically active. It has been shown previously that worms have a nutritional need for live, metabolically active microbes as a food source and that worms show dietary restriction–like effects – increased lifespan and decreased fecundity – when
grown on UV–killed bacteria (Lenaerts et al., 2008). For this reason, the use of UV–killed bacteria for all plates may still be far from ideal.

The problem of how to properly separate older worms from their progeny leads to another possible source of difference between the two treatments. Worms not transferred to FUdR plates require filtering immediately before harvesting to remove young worms from the sample. While worms transferred to FUdR plates do not need to be filtered, all worms were subjected to the same treatment to control for any possible effect the filtering process may have on the metabolic profile of the worms. However, filtering may not completely remove all young worms from the sample, and therefore those samples where young worms are present, i.e., for those worms transferred to control plates, more variance in metabolic profile may be expected due to the variation in metabolic profile with age (as described in Chapter 5). However, the small size and number of young worms in each sample relative to the total biomass should have a very small overall effect. In addition to the problem of young worms in samples, however, is the increased chance of bacterial or fungal contamination on control plates compared with FUdR plates. This is due to the fact that any bacterial or fungal contaminants are unable to propagate in the presence of FUdR. While great care is taken to avoid this type of contamination on any plate, the transfer of worms at adulthood using M9 buffer increases the chance of contamination. Again, this may increase the variation in metabolite levels in worms transferred to control plates compared with worms transferred to FUdR plates. However, no evidence of contamination was seen in this experiment. Together, these factors might help explain the fact that while, in a FUdR–only PCA model, genotype is the greatest source of variation, in a control plate–only model, strains are
4.3 Discussion

It is clear that using 5-fluoro-2-deoxyuridine simply for the maintenance of synchronous populations of worms may cause problems, however it is useful as a tool to investigate the effects of the suppression of reproduction. While FUdR does not significantly affect the lifespan of either of the two strains studied here, it is clear that the chemical has a considerable impact on the *C. elegans* metabolome. In this case, FUdR has more of an impact on metabolic profile than does the genotype of the worm. As FUdR suppresses reproduction, this may not seem surprising. Some reduction in fecundity of the *daf-2(m41)* strain compared with wild type worms has been reported (Gems et al., 1998), and so it might be expected that the removal of one of the probable sources of variation between the strains (with the addition of FUdR) may close the gap between the metabotypes of the strains. Based on principal component analysis (Figure 4.3), it might seem that this is the case, as the difference between the strain means seems slightly smaller for worms transferred to FUdR plates than the equivalent distance for control worms. However, a Euclidian distance of 5.4 between strains on control plates compared with 7.1 between strains transferred to FUdR plates (calculated by hierarchical clustering of group medians using standardised variables) implies that there is a greater difference between strains on FUdR plates than on control plates. It would be interesting to repeat this experiment using different *daf-2* alleles with fecundity affected to either a greater or lesser degree, as well as with other ageing mutants with or without altered fecundity. In addition to this, other methods used to produce sterile worms – such as *fer*– mutations – should be investigated for possible interactions with other mutations. This may help make informed decisions about which
method would be the most appropriate in a given study. One example of a case where a possible interaction between mutation and FUdR was discovered as part of a larger experiment is found in Vanfleteren & Vreese (1995). Here, it was noted that the presence of FUdR increased the levels of superoxide dismutase significantly in another daf-2 allele, daf-2(e1370), whereas this was not the case with the use of a temperature sensitive fer– mutation. In this case, use of the chemical was discontinued due to this effect. Given the possible links between life history traits such as longevity or ageing and reproduction, it may be the case that FUdR, or the suppression of reproduction, does not interact with all types of mutation – associated with longevity or otherwise – in the same way. The age at which worms are sampled may also be important in how great the interaction effect might be. The effect in much older worms, past reproductive age, may be greatly reduced.

Conclusion

This experiment was designed to test the effect of 5-fluoro-2-deoxyuridine on phenotypes other than the previously tested fecundity, ageing or longevity, by investigating the impact it has on the metabolic profile of C. elegans. It also demonstrates potential interactions with a commonly used mutation affecting longevity. The results presented here suggest that the chemical should be used with care, and that its effects are not necessarily easy to control for, or factor out completely.
5 Metabolomic analysis of the

\textit{C. elegans} life cycle

5.1 Introduction

The life cycle of wild type \textit{C. elegans} has been well characterised (Byerly \textit{et al.}, 1976) and consists of an embryonic stage, four larval stages (L1–L4) and an adult stage. Each stage ends with a cuticle molt followed by the formation of a new cuticle. Depending on culture temperature, the development of \textit{C. elegans} from egg to adult takes 3–5 days. Table 5.1 shows the growth parameters of wild type \textit{C. elegans} at 16, 20, and 25°C (Hirsh & Vanderslice, 1976), with most rapid growth and maximum fecundity at 20°C (Epstein & Shakes, 1995). This process is described in more detail in Chapter 1. At adulthood, the hermaphrodite begins to lay eggs, with egg laying continuing until around 6 days post–hatching. Wild type hermaphrodite worms have a lifespan of around three weeks and show signs of ageing including muscle deterioration and slowing of movement.

There have been several studies investigating the changes in gene expression that occur during \textit{C. elegans} larval development (Reinke \textit{et al.}, 2000; Thoemke \textit{et al.}, 2005; Hill \textit{et al.}, 2000; Jiang \textit{et al.}, 2001; Kirienko & Fay,
5 Life cycle analysis

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>L1–L2</th>
<th>L2–L3</th>
<th>L3–L4</th>
<th>L4–adult</th>
<th>First eggs laid (h post hatch)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>24</td>
<td>39</td>
<td>54.5</td>
<td>74.5</td>
<td>94–97</td>
</tr>
<tr>
<td>20</td>
<td>15</td>
<td>24</td>
<td>34</td>
<td>46</td>
<td>59–60</td>
</tr>
<tr>
<td>25</td>
<td>11.5</td>
<td>18.5</td>
<td>26</td>
<td>35.5</td>
<td>45–46</td>
</tr>
</tbody>
</table>

Table 5.1: C. elegans growth parameters

2007). Capra et al. (2008) compared the developmental expression profiles of two C. elegans isolates – Bristol N2 and Hawaii CB4856. Microarrays were used to obtain profiles for six stages of development (egg, L1, L2, L3, L4 and young adult) for each strain. The authors found that the expression of most transcripts vary with stage rather than with strain and that the greatest variation between strains occurs at the L4 stage, consistent with the idea that selection relaxes during later developmental stages (Cutter & Ward, 2005). Of the 13,474 genes whose expression was measured, 2211 genes were expressed at different levels in the two stains at least one of the stages. Some gene sets were expressed mostly during the egg stage in both strains; some genes were mostly expressed in either N2 or CB4856, and one gene set was turned on and off roughly one stage earlier in CB4856 than in N2. One of the gene sets that showed these patterns is linked with innate immunity and the authors suggest that the different expression patterns seen in the two strains may reflect the different pathogens found in the environments of the two strains. The low expression of these genes in both strains at the egg stage may reflect the fact that the egg shell prevents exposure to pathogens and that most pathogens are ingested and therefore do not affect worms before they are able to feed.

The effects on metabolism with age (without the direct measurement of metabolites) in C. elegans have been reviewed by Braeckman et al. (2000).
Increasing age is associated with a decline in enzyme activity, which seems to be a result of an increase in altered and damaged proteins due to reduced protein turnover. In addition to this, the respiration rates of the long-lived dauer-constitutive mutant *age-1* and wild type worms are the same in young adulthood, and begin to decline similarly with age. However, at 8.5 days, the decline in respiratory activity in mutant worms slows, and they retain a significantly higher rate of respiration, suggesting that mutant worms are biologically younger than predicted by chronological age (Vanfleteren & Vreese, 1996).

The changes in metabolic profile with age have already been investigated in organisms other than *C. elegans*. Wang *et al.* (2007) looked at the effects of ageing and dietary restriction (DR) on the metabolome of Labradors throughout their lifetime using the urinary metabolic profiles of both control-fed and diet-restricted dogs. Age related changes have also been studied in rats (Williams *et al.*, 2005), where $^1$H NMR and HPLC-TOF MS were used to look at endogenous metabolites excreted in urine. Longitudinal studies of ageing in *C. elegans* have already been carried out using gene expression data. Lund *et al.* (2002) looked at infertile worms from three to 19 days old and found that the expression of very few genes – making up less than 1% of the genome – change with age. Those that do change include those encoding heat shock proteins (decreased expression with age) and those encoding some transposases (increased expression with age). An increase in transposases may be responsible for the destabilisation of the genome in older worms. It has also been found that gene network integrity declines with age (Vinuela *et al.*, 2010). Many of the same age related gene expression changes in *C. elegans* can be observed across species, and the
transcriptional profiles of ageing in *C. elegans* and Drosophila have been compared (Mccarroll *et al.*, 2004).

By looking at individual worms, age related changes in gene expression can be studied without the use of fertility mutants or chemicals like FUdR – single worms can be easily transferred to new plates regularly for separation from their progeny. Microarrays using individual worms have been used to link age–related behaviours to gene expression (Golden *et al.*, 2008) and also to compare wild type worms with a long–lived mutant (Golden & Melov, 2004). In this comparison, four adult ages were sampled in both wild type and *daf-2(e1370)* between days four and 19. Again, changes in the expression of genes associated with heat shock proteins are seen, and this is the case in both strains. Other genes were found that differentiate the ageing process in wild type from ageing in *daf-2(e1370)*, including genes associated with proteins with anti–fungal properties and those that encode for electron transport chain components of mitochondria (more highly expressed in old wild type worms compared with old *daf-2* worms). In addition to this, genes that code for proteasome components increase in expression in wild type worms with age but not in *daf-2* worms. The authors suggest that this may be because wild type worms require a greater capability for protein degradation as they age due to an increase in the occurrence of malformed proteins, and that this process is accelerated in wild type relative to *daf-2* worms. It may be the case that these gene expression changes occur in *daf-2(e1370)* worms after the final, 19–day sampling time point.

Like *daf-2(e1370)*, *daf-2(m41)* is a long–lived, temperature–sensitive, dauer–constitutive IIS mutant. It has a smaller adult body size than other *daf-2* mutants (McCulloch & Gems, 2003) and a mean brood size that
5.2 Results

is around 11% lower than wild type mean brood size. Here, I have used proton NMR spectroscopy to study the changes that occur in this mutant during normal development and ageing in comparison to the changes that occur in wild type worms, sampling at all larval stages, young adults of just reproductive age, and six other adult time points.

5.2 Results

Synchronised populations of both strains were grown at 20°C on NGM plates until early adulthood. Each strain was sampled at each larval stage (L1, L2, L3 and L4) and at young adulthood, which was defined as the point at which some eggs were seen on plates but no second generation worms had hatched. At this point, worms were transferred to plates containing fluorodeoxyuridine (FUdR). As described in Chapter 4, this results in sterile worms and so ensured that the ageing populations continued to be synchronous without daily transfer to new plates, so that a single generation of ageing worms was maintained. After transfer to FUdR plates, worms were sampled at days 6, 8, 10, 13, 16 and 20 days post–hatching for each strain. Due to the slight difference in development time between the wild type and mutant, the times, post–hatching, at which the sampling of developmental stages occurred varied slightly between strains.

As wild type worms reached adulthood before *daf-2(m41)* worms, the two strains were transferred to FUdR plates at different time points to ensure that both strains were fully grown before the inhibition of DNA synthesis. The sampling and transfer times for both strains are given in Figure 5.1. Due to the large scale of the experiment and the biomass required for each
Figure 5.1: Sampling time points for wild type and \textit{daf-2(m41)} mutant worms during development (A) and adulthood (B), reflecting the differences in development rates between strains.
5.2 Results

sample, it was not possible to carry out the entire experiment using the same population of worms. Therefore, the experiment was carried out in two stages. The first stage included larvae and young adults (all grown on standard NGM plates) and the second stage consisted of all other adult time points (all grown on NGM plates containing FUdR).

At each sampling time point, worms were washed from plates with M9 buffer and allowed to settle at the bottom of a 15 ml centrifuge tube for three minutes. The M9 supernatant was then removed and the worm pellet transferred to small centrifuge tube and snap–frozen with liquid nitrogen. Remaining worms were washed from plates and transferred to fresh, E. coli–seeded plates on day ten. Extra E. coli OP50 was washed in M9 buffer and then pipetted onto plates (after sampling) at days 6, 8, 13 and 16 to ensure the worms were not starved at any time. Samples were stored at -80°C before extraction with 80% methanol and analysis by NMR spectroscopy. Six biological replicates were analysed per time point, per strain. Samples were randomised for harvesting, sample preparation, and analysis by NMR spectroscopy in order to eliminate batch effects. Individual metabolites were identified and quantified for each sample as described in Chapter 2 and the resulting data normalised using median fold change normalisation.

The C. elegans metabolome changes considerably with age

Figure 5.2 shows the relative concentrations for all metabolites identified for both strains at all time points. The normalised data have been scaled to unit variance and a hierarchical cluster analysis was used to cluster metabolites along the x axis. The Z–value refers to the number of standard deviations
Figure 5.2: Heat map showing the relative concentrations for all metabolites identified for both strains at all time points. The normalised data have been scaled to unit variance and a hierarchical cluster analysis was used to cluster metabolites along the x axis. The Z-value refers to the number of standard deviations from the mean value. During the life cycle of the worm, many metabolites show changes in relative concentration, with a clear overall change in metabolic profile occurring at the larval / adult boundary in both strains.
5.2 Results

from the mean value. During the life cycle of the worm, many metabolites show changes in relative concentration, with a clear overall change in metabolic profile occurring at the larval / adult boundary in both strains. The branched–chain amino acids (BCAAs), isoleucine, leucine and valine are seen to decrease in relative concentration at adulthood regardless of genotype, although the rate of decrease in the daf-2(m41) worms appears to be slower and than that seen in wild type worms. In wild type worms, a clear shift is seen in these metabolites at adulthood. Conversely, phosphocholine, glycerophosphocholine and glycine are all seen to increase in relative concentration at adulthood.

Wild type and daf-2(m41) mutant worms follow different developmental trajectories

Figure 5.3: For larval stages and young adults, principal component analysis (PCA) was carried out on unit variance scaled data. In the two component model, the first component explains 32% of the total variance in the data and the second component explains 11.2% of the variance (Q² (cumulative) = 0.256). A shows the mean score on each principal component for each time point for a given strain, along with trajectory lines for each strain. Error bars represent the standard error of the mean. B shows all data points for both strains.
For larval stages and young adults, principal component analysis (PCA) was carried out on unit variance scaled data. In the two component model, the first component explains 32% of the total variance in the data and the second component explains 11.2% of the variance ($Q^2$ (cumulative) = 0.256). The scores on both components are shown in Figure 5.3. Here, B shows all data points for both strains, but A shows the mean score on each principal component for each time point for a given strain, along with trajectory lines for each strain. At the L1 stage, wild type and $daf-2$ mutants have similar scores in PC1 and PC2, but as development progresses, the trajectories of the two strains separate. At L2, the mutant worms have a considerably higher score on PC1 than wild type worms, although the strains have similar PC2 scores. Both strains move to a lower score on PC1 between L2 and L3 but then move in different directions along PC1 between L3 and L4. The greatest difference between strains is seen in the progression from L4 to young adulthood. A relatively small shift along PC1 with time is seen in mutant worms, with little change in PC2 score. In wild type worms however, only a small change in PC1 score is seen but there is a large shift in a negative direction along PC2. This results in the young adult wild type and mutant worms having different scores on both principal components. Figure 5.4 shows the corresponding loadings for this analysis.

Wild type and $daf-2(m41)$ worms are metabolically distinct for the whole development period

In order to look at larval stages and young adults for both strains in more depth, all data were mean-centred and log transformed before an Orthogonal Partial Least Squares (OPLS) regression (where the y variable was time (in
5.2 Results

Figure 5.4: Loadings corresponding to the PCA in Figure 5.3.

hours) post–hatching) was carried out, with the results shown in Figure 5.5 (R²X (cumulative) = 0.512, R²Y (cumulative) = 0.825, Q² (cumulative) = 0.656). Figure 5.5 A shows scores and Figures 5.5 B and C show the corresponding loadings. By looking at the time–correlated component, it can be seen that if we consider both strains simultaneously, development from L1 to young adult is associated with an increase in trehalose, serine, glutamate and alanine and a decrease in the branched–chain amino acids as well as phenylalanine, malate and choline. Given that the y–variable was set as time, this analysis was supervised with respect to age, but not with respect to strain. Despite this, a clear separation between strains is seen along the first orthogonal component at all stages, indicating that the two strains behave differently over the developmental period. By looking at the loadings associated with the first orthogonal component it can be seen that high levels of trehalose, tyrosine and o–phosphoserine are linked with daf-2
Figure 5.5: For larvae and Young Adults (YA), an OPLS analysis (y = hours post hatching) was carried out ($R^2_X$ (cumulative) = 0.512, $R^2_Y$ (cumulative) = 0.825, $Q^2$ (cumulative) = 0.656). Data were mean centred and log transformed prior to analysis. A shows the resulting scores, B shows loadings for the correlated component and C shows loadings for the first orthogonal component. This analysis was supervised with respect to age but not with respect to strain. Despite this, a clear separation between strains is seen along the first orthogonal component at all stages, indicating that the two strains behave differently over the developmental period.
worms compared with high levels of lactate, succinate and o-phosphocholine in wild type worms.

**Different metabolite changes are associated with development in wild type worms compared with long-lived daf-2(m41) mutants**

The same mean-centred and log transformed data were then analysed using an Orthogonal Partial Least Squares Discriminant Analysis (OPLS–DA), with the Y variable distinguishing between the two strains. The results are shown in Figure 5.6, with scores shown in A and loadings for the strain-correlated component and first orthogonal component in B and C respectively ($R^2_X$ (cumulative) = 0.504, $R^2_Y$ (cumulative) = 0.893, $Q^2$ (cumulative) = 0.802). The different developmental stages are less distinct from each other and more closely clustered together in daf-2 mutants than in wild type worms. By looking at the strain-correlated component loadings (Figure 5.6 B), it can be seen that daf-2(m41) larvae have relatively high levels of the BCAAs, tyrosine, lysine and phosphoserine, whereas wild type larvae have relatively high levels of succinate, glutamate, betaine, putrescine and o-phosphocholine. In contrast to the OPLS regression in Figure 5.5, here the analysis is supervised with respect to genotype but unsupervised with respect to age. However, a progression from L1 to young adult can be seen in both strains in the same direction along the first orthogonal component. The loadings relating to the first orthogonal component show that if we consider the two strains together, L1 worms have relatively high levels of choline, malate, GPC and the BCAAs, whereas by young adulthood, worms have higher levels of trehalose, betaine and serine.
Figure 5.6: Mean centred, log transformed larvae and young adult (YA) data were analysed using OPLS–DA, where data were classed according to genotype (i.e. \textit{daf-2(m41)} or wild type). (\(R^2_X\) (cumulative) = 0.504, \(R^2_Y\) (cumulative) = 0.893, \(Q^2\) (cumulative) = 0.802). A shows the resulting scores, B shows loadings for the correlated component and C shows loadings for the first orthogonal component. Here the analysis is supervised with respect to genotype but unsupervised with respect to age. However, a progression from L1 to young adult can be seen in both strains in the same direction along the first orthogonal component.
Daf-2(m41) mutants and wild type worms follow different ageing trajectories

Principal component analysis (PCA) was carried out using adult worm data only (beginning at 6 days post hatching for each strain and ending at 20 days post hatching). This resulted in a four component model, however only the first three components are shown in Figure 5.7. The first, second and third components explain 28%, 14.3% and 10.4% of the variance respectively ($R^2_X$ (cumulative) = 0.597, $Q^2$ (cumulative) = 0.339). Here, A, C and E show the mean score on each principal component for each time point for a given strain, along with trajectory lines for each strain. B, D and F show all data points for both strains. If we consider only the first two components (Figures 5.7 A and B), some similarities in the ageing trajectories between strains can be seen. Both strains mostly progress with a similar trajectory with respect to direction between time points in both components. However, in most cases, the magnitude of the change of position between time points is much greater in wild type worms than in daf-2(m41) worms. The overall range in both components is also smaller in daf-2 worms than in wild type worms. If we consider a third component (Figures C–F), differences between the trajectories of the two strains as they age become much more apparent. The corresponding loadings plots are given in Figure 5.8.

Young adults, able to reproduce, may be included in an analysis of ageing in sterile adults

As the experiment was carried out in two stages, and no samples at the same time point were included in both experiment sets, it is possible that it would not be appropriate to analyse time points from the first part of the
Figure 5.7: For adults (days 6 to 20 post hatching), principal component analysis was carried out on unit variance scaled data. This resulted in a four component model, however only the first three components are shown here. The first, second and third components explain 28%, 14.3% and 10.4% of the variance respectively ($R^2$ (cumulative) = 0.597, $Q^2$ (cumulative) = 0.339.) A, C and E show the mean score on each component for each time point for a given strain, along with trajectory lines linking each strain. Error bars represent the standard error of the mean. B, D and F correspond to A, C and E respectively, and show all data points.
Figure 5.8: Loadings corresponding to the Principal Component Analysis score in Figure 5.7.
Figure 5.9: To determine whether the ‘young adult’ stage from the first part of the experiment may be grouped with subsequent adult time points in further analysis, a PLS regression was carried out using all adult time points. The data were mean-centred and log transformed prior to this analysis and the results are shown in Figure 5.9. A shows both strains in a regression using wild type as a training set, where $Y = \text{time}$ and B shows the results of two cross-validation methods in addition to this. The plots demonstrate that young adults (here, the first time point) fall close to the line $y=x$, suggesting that it is appropriate to include this.
5.2 Results

time point in further analysis of the ageing adult data. It can also be seen that *daf-2(m41)* mutants fall further from the line *y=x* than either of the data points obtained in cross validation analysis (Figure 5.9 B).

*Daf-2(m41)* and wild type worms are metabolically distinct throughout adulthood

Figure 5.10: Adult data (including Young Adults (YA)) were mean centred and log transformed before an OPLS analysis (where *y* = age in hours post hatching) was carried out. (*R*² (cumulative) = 0.496, *R*²*Y* (cumulative) = 0.904, *Q*² (cumulative) = 0.871). A shows the resulting scores, B and C show the loadings for the correlated component and first orthogonal component respectively. this analysis was supervised with respect to age, but not with respect to strain. However, as with the larval data, a clear separation between strains can be seen in the first orthogonal component.
5 Life cycle analysis

All adult time points (including ‘young adults’) were included in an OPLS analysis similar to that carried out for larval data. Here, the y variable was equal to time (in hours) post–hatching and the results of this analysis on mean–centred, log transformed data are shown in Figure 5.10 ($R^2_X$ (cumulative) = 0.496, $R^2_Y$ (cumulative) = 0.904, $Q^2$ (cumulative) = 0.871). A shows scores and B shows the corresponding loadings. The age–correlated component loadings show that the metabolomes of younger worms are defined by relatively high levels of succinate, cystathionine, glutamate, glutamine and the BCAAs isoleucine and valine, while older worms show increased levels of o-phosphoserine, o-phosphocholine, GPC, citrate and glycine. Given that in this model the regression was performed against time, this analysis was supervised with respect to age, but not with respect to strain. However, as with the larval data, a clear separation between strains can be seen in the first orthogonal component. Although the wild type and mutant worms can be separated in this way as they age, this effect does seem to lessen as time progresses, with the strains becoming less distinct with age. By considering the first orthogonal component loadings (Figure 5.10 C), it can be seen that the wild type adult metabotype features relatively high concentrations of glutamate, choline and o-phosphocholine while the $daf-2(m41)$ metabotype includes high levels of tyrosine, glycerol, o-phosphoserine and malate.

Some metabolite changes that occur during the ageing process are independent of genotype

The adult data were then analysed using OPLS–DA, with the Y variable grouping the data according to genotype. The results are shown in Figure 5.11, with scores shown in A and the corresponding loadings shown
Figure 5.11: Adult data (including Young Adults (YA)) were mean centred and log transformed before an OPLS–DA analysis was carried out, with classes based on genotype (i.e. *daf-2(m41)* and wild type). ($R^2_X$ (cumulative) = 0.485, $R^2_Y$ (cumulative) = 0.737, $Q^2$ (cumulative) = 0.668). A shows the resulting scores, B shows the loadings for the correlated component and C shows the loadings for the first orthogonal component. Here the analysis is supervised with respect to strain but unsupervised with respect to age, but a progression from young adult to 20–day–old adults can be seen in both strains from lower to higher score in the first orthogonal component.
5 Life cycle analysis

in B and C (\(R^2_X\) (cumulative) = 0.485, \(R^2_Y\) (cumulative) = 0.737, \(Q^2\) (cumulative) = 0.668). The strain–correlated component loadings (Figure 5.11 B) suggest that high levels of the BCAAs, glycerol, trehalose and tyrosine define an adult \(daf-2(m41)\) worm, whereas high levels of putrescine, o-phosphocholine, citrate, choline and betaine are associated with adult wild type worms. Here the analysis is supervised with respect to strain but unsupervised with respect to age, but a progression from young adult to 20–day–old adults can be seen in both strains from lower to higher score in the first orthogonal component. The first orthogonal component loadings can be seen in (Figure 5.11 C), and show that if we consider the two strains, young adults are are associated with relatively high levels of succinate, glutamate, acetate and cystathionine, but as worms age, there is an increase in relative concentration of o-phosphoserine, o-phosphocholine, trehalose and GPC.

Based on metabolic profile, \(daf-2(m41)\) worms are predicted to be younger than their chronological age

For each part of the \(C.\ elegans\) life cycle, i.e. development and ageing, an OPLS regression was carried out, with time as a Y variable. In both models, data were scaled to unit variance and the Y variable was equivalent to time (in hours or days for larvae and adults respectively). Figure 5.12 shows the results of this analysis for larvae (A) and adults (B), with models generated based on wild type worms only. For larvae, (\(R^2_X\) (cumulative) = 0.506, \(R^2_Y\) (cumulative) = 0.945, \(Q^2\) (cumulative) = 0.898 B. For adults, \(R^2_X\) (cumulative) = 0.322, \(R^2_Y\) (cumulative) = 0.938, \(Q^2\) (cumulative) = 0.916. The line y=x is shown in each plot. ‘Chronological age’ refers to
5.2 Results

Figure 5.12: An OPLS regression was carried out for both larvae ($R^2_X$ (cumulative) = 0.506, $R^2_Y$ (cumulative) = 0.945, $Q^2$ (cumulative) = 0.898) and adult data (days 6 to 20 post–hatching) ($R^2_X$ (cumulative) = 0.322, $R^2_Y$ (cumulative) = 0.938, $Q^2$ (cumulative) = 0.916) shown in A and B respectively. Data were scaled to unit variance before the analysis and the line $y=x$ is shown in each plot. Models were generated based on wild type worms only. 'Chronological age' refers to the time at which worms were sampled and ‘predicted age’ refers to the age predicted by the model based on metabolome. based on these metabolites, both adult and larvae daf-2(m41) worms are predicted to be younger than their chronological (sampling) age.

Figure 5.13: Loadings corresponding to Figure 5.12. A and B show which metabolites determine wild type age in larvae and adults respectively.
the time at which worms were sampled and ‘predicted age’ refers to the
age predicted by the model based on metabolome. Figure 5.13 shows the
Corresponding loadings; A and B show which metabolites determine wild
type age in larvae and adults respectively. In larval and young adult wild
type worms, development is associated with a decrease in the concentration
of BCAAs, choline, phenylalanine and malate, and an increase in betaine,
glutamate, arginine and glycine. For wild type adult worms, the ageing
process involves a decrease in glutamate, glutamine, cystathionine, histidine
and trehalose and an increase in GPC, phosphoserine, putrescine, glycine
and citrate. Figure 5.12 shows that based on these metabotypes, 

\[ \text{daf-2(m41)} \]

worms are predicted to be younger than their chronological (sampling) age at
each time point during development. During adulthood, differences between
the two strains increase with age, with \text{daf-2} worms predicted to be just
younger than their chronological age from day 13, with clear differences
between predicted and chronological age from day 16.

**Figure 5.14:** An OPLS regression against time was carried out for both larval–stage
\( R^2_X \) (cumulative) = 0.28, \( R^2_Y \) (cumulative) = 0.732, \( Q^2 \) (cumulative) = 0.616) and
adult \( R^2_X \) (cumulative) = 0.333, \( R^2_Y \) (cumulative) = 0.81, \( Q^2 \) (cumulative) = 0.769) 
\text{daf-2} worms. A and B show the correlation between score and age for development
and ageing respectively.
5.2 Results

Figure 5.15: Loadings corresponding to the model in Figure 5.14. A and B show metabolites ordered in terms of importance for determining daf-2(m41) development and ageing respectively. To help identify which metabolites are of different importance, or behave differently, in determining age in the two strains, C and D show metabolites ordered according to importance in determining wild type development and ageing (i.e. as Figure 5.13).
Figure 5.16: The pattern of change in trehalose and tyrosine with age in adult worms. Mean values are shown, with error bars representing the standard error. In ageing *daf-2(m41)* worms, trehalose concentration is strongly positively correlated with an increase in age and tyrosine is negatively correlated with age. In wild type worms, the reverse is true; trehalose levels decrease, and tyrosine levels increase with age.
5.2 Results

For comparison, an OPLS regression against time was carried out for both larval-stage ($R^2_X$ (cumulative) = 0.28, $R^2_Y$ (cumulative) = 0.732, $Q^2$ (cumulative) = 0.616) and adult ($R^2_X$ (cumulative) = 0.333, $R^2_Y$ (cumulative) = 0.81, $Q^2$ (cumulative) = 0.769) daf-2 worms (Figure 5.14). The loadings for the correlated component are shown in Figure 5.15. A and B show metabolites ordered in terms of importance for determining daf-2(m41) development and ageing respectively. For daf-2 worms, development is associated with a decrease in the BCAAs, phenylalanine, acetate, lysine and methionine and an increase in glutamate, alanine, putrescine, trehalose, serine and cystathionine. Ageing in this mutant is associated with a decrease in histidine, succinate, the BCAAs and cystathionine and an increase in GPC, glycine, trehalose, o-phosphocholine and citrate with time. Many of these changes are similar to the changes seen in development and ageing in wild type worms as detailed above.

To help identify which metabolites are of different importance, or behave differently, in determining age in the two strains, figures 5.15 C and D show metabolites ordered according to importance in determining wild type development and ageing respectively (i.e. as Figure 5.13). It can be seen that choline, tyrosine and o-phosphoserine, which are associated with early development in wild type worms, are not strongly associated with this stage in daf-2 worms. Acetate and NAD, however, are much more important when defining early development in daf-2 worms than in wild type worms. Phosphocholine, glycine, arginine and betaine become increasingly abundant with development in wild type worms, but this is not the case in daf-2 worms. In ageing mutant worms (Figure 5.15 D), trehalose concentration is strongly positively correlated with an increase in age and tyrosine and lysine levels are negatively correlated with ageing. In wild type worms, the reverse
is true; higher than average levels of trehalose are associated with young adulthood and increasing relative concentration of both lysine and tyrosine is linked with increasing age. Figure 5.16 shows the patterns of change of trehalose and tyrosine with age. As in development, in ageing choline follows a different pattern of relative concentration in the two strains, being more important in defining young wild type worms than *daf-2(m41)* worms.

### 5.3 Discussion

It is clear to see that the *C. elegans* metabolome changes considerably with age, regardless of genotype (Figure 5.2), and this is unsurprising given the many processes that take place over the course over the life cycle of a worm. The most distinct change is seen at the development / ageing boundary and again, this was to be expected – the processes involved in the development of a worm from larva to adult will be very different from those that occur during the ageing process. More interestingly, the two strains remain distinct at all time points through both development and ageing (Figures 5.5 and 5.10). It can be seen that if the metabolic profile as a whole is considered, long–lived *daf-2(m41)* mutants follow different trajectories from wild type worms in terms of both development and ageing (Figures 5.3 and 5.7). To further investigate the main differences between the strains, it is necessary to consider changes in the relative concentrations of individual metabolites.
5.3 Discussion

Development

In the transition from L1 to young adult, the changes in the majority of the amino acids identified and quantified here are consistent with the balloon hypothesis (Swire et al., 2009), and this is the case for both strains. In this model, the need for the production of cell membrane components lessens relative to the production of cytoplasmic components over the course of development. This is due to the fact that in C. elegans, cells expand in volume without dividing during development so that body size increases without the increase in cell number (Flemming et al., 2000). The balloon hypothesis suggests that amino acid usage is not driven by age but by geometry, and that during development, the production of cytoplasmic proteins increases and the production of membrane proteins decreases. Amino acid pools are affected by the requirement for either mainly hydrophobic amino acids or hydrophilic amino acids to make up membrane or cytoplasmic proteins respectively.

During development, the changes seen in the levels of certain metabolites are different in wild type and daf-2(e14) worms. Phosphocholine, glycine, arginine and betaine all increase and choline and tyrosine decrease considerably in relative concentration with time in developing wild type worms, but all show much smaller changes in daf-2(e14) worms (Figure 5.15). Tyrosine is a precursor to melanin, which has been found to have a protective function in C. elegans as a Reactive Oxygen Species (ROS) scavenger (Calvo et al., 2008). Decreasing levels of tyrosine in wild type worms as they approach adulthood may mean they have a reduced capacity to form melanin and are therefore at increased risk of oxidative damage. The decrease in choline and the increase in phosphocholine, glycine and betaine (all choline metabolites)
5 Life cycle analysis

with age in wild type worms does not occur to such a great extent in mutant worms, pointing to differences in the changes in lipid metabolism and lipid turnover between the two strains as they age.

Ageing

Some metabolites show similar patterns of change during the ageing process in the two strains. Levels of the branched–chain amino acids (BCAAs) decrease as worms age in both wild type and daf-2 worms. However, it can be seen that BCAA levels are higher in daf-2 adults than in wild type adults (Figure 5.11) and this is consistent with previous results (Fuchs et al., 2010). For C. elegans, as for other animals, these amino acids are essential (Payne & Loomis, 2006), so differences in their pool sizes result only from changes in their catabolism, in protein turnover or uptake. Fujita & Volpi (2006) suggest that these amino acids may directly stimulate protein synthesis, as well as being precursors for this synthesis. A decreasing pool of amino acids with age may be linked with a decrease in protein synthesis. Supplementation with the BCAAs have been found to increase chronological lifespan in yeast (Alvers et al., 2009) and longevity in mice (D’antona et al., 2010), as well as resulting in improvement in some age–related diseases in both rats (Pansarasa et al., 2008) and humans (Solerte et al., 2008b). In mice it was found that an increase in lifespan with BCAA supplementation was accompanied by increased mitochondrial biogenesis and SIRT1 expression and that ROS defence system genes were upregulated, reducing oxidative damage. The reduced change in BCAAs with age observed in daf-2 worms compared with wild type worms may help explain their increased longevity.
Other metabolites that show decreasing relative concentration with age in both strains are histidine, glutamine, and glutamate. Histidine is another essential amino acid and a component of carnosine; histidine and carnosine may contribute to antioxidant protection in rats (Chan et al., 1994). As histidine seems to be one of the most important metabolites in determining age in both strains, the link with antioxidant protection may point to a reason for the decrease in this amino acid with age. Decreasing levels of glutamine and glutamate with age in both strains is consistent with findings in both rat (Banay-Schwartz et al., 1989; Benedetti et al., 1990) and human (Kaiser et al., 2005) brains. Phosphocholine and GPC increase with age in both strains (though this is more a feature of wild type ageing than of daf-2(m41) ageing), pointing to altered lipid metabolism with age. An increase in age is also correlated with an increase in putrescine, which is a product of the breakdown of amino acids, which corresponds to the decrease seen in many of the amino acids here.

Trehalose levels change in opposite directions with age in the two strains. In wild type worms, levels decreases with age, whereas in daf-2(m41) worms levels increase with age. The disaccharide is an important carbohydrate storage molecule, thought to confer stress resistance in many invertebrates (Behm, 1997; Pellerone et al., 2003). As described in Chapter 3, trehalose has long thought to be a ‘longevity assurance sugar’, with genes involved in its synthesis found to be upregulated in IIS mutants (Lamitina & Strange, 2005; McElwee et al., 2006), and this is consistent with the differences between strains seen here.

Honda et al. (2010) found that trehalose lengthens C. elegans lifespan. It was found that very shortly after beginning treatment in 20–day adult
worms, trehalose suppressed the age-related decline in survivorship, and that while treatment of 10-day adult age worms for a ten day period extended mean lifespan, treatment from the first day of adulthood for ten days did not increase longevity. The authors suggest that trehalose protects against the increased stress that occurs with age, but is unable to repair damage to proteins that has already occurred during the ageing process. This can be directly linked with the pattern of change in trehalose levels with age here. In wild type worms, trehalose levels start to decrease from around 10 days post-hatching – for comparison, this is the equivalent of around 7-day adults – with the rate of decline increasing at day 13 post-hatch (day 10 adults). Trehalose treatment at day 10 of adulthood would therefore replenish trehalose stores just as they seem to begin to naturally deplete. Feeding worms between days 1 and 10 of adulthood (days 4 to 13 post-hatching) has no effect on lifespan, possibly because trehalose levels are still naturally high in worms during this period (Figure 5.16). The natural increase in trehalose levels with age in daf-2(m41) mutants studied here might explain why the authors saw no effect of trehalose treatment on the lifespan of another daf-2 mutant.

Tyrosine also behaves differently with age depending on genotype, but in contrast to trehalose, relative concentration increases with age in wild type worms and decreases with age in daf-2 worms. As detailed above, tyrosine is a precursor to melanin, an ROS scavenger (Calvo et al., 2008). An decrease in tyrosine pools in daf-2 worms may represent increased melanin production with age, contributing to reduced oxidative damage and an increased lifespan. Tyrosine is a non-essential amino acid for C. elegans, and can be synthesised from phenylalanine, which follows the same pattern of change as tyrosine in both strains. This is consistent with the fact that many genes encoding
components of the catabolic pathways of phenylalanine and tyrosine are regulated in \( daf-2 \) worms (Dong et al., 2007; Fisher et al., 2008).

Some metabolites appear to be important in the ageing process in one strain but not in the other. For example, there is very little change in relative concentration of lysine in wild type worms, but ageing in \( daf-2(m41) \) worms is strongly associated with a decrease in this essential amino acid. Conversely, asparagine levels show only small changes in mutant worms but decrease with age in wild type worms.

**Based on metabotype, \( daf-2(m41) \) worms are predicted to be younger than their chronological age**

In both development and ageing, long-lived \( daf-2 \) worms have a metabotype that is younger than expected based on chronological age (Figure 5.12). During development, worms are predicted to be younger than their chronological (sampling) age at each time point. During adulthood, differences between the two strains increase with age, with \( daf-2 \) worms predicted to be just younger than their chronological age from day 13, with clear differences between predicted and chronological age from day 16. This is, in part, consistent with the findings of Vanfleteren & Vreese (1996), in which a long-lived dauer constitutive mutant is found to have a slowed rate of decline of respiratory activity with age, so that mutant worms appear biologically younger than their chronological age. Based on metabolic profile, it seems that throughout development, mutant worms are younger than predicted at all ages, while in adults, the difference between biological age and chronological age in mutant worms seems to increase with age. In addition to this, the respiration rates of the long-lived dauer-constitutive mutant \( age-1 \) and wild type worms are
5 Life cycle analysis

the same in young adulthood, and begin to decline similarly with age. However, at 8.5 days, the decline in respiratory activity in mutant worms slows, and they retain a significantly higher rate of respiration, suggesting that mutant worms are biologically younger than predicted by chronological age (Vanfleteren & Vreese, 1996). The differences in the metabolic changes that occur with the ageing process in the two strains highlight the importance of choosing carefully the time points used in wild type / mutant comparisons. This is especially important if only a single time point is to be chosen.

Experiment limitations and further work

Although a significant shift in metabolism is to be expected at the larval / adult boundary, with the end of development and the onset of ageing, it is possible that some changes may be an artefact of the transfer to different media (i.e. plates containing FUdR). However, as can be seen from the regression analysis in Figure 5.9, for both strains, the last time point on standard NGM plates – the young adults – seems to fit well with the later adult time points, which were sampled from plates containing FUdR. In addition to this, worms were transferred to fresh (FUdR) plates on day 10. No obvious change is seen at this point in either Figure 5.9 or Figure 5.2. As shown in Chapter 4, the use of FUdR when investigating ageing, and comparing mutants, is far from ideal as any effects of reproduction on ageing are not taken into account. In a preliminary experiment, adult worms were regularly filtered using nylon mesh to remove progeny in an attempt to maintain a synchronised population throughout adulthood. However, this method was unable to remove all young worms and so by day 20, populations were no longer synchronised and contained worms at many stages. The old
adult worms could not be separated from younger adult worms. In addition to this, the filtering method increases the likelihood of bacterial and fungal contamination on plates, and this problem increased with sampling age due to repeated filtration and extra time for contaminants to proliferate on plates. The combination of decreasingly synchronised populations and increasing contamination would have been likely to result in false trends with time. It was decided that this would have caused a greater problems in interpreting results than the use of FUdR which, with using previous results (Chapter 4) can to a certain extent be controlled for. When grown on FUdR plates, adult populations remain synchronous and the problem of contamination is effectively eliminated.

The timing of the transfer of worms to FUdR plates is extremely important. Worms should not be transferred to FUdR plates before they reach adulthood so that they are able to develop normally, but should be transferred before any hatched progeny appear on plates. The two strains have slightly different development times and for this reason, spent different periods of time in the presence of FUdR (a difference of 19 hours) as during adulthood, both strains were sampled based on number of days post–hatching (Figure 5.1). This problem is difficult to overcome, but one solution may have been to sample worms based on time post–maturity. However, the effect of being exposed to FUdR for different periods is likely to decrease with time, as the time difference becomes much smaller relative to sampling age. In addition to this, lifespan statistics are generally quoted in terms of absolute age (post–hatching), rather than age–post maturity so that comparisons based on chronological age based on time of hatching seem sensible.
This experiment investigated only normal development and did not include dauer larvae. The wild type dauer larvae metabolome has already been well characterised by NMR (Fuchs et al., 2010), however it would be interesting to compare the metabolic profile of wild type dauer larvae with that of daf-2(m41) dauers. In addition to this, wild type worms were sampled very close to their maximum lifespan of around 21 days in this laboratory (Chapters 3 and 4), however daf-2(m41) worms have a maximum lifespan of around 28 days (again, under the conditions of this laboratory). Continuing to sample daf-2 worms after the 20–day point may highlight further similarities and differences in the ageing process between the two strains.

Conclusion

As a first characterisation of the C. elegans metabolome throughout both development and ageing, these results provide an insight in the metabolic changes that occur with time. The comparison of these changes in wild type worms with the changes in a mutant known to show differences in both of these processes help to determine the point at which point long–lived mutants begin to acquire their long–lived phenotype. This has allowed the prediction of results of research published subsequently. For example, the importance of the timing of trehalose treatment in extending C. elegans lifespan (Honda et al., 2010) could have been predicted using relative changes in trehalose levels that occur naturally in worms as they age.
6 Metabolomic analysis of mutation accumulation in C. elegans

6.1 Introduction

New mutations are the source of genetic variation. Mutation Accumulation (MA) experiments have played a key role in determining what is known about how spontaneous mutations contribute towards genetic variation, in addition to their effect on fitness (reviewed by Halligan & Keightley, 2009). The purpose of a MA experiment is to reduce natural selection to as great a degree as possible, and this is achieved by splitting an ancestral line into a number of MA lines which are then allowed to accumulate mutations for a given number of generations, by reproducing in an environment lacking in selection pressure. Mutations that arise will then either be lost or fixed in subsequent generations and each line may accumulate different combinations of mutations. The majority of mutations appear to be either neutral or harmful, given that they perturb highly adapted systems (Lynch et al., 1999; Drake et al., 1998). In addition to the fact that mutations that result in a visible phenotype are usually deleterious, the high degree of conservation of
protein-coding sequences compared with non-coding DNA regions, suggests that most changes in amino acids are deleterious, as individuals with harmful mutations either die or fail to reproduce (Keightley & Eyre-Walker, 1999). For this reason, there is likely to be not only an increase in genetic variation between lines, but also a decrease in mean fitness in the MA lines compared with the ancestral line.

Many MA experiments are concerned with the rate at which new mutations accumulate in the genome (Denver et al., 2005a; Garcia et al., 2010; Lynch et al., 1995; Phillips et al., 2009; Baer et al., 2005, 2007), and the effect of those mutations on fitness traits, including reproduction (Estes et al., 2004; Baer et al., 2006), body size (Azevedo et al., 2002; Salomon et al., 2009) and behavioural responses (Ajie et al., 2005). They have also been used to investigate the recovery from deleterious mutations (Denver et al., 2010). Denver et al. (2005b) investigated the effect of mutation accumulation on the *C. elegans* transcriptome and found that the evolution of gene expression occurs at a greater rate than the evolution of genes themselves, probably in part due to the fact that a single mutation in one regulatory locus is likely to affect many other genes. It was also found that not all biological processes or functions at the molecular level are equally mutable. For example, the transcriptional stability of genes that are involved in signal transduction pathways are under relatively high levels of stabilising selection. In contrast, the transcription of genes involved in carbohydrate, amino acid and lipid metabolism pathways are under less stringent stabilising selection than many other sets of genes. Baer & Denver (2010) used microarrays to look at transcripts from four *C. elegans* MA lines, along with their common ancestor, and found that the transcriptome of MA lines varies less with environmental change than that of their common ancestor. This suggests
that deleterious mutations reduce gene expression sensitivity to small random environmental changes.

*C. elegans* is an ideal organism for mutation accumulation experiments. As the great majority of a population of worms will be hermaphrodites, its mode of reproduction by self-fertilisation results in what can essentially be considered clonal propagation. A minimum population size of one worm per generation allows for the elimination of natural selection, and only lethal mutations or mutations that result in sterile worms are able to escape propagation. In order to generate *C. elegans* mutation accumulation lines, one larva from the progeny of a single worm is selected at random and allowed to produce its own progeny. One of these second-generation larvae will be selected at random and allowed to reproduce, and this process will be repeated until the desired generation number has been reached (Figure 6.1).

It has already been shown that changes in the *C. elegans* metabolome are clearly related to traditional fitness components such as longevity (Chapters 3 and 5) and reproduction (Chapter 4). Using metabolomics to study mutation accumulation lines allows us to estimate the impact of spontaneous variation on many traits (metabolites) simultaneously. Here, Gas-Chromatography Mass-Spectrometry (GC-MS) has been used to analyse the metabolic profiles of over forty mutation accumulation lines and their common ancestor, assumed to be free of mutations. The aim of this experiment is to identify the metabolic basis of mutational fitness variation. It may be the case that the changes in some metabolites correlate with fitness, or that each line has a unique change in metabolome compared with its ancestor. It is also
Figure 6.1: Generation of *C. elegans* Mutation Accumulation lines. One larva from the synchronised progeny of a single worm is selected at random and allowed to produce its own progeny. One of these second–generation larvae will then be selected at random and allowed to reproduce. This process is repeated so that mutations are allowed to accumulate over \( x \) generations. After \( x \) generations, each of \( n \) lines is expected to have accumulated different combinations of mutations. As the majority of mutations are deleterious, this results in an overall decrease in fitness relative to a control line (the preserved common ancestor of the \( n \) MA lines.)
possible that changes may be seen in the metabolic profile as a result of mutation accumulation that have no impact on fitness.

Particular metabolites may be particularly susceptible to mutation. It may be the case that the propensity of a metabolite to change with mutation accumulation is in some way linked with its connectedness within the metabolic network, so that, for example, highly connected or critical metabolites are less likely to change with mutation than less-critical metabolites. Selvarasu et al. (2010) calculated values for the ‘centrality’ of metabolites in a genome-scale model of a mouse metabolic network. These values are freely available as supplementary data (Selvarasu et al., 2010, Supplementary Material 5), and will be used here to investigate a link between metabolite centrality and propensity to change with mutation accumulation. The authors’ analysis generated two values for the centrality of a given metabolite: ‘degree centrality’ and ‘betweenness centrality’, helping to identify highly linked and bridging metabolites. Degree centrality is a measure of the number of links incident upon a given metabolite, while betweenness centrality is a measure of a node’s centrality in a network as a whole. Betweenness centrality is useful as a measure of the total load placed on a given metabolite in a network, rather than just localised connectivity (Selvarasu et al., 2010).

The MA lines profiled here were acquired from the Baer lab (University of Florida). The creation of the lines (over a total of 200 generations) is described in detail in Baer et al. (2005). Lines were then allowed to accumulate mutations over an additional 50 generations, giving 250-generation MA lines for metabolomic analysis. The Baer lab measured fitness by counting the number of hatched eggs produced by each line over a three day period. This was carried out at 100 generations, 200 generations and 220 generations and
these data will be used in an attempt to link metabotype with reproductive fitness.

6.2 Methods

Worms received from the Baer lab were washed from plates on arrival, using M9 buffer and frozen (as described in Chapter 2) before storage at -80°C until use. To grow lines for sampling, worms were thawed on unseeded NGM plates and incubated overnight at 20°C. Plates were then ‘chunked’, with a small (approx. 1cm²) uncontaminated area containing a large number of worms onto NGM plates seeded with E. coli OP50. For each MA line, five of these plates were made, giving five biological replicates per line. For ancestral worms, ten replicates were made. All lines were then allowed to reproduce until plates had reached a point suitable for synchronisation by treatment with hypochlorite. Following synchronisation, worms were incubated at 20°C until young adulthood, defined here as the point at which some eggs were seen on plates but no second generation worms had hatched. At this point, worms were washed from plates with M9 buffer and allowed to settle naturally at the bottom of a 15 ml centrifuge tube for three minutes. The M9 supernatant was then removed and the worm pellet transferred to small centrifuge tube and snap–frozen with liquid nitrogen. Samples were stored at -80°C before extraction with 80% methanol and analysis by GCMS. Samples were randomised for harvesting, sample preparation, and analysis by GC–MS in order to eliminate batch effects. Individual metabolites were identified and quantified for each sample (as described in Chapter 2) and the resulting data normalised using median fold change normalisation.
6.3 Results

Overview

Empty samples and obvious outliers were removed, along with the metabolites with the lowest retention times, which could not be reliably quantified. Mean values were calculated for each metabolite for each line. Figure 6.2 shows the log transformed fold change of mean concentration relative to mean ancestral level for each metabolite. A two–way Hierarchical Cluster Analysis (based on Euclidian distance) was used to cluster metabolites along both axes. The dendrogram showing the clustering of the lines is shown, with clear separation of the lines into two groups.

Principal Component Analysis

To further investigate the grouping observed in Figure 6.2, a principal component analysis was carried out. The filtered dataset was scaled to unit variance and log transformed before PCA. This resulted in a six component model ($R^2_X$ (cumulative) = 0.725, $Q^2$ (cumulative) = 0.413), with the first four components explaining 28.7%, 18.4%, 8.9% and 6.8% of the variance respectively. Figures 6.3, 6.4 and 6.5 show the results of this analysis (first four components only). It can be seen that ancestral samples fall at the edge of the data, tending towards a high score on PC1, PC2 and PC3 and a score close to zero on PC4. It can also be seen that the data are separable into two populations on PC2, with all ancestral samples contained in one of these groups.
Figure 6.2: Mean values were calculated for each metabolite for each line. The log transformed fold change of mean concentration relative to mean ancestral level for each metabolite is shown. A two-way Hierarchical Cluster Analysis (HCA) was performed based on Euclidean distance, and the dendrogram showing the clustering of the lines is shown.
6.3 Results

Figure 6.3: Principal component analysis of unit variance scaled, log transformed data (six component model, where $R^2_X$ (cumulative) = 0.725, $Q^2$ (cumulative) = 0.413). A shows scores on PC1 vs scores on PC2 and B shows the corresponding loadings. Principal components 1 and 2 explain 28.7% and 18.4% of the variance respectively.
Figure 6.4: Principal component analysis of unit variance scaled, log transformed data (six component model, where $R^2X$ (cumulative) = 0.725, $Q^2$ (cumulative) = 0.413). A shows scores on PC2 vs scores on PC3 and B shows the corresponding loadings. Principal components 2 and 3 explain 18.4% and 8.9% of the variance respectively.
6.3 Results

Figure 6.5: Principal component analysis of unit variance scaled, log transformed data (six component model, where $R^2_X$ (cumulative) = 0.725, $Q^2$ (cumulative) = 0.413). A shows scores on PC2 vs scores on PC4 and B shows the corresponding loadings. Principal components 2 and 4 explain 18.4% and 6.8% of the variance respectively.
6 Mutation accumulation

The lines can be grouped into two populations

In order to help view the grouping of the data on PC2, error bars were added to PCA score plots, showing the standard error of the mean for each MA line for the given components (Figure 6.6 A, B and C). The two groups can now be seen more clearly and a histogram, showing the distribution of the scores on PC2 (Figure 6.6 D) confirms the bimodal distribution of scores on this component. In order to confirm that this grouping related to variance between but not within MA lines, a box and whisker plot was constructed (Figure 6.7), with lines ordered according to their median score on PC2. This demonstrates that the two groups observed on PC2 are related to individual lines. While some overlap and a slight gradient can be seen, generally lines either score high on PC2 or low or PC2, enabling the lines to be grouped into two sets. This grouping is consistent with that seen in an HCA in Figure 6.2.

Which metabolites separate these two groups?

Figure 6.8 shows the loadings for the second principal component, which separates the lines into two groups. It can be seen that a low score on PC2 is associated with relatively high levels of adenosine, xanthine, nicotinic acid, allose and mannose, whereas a low score is associated with relatively high levels of palmitic acid, aspartate, serine, stearic acid, 6-hydroxyhexanoic acid, citrate and 2-hydroxyproline.
6.3 Results

Figure 6.6: Principal component analysis scores (unit variance scaled, log transformed data, a six component model, where $R^2X$ (cumulative) = 0.725, $Q^2$ (cumulative) = 0.413). A, B and C show scores for PC1 vs PC2; PC2 vs PC3 and PC2 vs PC4 respectively, with the addition of error bars (showing standard error of the mean score) for each MA line. Viewing the data in this way allows two groups to be seen clearly in PC2. D, a histogram which shows two groups of scores in the distribution of PC2, confirms this.
Figure 6.7: A box and whisker plot (showing median, range and interquartile range) confirms that the grouping observed on PC2 relates to two groups of MA lines, rather than some other source of variation. Lines are ordered according to their median score on PC2. It can be seen that the two groups seen in PC2 are related to individual lines; while some overlap and a slight gradient can be seen, generally lines either score high or low on PC2. The dashed line shows the point at which there is no overlap between the two groups.
Table 6.1: A set of t-tests were carried out on the median fold change normalised, log transformed data, comparing the mean level of each metabolite in each MA line with its mean level in ancestral worms. Summing the number of changed metabolites allowed the identification of the lines with the greatest number of metabolite level changes compared with ancestral worms. The analysis was carried out at four different levels of significance; $p \leq 0.1$, $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$.  

<table>
<thead>
<tr>
<th>Line</th>
<th>Number of metabolites changed</th>
<th>($p \leq 0.1$)</th>
<th>($p \leq 0.05$)</th>
<th>($p \leq 0.01$)</th>
<th>($p \leq 0.001$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>503</td>
<td>11</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>504</td>
<td>23</td>
<td>20</td>
<td>14</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>505</td>
<td>14</td>
<td>11</td>
<td>5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>507</td>
<td>23</td>
<td>20</td>
<td>15</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>508</td>
<td>21</td>
<td>16</td>
<td>9</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>509</td>
<td>12</td>
<td>12</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>511</td>
<td>23</td>
<td>21</td>
<td>15</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>513</td>
<td>11</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>514</td>
<td>22</td>
<td>19</td>
<td>12</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>516</td>
<td>17</td>
<td>14</td>
<td>10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>517</td>
<td>9</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>520</td>
<td>11</td>
<td>11</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>522</td>
<td>22</td>
<td>18</td>
<td>12</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>523</td>
<td>16</td>
<td>13</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>524</td>
<td>10</td>
<td>8</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>525</td>
<td>12</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>528</td>
<td>16</td>
<td>13</td>
<td>8</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>529</td>
<td>22</td>
<td>18</td>
<td>13</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>530</td>
<td>14</td>
<td>9</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>536</td>
<td>18</td>
<td>11</td>
<td>10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>540</td>
<td>23</td>
<td>22</td>
<td>15</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>542</td>
<td>20</td>
<td>17</td>
<td>14</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>543</td>
<td>29</td>
<td>27</td>
<td>24</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>544</td>
<td>20</td>
<td>16</td>
<td>13</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>546</td>
<td>17</td>
<td>10</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>547-B</td>
<td>15</td>
<td>13</td>
<td>12</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>548</td>
<td>13</td>
<td>9</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>552</td>
<td>16</td>
<td>13</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>555</td>
<td>17</td>
<td>14</td>
<td>14</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>563</td>
<td>20</td>
<td>18</td>
<td>11</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>564</td>
<td>25</td>
<td>23</td>
<td>20</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>567</td>
<td>21</td>
<td>20</td>
<td>10</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>570</td>
<td>24</td>
<td>17</td>
<td>8</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>571</td>
<td>10</td>
<td>8</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>573</td>
<td>27</td>
<td>23</td>
<td>21</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>574</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>575</td>
<td>19</td>
<td>14</td>
<td>8</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>577</td>
<td>12</td>
<td>9</td>
<td>5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>581</td>
<td>25</td>
<td>20</td>
<td>13</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>582</td>
<td>8</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>592</td>
<td>21</td>
<td>17</td>
<td>10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>593</td>
<td>13</td>
<td>11</td>
<td>6</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>595</td>
<td>19</td>
<td>17</td>
<td>11</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>598</td>
<td>16</td>
<td>16</td>
<td>12</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>
Some lines have more metabolite levels changed with respect to ancestral levels than others

T–tests were carried out on the median fold change normalised, log transformed data, comparing the mean level of each metabolite in each MA line with its mean level in ancestral worms. Summing the number of changed metabolites allowed the identification of the lines with the greatest number of metabolite level changes compared with ancestral worms. The analysis was carried out at four different levels of significance: \( p \leq 0.1 \), \( p \leq 0.05 \), \( p \leq 0.01 \) and \( p \leq 0.001 \) and the results are shown in Table 6.1, and as histograms in Figure 6.9. At the 95% confidence level, the lines that have the most metabolite level changes are 543 (27), 564 (23), 573 (23), 540 (22), and 511 (21). The number of metabolites whose levels are significantly different from ancestral levels is given in brackets for each line. The lines with the fewest
6.3 Results

Figure 6.9: Histograms showing the distribution of the number of metabolites with levels significantly different from ancestral levels per line for four significance levels; $p \leq 0.1$, $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$. 
6 Mutation accumulation

metabolite level changes are 525 (5), 574 (5) and 582 (7). The number of metabolites that have significantly changed levels compared with control levels correlates negatively with the mean score on PC2 for a given line at all significance levels (Figure 6.10).

Figure 6.10: The number of metabolites that have significantly changed levels compared with control levels in a given MA line correlates negatively with the mean score on PC2. This is the case at all significance levels.
### 6.3 Results

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Number of lines in which metabolite changes</th>
<th>(p≤0.1)</th>
<th>(p≤0.05)</th>
<th>(p≤0.01)</th>
<th>(p≤0.001)</th>
</tr>
</thead>
<tbody>
<tr>
<td>serine</td>
<td></td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>glycine</td>
<td></td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>nicotinic acid</td>
<td></td>
<td>33</td>
<td>32</td>
<td>28</td>
<td>23</td>
</tr>
<tr>
<td>succinic acid</td>
<td></td>
<td>44</td>
<td>43</td>
<td>40</td>
<td>32</td>
</tr>
<tr>
<td>porphine</td>
<td></td>
<td>13</td>
<td>9</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>uracil</td>
<td></td>
<td>11</td>
<td>6</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>fumaric acid</td>
<td></td>
<td>18</td>
<td>17</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>6-hydroxyhexanoic acid</td>
<td></td>
<td>17</td>
<td>15</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>methionine</td>
<td></td>
<td>31</td>
<td>27</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td>alanine</td>
<td></td>
<td>9</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>aspartate</td>
<td></td>
<td>37</td>
<td>30</td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td>3-aminoisobutyric acid</td>
<td></td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2-hydroxyproline</td>
<td></td>
<td>33</td>
<td>26</td>
<td>22</td>
<td>17</td>
</tr>
<tr>
<td>malic acid</td>
<td></td>
<td>20</td>
<td>15</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>pyroglutamic acid</td>
<td></td>
<td>23</td>
<td>23</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>glutamate</td>
<td></td>
<td>7</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>phenylalanine</td>
<td></td>
<td>9</td>
<td>7</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>asparagine</td>
<td></td>
<td>16</td>
<td>11</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>lyxose</td>
<td></td>
<td>21</td>
<td>18</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td>ribose</td>
<td></td>
<td>23</td>
<td>21</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>N-methyl-DL-glutamic acid</td>
<td></td>
<td>21</td>
<td>16</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>putrescine</td>
<td></td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>citric acid</td>
<td></td>
<td>10</td>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>adenine</td>
<td></td>
<td>14</td>
<td>11</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>allose</td>
<td></td>
<td>39</td>
<td>33</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>benzoic</td>
<td></td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>mannose</td>
<td></td>
<td>37</td>
<td>32</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>lysine</td>
<td></td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>tyrosine</td>
<td></td>
<td>16</td>
<td>10</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>pantothenic acid</td>
<td></td>
<td>32</td>
<td>30</td>
<td>18</td>
<td>13</td>
</tr>
<tr>
<td>xanthine</td>
<td></td>
<td>24</td>
<td>21</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>palmitic acid</td>
<td></td>
<td>22</td>
<td>12</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>uric acid I</td>
<td></td>
<td>12</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N-acetyl-L-histidine</td>
<td></td>
<td>10</td>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>heptadecanoic acid</td>
<td></td>
<td>31</td>
<td>31</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>tryptophan</td>
<td></td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>oleic acid</td>
<td></td>
<td>34</td>
<td>34</td>
<td>23</td>
<td>14</td>
</tr>
<tr>
<td>stearic acid</td>
<td></td>
<td>18</td>
<td>13</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>adenosine</td>
<td></td>
<td>31</td>
<td>30</td>
<td>26</td>
<td>22</td>
</tr>
<tr>
<td>trehalose</td>
<td></td>
<td>15</td>
<td>10</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

**Table 6.2:** Using t–test results comparing the mean level of each metabolite in each MA line with its mean level in ancestral worms, the number of lines in which the level of a given metabolite changed significantly compared with ancestral worms was calculated. This allowed the identification of the metabolites whose levels were most likely to change with the accumulation of mutations. Again, this was carried out at four different levels of significance; p≤0.1, p≤0.05, p≤0.01 and p≤ 0.001.
6 Mutation accumulation

Figure 6.11: For each metabolite identified and quantified, the fold change relative to the ancestral line was calculated. The log₂ fold change ratios for metabolites with relative concentration most likely to change with mutation accumulation are shown here. Of the metabolites with relative concentrations most likely to change with mutation accumulation, succinate, oleic acid and nicotinic acid all increase relative to the ancestral line while aspartate, methionine and 2-hydroxyproline all decrease relative to the ancestral line.
The levels of some metabolites are more likely to be affected by mutation accumulation than others

Using the same t-test results, the number of lines in which the level of a given metabolite changed significantly compared with ancestral worms was calculated. This allowed the identification of the metabolites whose levels were most likely to change with the accumulation of mutations. Again, this was carried out at four different levels of significance; \( p \leq 0.1 \), \( p \leq 0.05 \), \( p \leq 0.01 \) and \( p \leq 0.001 \). The results are shown in Table 6.2. At the 95% confidence level, the metabolites with levels that are most likely to change with mutation accumulation are succinate, oleic acid, allose, nicotinic acid, mannose, aspartate, 2-hydroxyproline and methionine. The metabolites with levels that are the least likely to change are lysine, tryptophan and glycine. Putrescine and 3-aminoisobutyrate both change in just one line. For each metabolite identified and quantified, the fold change relative to the ancestral line was calculated. The log\( _2 \) fold change ratios for metabolites with relative concentration most likely to change with mutation accumulation are shown in Figure 6.11. Of the metabolites with relative concentrations most likely to change with mutation accumulation, succinate, oleic acid and nicotinic acid all increase relative to the ancestral line while aspartate, methionine and 2-hydroxyproline all decrease relative to the ancestral line.

**Metabolite centrality**

In order to test the hypothesis that the propensity of the levels of a given metabolite to change as a result of mutation accumulation is correlated with the centrality of that metabolite, metabolite data from a mouse model were used (Selvarasu et al., 2010). The Spearman’s rank correlation coefficient (\( \rho \))
Figure 6.12: The Spearman’s rank correlation coefficient (\( \rho \)) and corresponding p-value were calculated to test the relationship between the propensity of a metabolite to change (the number of lines in which levels of a metabolite are changed relative to ancestral levels) and the centrality of that metabolite. Values were calculated for each significance level given in section 6.3. Betweenness centrality was found to be significantly negatively correlated with propensity to change with mutation (\( p = 0.048 \)) using \( p \leq 0.1 \) values for propensity to change. At \( p \leq 0.05 \), \( p \leq 0.01 \) and \( p \leq 0.001 \), p-values for correlation were 0.055, 0.078 and 0.085 respectively. A Reduced Major Axis (RMA) regression line is shown for each analysis.
and corresponding p-value were calculated to test the relationship between the propensity of a metabolite to change (the number of lines in which levels of a metabolite are changed relative to ancestral levels) and the degree and betweenness centrality of that metabolite. Values were calculated for each significance level given in section 6.3. Betweenness centrality was found to be significantly negatively correlated with propensity to change with mutation ($p = 0.048$) using $p \leq 0.1$ values for propensity to change. At $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, p-values for correlation were 0.055, 0.078 and 0.085 respectively. For significance levels $p \leq 0.1$, $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, $\rho$ values were -0.370, -0.360, -0.332 and -0.326 respectively.

For each of these significance levels, the correlation between betweenness centrality and propensity to change is shown in Figure 6.12. A Reduced Major Axis (RMA) regression line is shown for each analysis.

No significant correlation was found between propensity of a metabolite to change and degree centrality.

**Correlation between metabotype and fitness**

The Spearman's rank correlation coefficient ($\rho$) and corresponding p-value were calculated to test the relationship between each metabolite and fitness (based on total lifetime egg production). Fitness data were provided for the lines at three time points: after 100, 200 and 220 generations of mutation accumulation. As this study looked at the lines after 250 generations of mutation accumulation, this analysis was carried out using the fitness data for the closest time point of 220 generations. However, this fitness assay was carried out at 25°C, while populations grown for metabolomic analysis were incubated at 20°C. To take this temperature difference into
Figure 6.13: The Spearman’s rank correlation coefficient ($\rho$) and corresponding p-value were calculated to test the relationship between each metabolite and fitness (based on total lifetime egg production). This analysis was carried out on two fitness datasets: an assay at 20°C after 200 generations of mutation accumulation and an assay at 25°C after 220 generations of mutation accumulation. The relative concentration of only one metabolite, N-acetyl-L-histidine, was found to correlate with fitness in either assay ($p = 0.000096$ and $p = 0.00060$ for 200 generations and 220 generations respectively). A linear, Y on X regression and corresponding r value is shown for each analysis.
account, correlation analysis was also performed using fitness data recorded at 200 generations, as this assay was carried out at 20°C. This analysis was performed using mean values for total lifetime egg production and mean relative metabolite concentrations as well as mean scores on the principal components from the earlier PCA. A Bonferroni correction was applied to a 95% significance level, giving a corrected threshold of $p = 0.001$. At this level, the relative concentration of only one metabolite, N-acetyl-L-histidine, was found to correlate with fitness in either assay ($p = 0.000096, \rho = 0.634$ and $p = 0.00060, \rho = 0.544$ for 200 generations and 220 generations respectively). Figure 6.13 shows this positive correlation, with $r$ values based on a linear, $Y$ on $X$ regression. There was no correlation between fitness and the score on PC2, the principal component separating the lines into two groups.

6.4 Discussion

It is clear that the accumulation of mutations over 250 generations results in significant changes to the *C. elegans* metabolic profile. With the change in fitness that is observed as a result of this mutation accumulation, this is not surprising. However, rather than all MA lines having metabolomes that are changed in the same manner, the change in metabolome appears to tend towards two distinct end points, with the MA lines forming two groups in a PCA analysis along the second principal component (Figure 6.6). One group, consisting of ancestral worms and 24 of the 47 MA lines have a high score on PC2 while the remaining MA lines have lower PC2 scores. The mean score on PC2 is negatively correlated with the number of metabolites that have significantly changed over the 250 generations of mutation accumulation (Figure 6.10), indicating that one group contains lines that have changed
relatively little over the 250 generations in terms of metabolic profile and the second group contains lines which have metabolomes that have been more greatly affected by the accumulation of mutations. The grouping of the lines is consistent with the HCA in Figure 6.2, where one group can be seen to have a greater number of high fold change values than the other.

The separation of lines into two distinct groups is a surprising result. There are two outcomes that would have seemed more likely: firstly, that each MA line was metabolically distinct from the ancestral line in its own way. Secondly, that there would be a continuous spectrum of metabolic change, from ancestral worms to the lines with the most metabolites affected by mutation. One possible explanation is that the groups separate lines based on their developmental age. As described, lines were sampled at young adulthood, defined here as the point at which some eggs were seen on plates but no second generation worms had hatched. Once all lines had started laying eggs, all lines were sampled at one time point, so that all lines sampled were at the same chronological age. However, the slight variation in development times between lines may have resulted in slight variation in age in terms of development. It is possible that this may have contributed to the grouping of the lines, with worms with a delayed onset of reproduction looking metabolically less like ancestral worms than worms with development times close to those of ancestral, mutation–free worms. As stated, samples were randomised for harvesting, sample preparation, and analysis by GC–MS in order to eliminate as many external factors that may lead to grouping as possible. It is possible that other measures of fitness, such as body size, would correlate with score on PC2.
The groups are associated with two distinct metabotypes. High scores on PC2 (as seen in ancestral worms) are associated with relatively high levels of metabolites such as 2-hydroxyproline, serine and aspartate, and low levels of adenosine, xanthine and nicotinic acid. Low scores on PC2 (lines where more metabolites are affected by mutation accumulation) are associated with high levels of adenosine, xanthine and nicotinic acid, which may be related to changes in nucleotide turnover and degradation, and low levels of 2-hydroxyproline, serine and aspartate. Serine and aspartate are involved in sphingolipid metabolism, and relatively low levels of these metabolites in lines that have low scores on PC2 (and a greater number of metabolites differing from ancestral levels) point to an alteration of lipid metabolism in this group. It is possible that this is linked with a greater number of mutations in these lines relative to the ancestral line.

Some metabolites identified are more likely to change with mutation accumulation than others. These include succinate, oleic acid and nicotinic acid, which all increase relative to the ancestral line, and aspartate, methionine and 2-hydroxyproline, which decrease relative to the ancestral line. The relative concentrations of these 'most likely to change' metabolites appear to change in a largely uniform direction, i.e. they either increase or decrease in the great majority of MA lines. Nicotinic acid, involved with the metabolism of NAD+ and nicotinamide, has been linked with regulation of reproduction in *C. elegans* (Vrablik *et al.*, 2009), with delays in gonad development and defects in egg laying behaviour associated with depletion of NAD+ and accumulation of nicotinamide respectively.

Nicotinic acid is found at increased concentrations in those MA lines that appear to be more affected by mutation accumulation than others,
6 Mutation accumulation

and this change may be associated with a decrease in fitness in these lines. 2-hydroxyproline is a posttranslationally modified amino acid, which has been detected in dauers (Fuchs et al., 2010) and high levels are likely to be the result of protein degradation. Hydroxyproline is a major component of collagen and is one of the metabolites that is most likely to change (and decrease) in MA lines, and may point to changes in collagen production or degradation in MA lines. The increase in succinate relative to the ancestral line in all MA lines, suggesting that carbohydrate metabolism may be altered in a similar way in all lines. Oleic acid is a monounsaturated fatty acid, found to suppress stress resistance and reduce viability in C. elegans fatty acid metabolism mutants (Horikawa & Sakamoto, 2009). Oleic acid is increased in all but two MA lines relative to the ancestral line and this, along with the suggested involvement of fatty acid metabolism in regulating stress tolerance mechanisms may be linked with the decreased fitness seen in MA lines.

The levels of other metabolites are apparently more stable. For example, levels of the amino acids lysine, tryptophan and glycine change with mutation accumulation in very few lines and the level of putrescine changes in just one line. Putrescine is a product of the breakdown of amino acids, suggesting that this process may not be affected by mutation accumulation. It may be the case that these stable metabolites are critical for the survival of the worm.

A weak, negative correlation was observed between the propensity of the levels of a metabolite to change with mutation and its centrality (betweenness centrality only), or its connectedness, within a metabolic network. However, the centrality data used here were obtained from a mouse model (Selvarasu et al., 2010), and it may be the case that centrality values for metabolites in
a *C. elegans* metabolic network would yield more significant correlations in this type of analysis. These results suggest however, that highly connected, or critical metabolites may be less likely to change with mutation than less-critical metabolites. While correlation was seen between betweenness centrality and propensity to change, no significant correlation was observed between degree centrality and propensity of a metabolite to change. This suggests that the number of links incident on a given metabolite may not be a good indicator of how likely that metabolite is to change with mutation, and that the overall load placed on a metabolite in a network appears to be a more important factor.

Changes in only one metabolite, N-acetyl-L-histidine, were found to significantly correlate with fitness, here based on fecundity, and defined as the total number of eggs produced in the lifetime of a worm. It is not clear what role this metabolite has in *C. elegans*. N-acetyl-L-histidine is known to be present in the vertebrate nervous system, but its function is unknown (Baslow, 1997).

Estes *et al.* (2004) found that DNA sequencing of *C. elegans* mutation accumulation lines was able to reveal many evolutionary relevant mutations that had no measurable effect on laboratory fitness. The fact that no correlation was found between fitness and all but one of the metabolites identified demonstrates that metabolic profiling is able to provide new insights into the ‘silent’ effects of mutation accumulation.

Some of the MA lines received from the Baer lab did not survive the freezing process required for storage prior to this experiment. It is possible that these lines may have been some of the least fit MA lines. In addition to this, some lines did not produce enough progeny to provide sufficient biomass
for metabolomic analysis. The synchronisation of lines by hypochlorite treatment may also have resulted in the loss of worms unable to survive the process. However, this method provides the large number of synchronised worms required and also ensures that plates are free of contamination, and therefore was favoured over other synchronisation methods, such as allowing individuals to lay eggs over a given period and then removing the first-generation adults. These factors have contributed to the fact that this analysis was unable to include some of the most unfit lines. Metabolic profiling of these very unfit lines could have provided further insight into the effect of mutation accumulation on the *C. elegans* metabolome, and may have resulted in additional groupings being identified.

Additionally, on receiving the lines from the Baer lab, some bacterial and fungal contamination could be seen on many of the plates. In order to ensure only clean worms were frozen for storage, some worms from uncontaminated areas of each plate were transferred to clean plates and allowed to reproduce to give populations that were free of contamination. Similarly, for sufficient biomass, worms were transferred by ‘chunking’ onto new plates before synchronisation. It is possible that by selecting more than one worm at these transfers, some lines were able to recover to some degree as a result of natural selection. *C. elegans* MA lines are known to recover quickly on the reintroduction of natural selection (Estes & Lynch, 2003; Estes et al., 2011). However, at the time of sampling, all lines appeared to have slightly smaller body sizes, reduced speed of movement and reduced fecundity compared with the ancestral line, although no fitness assays were carried out at this point. Ideally, fitness data should be obtained at this point in order to help the interpretation of metabolomic data. While very little correlation was identified between metabolomic data and the fitness data.
provided by the Baer lab, it may be the case that other measures of fitness, such as body size, would better correlate with metabolomic data.

Conclusion

The accumulation of mutations over 250 generations results in significant changes to the *C. elegans* metabolome. Using the fitness data available for these MA lines, it is not possible to find links between these metabolic changes and the fecundity phenotype. However, not all MA lines change in the same way in terms of metabolism – it appears that the metabolism of some lines is less affected by mutation accumulation than that of other lines. These lines can be separated into two groups: one group containing lines that have changed relatively little over 250 generations, and a second group containing lines with metabolomes that have been more greatly affected by the accumulation of mutations. In addition to this, the levels of some metabolites appear to be more susceptible to change than others, with the levels of some, presumably critical, metabolites remaining stable with mutation accumulation. Initial analysis suggests that the propensity of a metabolite to change, however, may not be linked with its centrality or connectedness in a metabolic network. While changes in metabolic profile were not found to correlate with the majority of the fitness data available for these MA lines, this analysis has shown that metabolomics provides new phenotypes and therefore novel information about variation that occurs as a result of spontaneous mutation.
6 Mutation accumulation
7 Discussion

Metabolomics for the study of ageing

While there is a clear link between changes in metabolism and changes in longevity (discussed in section 1.3), there has been relatively little ageing–related research investigating metabolism by measuring metabolites directly. Research has generally concentrated on genes that control metabolism, but this does not take into account regulation at post–transcriptional or post–translational levels. Metabolomics – measuring low molecular weight metabolites directly – has an advantage over other ‘omics’ techniques, in that it directly samples the metabolic changes in an organism, and allows for variation at the gene, transcript and protein levels, as well as post–translational modification (ter Kuile & Westerhoff, 2001). In addition to this, metabolites, as clearly defined chemical compounds, are directly comparable between species, while genes and gene transcripts are often species–specific.

Metabolic profiling not only allows the direct analysis of the metabolic changes that occur with age, or may contribute to a long life, but also provides new, intermediate phenotypes that may help distinguish between individuals with a superficially similar ‘end–point’ phenotype – longevity.
This is clearly demonstrated in Chapter 3, where three strains with lifespans that are not seen to differ significantly from one another have distinct metabolic profiles. Even daf-16 mutants, with wild type longevity, are separable from wild type worms based on metabolic profile. While it has been shown that low levels of DAF-16 may be found in the nuclei of wild type worms (Cypser & Johnson, 2003; Weinkove et al., 2006), this experiment shows just how sensitive metabolism may be to changes in an important regulator of lifespan, even if no change in longevity is seen. In addition to this, identifying those changes in metabolism that are dependent on the function of this key regulator of ageing should help to focus future research, by aiding the generation of testable hypotheses. By examining the metabolic profile of mutants that are long–lived via a variety of different mechanisms – IIS mutants, dietary restriction mutants, fertility mutants and so on, it may be possible to identify a common metabolic signature of long life, or cluster long–lived mutants into distinct groups.

A metabolic signature of long life

Branched–Chain Amino Acids

One metabolic signature that is seen to be associated with long life across all experiments here, is an increase in the relative abundance of the Branched–Chain Amino Acids (BCAAs) – isoleucine, leucine and valine. These are seen to decrease with age in both wild type worms and in at least one daf-2 mutant (Chapter 5), and are found at increased concentration at a given age in two of the daf-2 mutants tested here (Chapters 3 and 5) as well as in other long–lived mutants (Fuchs et al., 2010). In addition to this, it has been
shown that these changes are DAF-16 dependent (Chapter 3), suggesting that these metabolites have a causal role in ensuring a long life. This finding has since been replicated in *C. elegans* (Martin *et al.*, 2011). The fact that BCAAs are found at increased levels in protein translation mutant *ife-2* (Fuchs *et al.*, 2010), which has increased longevity that does not require DAF-16, but appears to work either downstream or in parallel to DAF-2 to regulate longevity, is an indication that these amino acids are important for long–life, regardless of how long–life is conferred.

BCAAs have found to be important in the longevity of both yeast and mice (Alvers *et al.*, 2009; D’antona *et al.*, 2010), where supplementation with BCAAs has been found to increase lifespan. In a mouse model, an increase in lifespan with BCAA supplementation is associated with increased mitochondrial biogenesis, with no lifespan extension seen in mice with mutations impairing mitochondrial biogenesis. The treatment is also associated with the up–regulation of ROS defence system genes, both processes contributing to an overall decrease in oxidative damage. BCAA supplementation in *C. elegans* also increases lifespan, although this has not yet been replicated (Valbuena, pers. comm., 2011).

*C. elegans* is not able to synthesise BCAAs and so changes in pool sizes depend on changes in the catabolism of BCAAs or in protein turnover. The down–regulation of genes involved in the catabolism of BCAAs may be responsible for the changes in BCAA pool sizes in long–lived *daf-2* worms (Fuchs *et al.*, 2010), and it has been found that the transcripts of genes associated with BCAA metabolism vary in other long–lived *C. elegans* mutants (Martin *et al.*, 2011).
Discussion

BCAA supplementation in mice increases the activity of mammalian target–of–rapamycin (TOR) complex 1 (mTORC1), a key regulator of cell growth and protein synthesis in response to dietary amino acids (Valerio et al., 2011). TOR protein kinases are involved in signalling in response to environmental nutrient availability, and the *C. elegans* TOR homologue regulates dauer diapause (Kim et al., 2002; Hara et al., 2002). It appears that in mice, mTOR signalling activated by BCAA supplementation may enhance biogenesis of mitochondria by increasing the generation of NO, with endothelial nitric oxide synthase (eNOS) gene silencing decreasing the activation of mTOR by BCAA supplementation (D’antona et al., 2010). eNOS–derived nitric oxide directly increases mitochondrial biogenesis (Nisoli et al., 2004) and eNOS Null mutant mice have age–related diseases at a young age, a reduced lifespan, reduced mitochondrial biogenesis and reduced SIRT1 expression (Li et al., 2004). While changes in mTOR signalling have been suggested as a possible mechanism involved in increased longevity by dietary restriction, whether signalling is up– or down– regulated may depend on age and type of dietary restriction applied (reviewed in Valerio et al., 2011).

One hypothesis has been put forward to attempt to explain why dietary supplementation with BCAAs promotes mitochondrial biogenesis. Valerio et al. (2011) suggest that an increase in dietary BCAAs induces mitochondrial biogenesis in order to promote amino acid catabolism itself. Amino acids are key precursors of components of the citric acid cycle, and the catabolism of amino acids lead to ammonia production. Ammonia is metabolised via the urea cycle, which occurs, in part, in the mitochondrial matrix.
Leucine in particular appears to be important in mTOR signalling, with leucine deprivation in mammalian cell culture media inhibiting mTOR signalling (Haar et al., 2007). Leucine also acutely stimulates the secretion of insulin from pancreatic B cells (Yang et al., 2010), and rats fed leucine show an increase in serum insulin (Garlick, 2005). BCAAs are found to be increased in worms with mutations a gene encoding insulin–like peptide, daf-28 (Fuchs et al., 2010) as well as in daf-2 worms, with mutations affecting the insulin–like receptor. This link between leucine and insulin signalling could point to a mechanism behind the increased pools of this BCAA seen in insulin signalling mutants.

**Trehalose**

Trehalose levels were found not to be DAF-16 dependent with either NMR or GC–MS analysis (Chapter 3). This is consistent with findings that lifespan extension with trehalose in wild type worms does not require DAF-16, and that trehalose treatment does not promote the translocation of DAF-16 to nuclei, or increase the expression of sod-3, a known target of DAF-16 (Honda et al., 2010). However, daf-2 worms show no increased longevity with trehalose treatment, and RNAi inactivation of trehalose biosynthesis genes in daf-2 worms results in a large decrease in lifespan, compared with a small decrease in lifespan in wild type worms (Honda et al., 2010). This suggests that daf-2 mutation and treatment with trehalose extend lifespan via a common mechanism, with daf-2 regulating levels of trehalose via a transcription factor that acts parallel to DAF-16, and that trehalose functions as a suppressor of ageing under control of IIS (Honda et al., 2010).
In addition to being elevated in the daf-2 mutants studied here, trehalose levels have been found to be elevated in age-1 mutants, dauers, and the long–lived translation–defective mutant (where longevity is not DAF-16 dependent) described above (Fuchs et al., 2010), and genes involved in its synthesis are known to be upregulated in IIS mutants (Lamitina & Strange, 2005; McElwee et al., 2006), implying that this metabolite may still have a role in longevity. Trehalose levels change differently in wild type and daf-2 mutants as they age (Chapter 5). In wild type worms, trehalose levels decrease with age, while in long–lived daf-2(m41), levels increase with age.

Trehalose treatment has been shown to enhance thermotolerance, and RNAi inactivation of trehalose biosynthesis genes increase the sensitivity of C. elegans to heat stress (Honda et al., 2010). Many links between enhanced heat–stress tolerance and increased lifespan have been found (Lithgow et al., 1995; Gems et al., 1998; Hertweck et al., 2004), with trehalose–mediated thermotolerance mechanisms thought to be a result of the stabilising of protein structures; trehalose prevents both protein denaturation as a result of heat, and the aggregation of misfolded proteins (Singer & Lindquist, 1998; Jain & Roy, 2008).

Honda et al. (2010) found that trehalose appears to lengthen C. elegans healthspan as well as lifespan, with both mean and maximum lifespan increased with trehalose treatment, along with a lengthened reproductive span and slowing of age–associated phenotypes such as the decline in pharyngeal pumping rate. Based on age–specific mortality rates, the authors conclude that trehalose lowers age–dependent vulnerability to both internal and external stresses, rather than reducing the intrinsic rate of ageing. The
authors suggest that trehalose protects against the increased stress that occurs with age, but is unable to repair damage to proteins that has already occurred during the ageing process. This can be directly linked with the pattern of change in trehalose levels with age observed here (Chapter 3, Figure 5.16).

**Metabolomics for Mutation Accumulation studies**

The type of analysis carried out using mutation accumulation (MA) lines here differs from that used in the majority of MA experiments. Most MA experiments are concerned with determining the rate of spontaneous mutation, however the purpose of using metabolic profiling to study MA lines here was to study the phenotypic impact of mutations, rather than the rate of mutation. While determining the rate of spontaneous mutation is important in understanding many biological processes, including genetic disease and the evolution of sex, in addition to the evolution of ageing, there is debate over the use of mutation accumulation experiments over many generations as a reliable way to measure per–generation mutation rate (reviewed by Kondrashov & Kondrashov, 2010). Measuring mutation rate is difficult for a number of reasons. Firstly, the per–nucleotide rate of mutation is very low, particularly in eukaryotes. In addition to this, the random nature of mutation makes it difficult to know when and where mutation might occur. The rates of different mutation–types (such as point mutations and large–scale mutations) need to be measured separately, and ideally mutation rates should take into account varying genetic backgrounds,
in addition to various mutation–types. Lastly, the mutation rate is heavily influenced by the environment, and the rate of mutation itself is subject to selection and evolution (Kondrashov & Kondrashov, 2010).

Mutation rate may be measured in various ways (reviewed by Kondrashov & Kondrashov, 2010), including the use of fitness data from MA experiments to estimate per–generation mutation rates. However mildly deleterious mutations may not be detected, and it is known that many evolutionary relevant mutations have no measurable effect on laboratory fitness (Estes et al., 2004). This first metabolomic analysis of mutation accumulation has demonstrated that metabolic profiling is able to reveal new phenotypes that are affected by mutation accumulation in *C. elegans*, and that these phenotypes are not necessarily associated with fitness (Chapter 6). However, estimating per–generation mutation rates with these data would require more than the single ancestral line profiled here as control lines (Baer et al., 2006).

It is known that the most highly connected proteins in a cell are the most important for survival, with the deletion of these proteins associated with a lethal phenotype (Jeong et al., 2001). However, the propensity of a given metabolite to change with the accumulation of mutations does not correlate with the available values (based on a mouse model) for metabolite centrality or connectedness. Analysis by Mahadevan & Palsson (2008) suggests that in genome–scale metabolic networks, even the least connected metabolites are as critical to the overall network as the most connected nodes, and this may explain these findings. Despite this, it may be the case that results based on a *C. elegans*–specific metabolic network may be different. The fact that changes in metabolite levels did not correlate with fitness (for all but one
metabolite) adds to the difficulty in interpreting these results. Additionally, the propensity to change may correlate with other network properties of a metabolite. Further work in this area is needed, but metabolomic analysis of mutation accumulation may still provide information about the network properties of metabolites.

**How can we interpret metabolomic data?**

Metabolic profiling generates large amounts of data. One of the issues raised with this approach is the question of how to interpret this data – it is often difficult to link changes in metabolite levels with the reasons and mechanisms behind these changes. The fact that there are fewer metabolites than there are genes or gene products means that it is not possible to find direct relationships between metabolites and genes (Raamsdonk et al., 2001). Mutations may have a small, or silent effect on phenotypes such as growth rate because the concentration of intracellular metabolites are altered to compensate for the mutation. By quantifying the relative changes of metabolite concentrations that occur as a result of ‘silent’ mutations, it should be possible to identify the site of action of the product of a gene, and to identify new, silent, regulatory genes (Raamsdonk et al., 2001).

The number of metabolites identified and quantified here, with this untargeted analysis, is generally not sufficient for linking changing pool sizes with pathways. Identifying more metabolites may help this type of pathway-based analysis. One way to go about interpreting metabolic data in terms of networks and pathways is the use of mathematical modelling. Broadly, there are two approaches to modelling: genome-scale modelling, and kinetic
modelling. In addition to this, results may be interpreted using a statistical approach.

**Genome–scale modelling**

Genome–scale metabolic modelling involves reconstructing a model of the metabolic network of a given organism based on an annotated genome sequence (Fell et al., 2010). The ideal would be a database of all reactions and metabolites that could possibly occur in an organism, complete with their linking all enzymes and proteins to their associated genes.

The definition of the stoichiometries of many reactions, and the grouping of shared metabolites have helped to define traditional pathways (Papin et al., 2003). Constraint–based genome–scale modelling determines the optimal path through a stoichiometric network within certain physicochemical constraints (Smallbone et al., 2007). Genome–scale modelling makes use of genome annotation to describe the components of a metabolic network, along with parameters such as cellular growth and flux (Papin et al., 2003). Metabolic flux is the way in which metabolites passage through a reaction with time (Zamboni & Sauer, 2009), and fluxes vary less than do metabolite pools with mutation (Raamsdonk et al., 2001). Metabolic flux can be determined directly, using time course data, where pathway intermediates are enriched with stable, dynamic isotopes. They may also be inferred using macroscopic uptake and production rates, with steady state isotope–labelling of metabolites. Both metabolic flux analysis (Bonarius et al., 1997) and flux balance analysis (Varma & Palsson, 1994) use steady state conditions, where, in a given network, all metabolite concentrations are constant, though there may be flux through the reactions (Fell et al., 2010).
Schilling et al. (1999) used this approach in an attempt to predict genotype–phenotype relationships in *E. coli*, creating a stoichiometric model to describe all known reactions in *E. coli* cells, and predicting phenotypes for various deletion mutants. Predictions made using this model were accurate in around 90% of deletion mutants examined.

This type of modelling does not make use of metabolite concentrations or enzyme connections in its parameters, so requires minimal biological data, however this means that it is not able to predict changes in metabolite or enzyme concentrations (Smallbone et al., 2007).

**Kinetic modelling**

Kinetic modelling aims to simulate the dynamics of a system (Smallbone et al., 2007). It combines mechanistic rate equations with kinetic parameters such as metabolite concentrations, enzyme concentrations and *V*\(_{\text{max}}\) (the maximum rate achieved by a system at saturating substrate concentrations) to fully characterise the mechanics of each reaction in a network. This type of modelling attempts to predict how changes in metabolite concentrations may affect local reaction rates.

This approach has been used to model many processes in microbes, including glycolysis (Rizzi et al., 1997), the pentose phosphate pathway (Vaseghi et al., 1999) and central nitrogen metabolism (van Riel et al., 1998) in the yeast *S. cerevisiae*. These models apply to very short time scales, however (Rizzi et al., 1997), and therefore do not take into account enzyme synthesis and degradation, which would be an important factor over larger timescales.
In contrast to genome–scale modelling, kinetic modelling requires a large amount of data, with models requiring many parameters, and this is generally both time–consuming and expensive. In addition to this, is it often difficult to determine many parameters experimentally (Smallbone *et al.*, 2007).

**Statistical interpretation**

One way to interpret data involves the use of enrichment or over–representation analysis. Enrichment analysis is a computational method that determines whether a defined set of metabolites differ significantly between two phenotypes. This type of approach is common in the analysis of gene expression data sets (Khatri & Draghici, 2005), but may also be used for metabolomic analysis (Subramanian *et al.*, 2005; Xia & Wishart, 2010; Chagoyen & Pazos, 2011). A set of metabolites that are found to be correlated with a phenotype of interest is selected and compared with a priori–defined sets of metabolites, i.e., metabolites that are grouped based on prior biological knowledge, using databases of metabolite annotations specific to a particular organism (Chagoyen & Pazos, 2011). This helps to identify sets of metabolites that show greater overlap with metabolites associated with a particular phenotype than would be expected by chance, generating a list of significantly over–represented or ‘enriched’ pathways or metabolite sets that may be used to aid the biological interpretation of the data (Cavill *et al.*, 2011).

**Are these techniques applicable to *C. elegans* metabolomics studies?**

While the modelling methods described are clearly very useful for the biological interpretation of metabolomics data, the main obstacle in using
these approaches in *C. elegans* metabolomics studies is that there are no *C. elegans* metabolic models available, and no agreed metabolic networks. This type of approach would require the generation of metabolic networks specific to *C. elegans*, as well as the stoichiometry associated with each reaction. Additionally, the problems associated with the modelling of whole multicellular organisms would need to be addressed. Both genome–scale modelling and kinetic modelling work best in single–cellular organisms such as yeast or bacteria, and are more complex and less effective in multicellular organisms such as *C. elegans*, where there are a variety of different cell types containing enzymes specific to a given cell type.

Constraints–based modelling has been applied to ageing in *Drosophila* (Feala *et al.*, 2007; Coquin *et al.*, 2008), with the investigation of the decline in hypoxia tolerance with age. Here, metabolomics, the annotated *Drosophila* genome, and existing *Drosophila* metabolic models were combined to generate hypotheses for the biological mechanisms behind this changing phenotype. The problems of modelling in organisms other than microbes were overcome by using just one tissue–type – muscle.

In addition to this, these models are better suited to examining rapid changes, rather than slow changes such as development, ageing, or the accumulation of mutations, and most functional analysis focuses only on changes produced by the mutation of a single enzyme. The genetic control of ageing is linked with receptors, such as DAF-2, and transcription factors, such as DAF-16. Mutations in the genes associated with these proteins produce multiple changes, unlike a mutation in a single enzyme. While the work required for modelling *C. elegans* metabolism is beyond the scope of this thesis, further studies would benefit from this approach.
Of the methods of interpreting metabolomic data described, enrichment or overrepresentation analysis is most applicable to *C. elegans* metabolomics studies. However, the relatively small number of metabolites identified here, even with the use of a relatively sensitive platform such as GC–MS, means that there is little power to obtain a substantial amount of additional insight using these techniques. The identification and quantification of a larger number of metabolites would give more power to this approach.

Additionally, it is not always possible to link changes identified in a particular pathway with the mechanisms behind these changes, as metabolites often contribute to multiple pathways. It is also difficult to define pathways, and although pathway databases such as KEGG (Kyoto Encyclopedia of Genes and Genomes (Kanehisa, 2006)) exist, pathway definitions are often arbitrary.

The use of metabolomics for *C. elegans* ageing research: advantages and limitations

The metabolic profiling of *C. elegans* here has allowed the prediction of results of research published subsequently (D’antona et al., 2010; Honda et al., 2010). Epistasis analysis and life cycle analysis both predicted the importance of branched–chain amino acids in longevity. That these metabolites, when fed to mice (D’antona et al., 2010), would increase lifespan, could have been predicted using metabolomic analysis. Similarly, the importance of the timing of trehalose treatment in extending *C. elegans* lifespan (Honda et al., 2010) could have been predicted using relative changes in trehalose levels that occur naturally in worms as they age. It is possible to see these changes
without modelling or complex statistical analysis, and this demonstrates the
usefulness of metabolomic analysis in ageing research; it identifies longevity–related metabolites, and helps to generate easily–testable hypotheses. A next step would be to test the effects of other DAF-16 repressible metabolites such as dopamine, putrescine, adenosine, citrate and phenylalanine (Chapter 3, section 3.2.2) on *C. elegans* lifespan.

Here, the epistatic interaction between *daf-2* and *daf-16* helped to focus on metabolites that appeared to have a causal role in longevity, based on the known importance of DAF-16 in longevity. By studying other epistatic interactions linked with longevity, for example, the *eat-2 / pha-4* pathway thought to be involved in lifespan extension by dietary restriction (Panowski *et al.*, 2007), it should be possible to identify common, or new metabolites important for increased lifespan. This type of approach, along with the identification of a greater number of metabolites, may help establish additional signatures of longevity.

As discussed, to find a true ‘signature of longevity’, many different life span mutants, with longevity altered by a variety of mechanisms, would need to be analysed. Any common metabolic features across mutants would help provide a real insight into what, on a metabolic level, helps promote long life. However, this would require the identification and quantification of many more metabolites that have been detected here, so that metabolites important in longevity are not overlooked – currently it is difficult to know how many of the total metabolites in an organism are being detected (Weckwerth & Morgenthal, 2005).

Metabolomics also yields interesting results with pattern recognition–type analysis. For example, using a model wild type metabolic profile, *daf-2*
worms are predicted to be younger than their chronological age in both development and adulthood, and this is consistent with the fact that long–lived daf-2 worms can be seen to age more slowly than wild type worms, remaining fertile and more active for longer than wild type worms (Kenyon et al., 1993; Morris et al., 1996; Kimura, 1997), with the increase in lifespan relative to wild type worms in long–lived mutants including age-1, clk-1 and daf-2 occurs post–reproduction (Friedman & Johnson, 1988; Chen et al., 2007). Assessing the changes in metabolism with age and the differences in these changes in long–lived individuals allows us to begin to answer the question of whether long–lived mutants simply have a reduced rate of ageing, or whether they follow completely different ageing trajectories, with different metabolic changes. These type of analyses are useful, despite the fact that the results may not be interpretable mechanistically.

Conclusion

In this thesis, I have demonstrated that metabolic profiling provides a new and useful phenotyping tool for studying ageing in the nematode Caenorhabditis elegans. Using two platforms, I have identified metabolites that are linked to long life. I have carried out the first characterisation of the C. elegans metabolome throughout both development and ageing. Comparing these metabolic changes in wild type worms with those seen in a long–lived mutant aid the understanding of when and how mutant worms acquire their long–lived phenotype. In addition to this, I have examined the effects on metabolism of a commonly–used technique in C. elegans ageing research: the inhibition of DNA synthesis to maintain synchronous populations. This provided a way to control for the effects of this technique when used in my
work, but also demonstrated that its use may result in artefacts in data. I have also carried out the first study of the effect of mutation accumulation on the *C. elegans* metabolic profile. I have shown that metabolomics provides a way to obtain new phenotypes in this type of experiment, and novel information about the variation that occurs as a result of spontaneous mutation.
7 Discussion


Banay-Schwartz, M., Lajtha, A., & Palkovits, M., 1989. Changes with aging in the levels of amino acids in rat CNS structural elements. i. glutamate


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography

102(43):15545–50.


