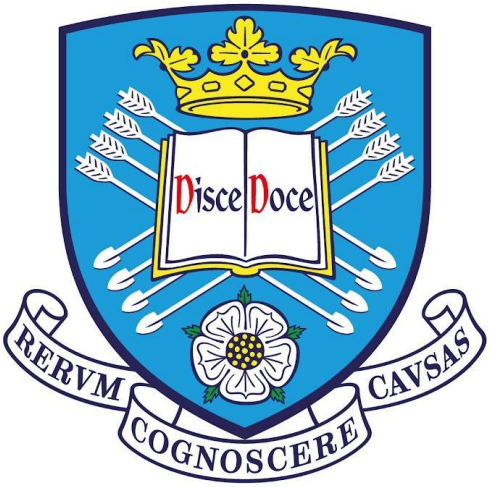

Metabolomics and proteomics of dietary restriction in *Drosophila*

by

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Dulcius ex asperis

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Declaration of intellectual contribution

I, the author, confirm that the Thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has not been previously presented for an award at this, or any other, university. Each chapter contains a list of author contributions.

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Abbreviations

Acronym	Definition	Acronym	Definition
AICAR	5-Aminoimidazole-4-carboxamide ribotide	m/z	Mass to charge
ATP	Adenosine 5'-triphosphate	MS	Mass spectrometry
BPC	Base peak chromatogram	MTHF	(6R)-5,10-Methylene-THF
CI	Confidence interval	NAD	Nicotinamide adenine dinucleotide
CPS	Counts per second	NGS	Next generation sequencing
CR	Caloric restriction	NIA	National institute on ageing
DNA	Deoxyribonucleic acid	NMN	Nicotinamide mononucleotide
DR	Dietary restriction	NR	Nicotinamide riboside
dTMP	2′-deoxy-thymidine-5′-monophosphate	OMP	Orotidine-5’-monophosphate
dUMP	Deoxyuridine monophosphate	P:C	Protein to carbohydrate ratio
EIC	Estimated ion chromatogram	PA	Palmitic acid (Palmitate)
FA	Formic acid	PC	Principal component
FC	Fold change	PP-IX	Protoporphyrin-IX
FDR	False discovery rate	RNA	Ribonucleic acid
GFN	Geometric framework of nutrition	RT	Retention time
GPI	Glycosylphosphatidylinositol	SE	Standard error
IMP	Inosine monophosphate	TCA cycle	Tricarboxylic acid cycle
IPO	Isotopologue parameter optimisation	TMT	Tandem mass tag
ITP	Interventions Testing Program	UDP	Uridine diphosphate
KEGG	Kyoto Encyclopedia of Genes and Genomes	UMP	Uridine-5’-monophosphate
LC-MS	Liquid chromatography mass-spectrometry	WGS	Whole-genome sequencing
LT	Leukotriene		

Contents

Summary

The life-extending paradigm of Dietary Restriction (DR) is one of the most powerful yet poorly understood geroprotective mechanisms known to modern man. DR restricts a specific dietary component, often total protein content, to reduce mortality without malnutrition. Though progress continues to be made in disentangling the multi-faceted effects of dietary restriction, not one mechanism associated with DR physiology recapitulates its benefit in totality. Therefore, my focus is to better understand the mechanisms underpinning the DR effect. In this thesis, I employ a mechanistic approach combining demographic, metabolomic, and proteomic approaches to better understand the biology of DR. I begin with the introduction, discussing aspects of ageing and the current state of DR within the current literature and discusses how genomic approaches have become relatively commonplace, whilst mass-spectrometry-based technologies continue to be underutilised. In chapter one, I explore the demographic response to the application of short- and long-term DR within *Drosophila melanogaster*. I discuss the immediate reduction in age-specific mortality in response to DR and the recently discovered mortality overshoot phenomenon. In chapter two, I explore the metabolomic mechanisms which underpin the switching paradigm in *Drosophila melanogaster* and identify known (ceramide, NAD+ and uridine) and potentially novel (xylitol, palmitate) mediaries of DR. In chapter three, I compare the effects of DR on the metabolome across a further three species of fly (*Drosophila ananassae*, *Drosophila biarmipes* and *Drosophila virilis*), identifying conserved interspecies mediators of longevity (palmitate and uridine). In chapter four, I return to the switching paradigm, investigating the proteomic responses to short- and long-term DR in *Drosophila melanogaster* to complement the metabolomic findings identified previously. We discover a significant (>50% proteome) long-term adjustment to DR and a strong response to refeeding. Surprisingly, we find no evidence of proteomic change in response to DR which contravenes biological expectations. Finally, I critically evaluate and discuss my research findings within the context of the current literature and highlight the overall importance of the work.

General Introduction - The healthspan extending paradigm of dietary restriction.

This section provides an introduction to dietary restriction (DR). I begin by outlining the fundamental concepts which underlie studies of ageing more broadly before focusing on the concept and real-world applications of dietary restriction and how it is yet fully understood. Specific attention is given to modern omics approaches and how certain technologies are favoured over others, possibly at the cost of a more complete understanding.

Ageing as a target to improve human health

Ageing is a field of research affecting all members of society. Technological advancement and improvements in healthcare provision have driven an exponential increase in global life expectancy in the 20th century (Riley 2005; Infra *et al.* 2015). However, the exact biological causes of ageing remain elusive. Ageing may be defined simplistically as the process that converts healthy young adults, into older ones, with an increased risk of illness and death (Miller 2002). An umbrella cause of ageing is thought to be the imbalance between accrued damage and repair which causes organisms to deteriorate over time (Haigis and Yankner 2010). The inevitable toll of ageing can be observed in almost all life forms, usually from the onset of sexual maturity (Medawar 1946). Ageing drives tissue dysfunction, decreased reproductive output and disease (Kirkwood 2005; Hayflick 2007; Kirkwood and Shanley 2010). Living longer and functionally healthier lives are key to both medical science and the alleviation of the economic burden of an ageing population (Goldman 2016). A highly debated question in gerontology has been whether ageing should be classified as a disease or not (Caplan 2005; Bulterijs *et al.* 2015; Faragher 2015; Stambler 2017). I would argue that ageing should be defined as a disease, even though it is a biological norm, as cumulative pathological risk increases exponentially with age. As such, ageing is the single greatest risk factor for the leading causes of death in modern society (Kaeberlein 2013). Therefore, as we get older it becomes increasingly beneficial to directly target the molecular processes of ageing to proactively protect against and delay future costs to health (Olshansky *et al.* 1990; Hopkin *et al.* 2003).

Hallmarks of Ageing

There are several known hallmarks of ageing, which may lead to the breakdown of cellular function that is observed during ageing (López-Otín *et al.* 2013). These ‘hallmarks’ are predominantly expected to result from the accumulation of damaged macromolecules such as lipids and proteins and damage to the DNA of the cell and mitochondria (Partridge and Gems 2002). The balance of cellular homeostasis through, for example, signalling pathways or the action of antioxidants and free radicals is essential for proper physiological function (Lobo *et al.* 2010; Hartl 2016). Such imbalances are interconnected and cause or effect is often unclear. For example, the production of such chemically reactive species increases with age (Haigis and Yankner 2010), but whether this is a result of dysregulation of antioxidant machinery or an inherent feature of ageing remains to be determined. Somatic DNA damage has traditionally been thought of as a major force in ageing (Cagan *et al.* 2022), but evidence in support of this mechanism is not equivocal (Franco *et al.* 2022). However, damage in the form of somatic mutations, fits many of the phenotypes and mechanisms of ageing. For example, the accruement of DNA damage may disrupt gene expression, resulting in proteotoxicity (due to damage-induced protein misfolding), or jeopardise the structural stability of the cell (Kenyon *et al.* 2010; Seviour and Lin 2010; Koga *et al.* 2011; Goloubinoff 2016). Such hallmarks define the consequences of cellular senescence, a process by which cells stop dividing and enter a state of permanent growth arrest (López-Otín *et al.* 2013). Although the accumulation of senescent cells is perceived as a deleterious consequence of ageing (Baker *et al.* 2011), the process of cellular

senescence is in fact, vital, as it protects against the immediate consequences arising from the unrestricted cellular division of damaged cells (Hayflick and Moorhead 1961; Bodnar *et al.* 1998).

Therefore, the accumulation of damage in the face of entropy, or “wear and tear” over time is considered the main major driving force of ageing (Niccoli and Partridge 2012). However, the tightly regulated environment of cellular dynamics has been carefully selected to maximise Darwinian fitness. Therefore, genes that extend longevity after reproduction, or later in life, do not improve organismal fitness necessarily, and thus the force of selection diminishes (Medawar 1946; Medawar 1952; Kirkwood 2005). The evolutionary benefit of a longer lifespan is inconsequential if an organism does not gain fitness from this adaptation, due to extrinsic mortality for example or other ecological reasons. This suggests that the benefits of modern lifestyles and medical interventions may highlight a suboptimal solution to an extended lifespan. This is supported by the fact that the observed extension of longevity is not accompanied by an increase in disease-free life expectancy (Franco *et al.* 2007). Therefore research into ageing has the greatest potential return on investment as it may improve public health to a far greater extent (at a much-reduced economic cost) than targeting other end-point diseases individually (Miller 2002).

Experimental manipulation of ageing

One way to understand ageing is to study ways to extend lifespan and understand the mechanisms of how this is achieved. Approximately 25% of the natural variation in human lifespan is determined by genetics (Passarino *et al.* 2016). The genetic mediators of natural ageing are predominantly controlled by homeostatic and stress response pathways (Tatar *et al.* 2003; Rattan 2008; Haigis and Yankner 2010; Kenyon *et al.* 2010; Koga *et al.* 2011; Hartl 2016). Though genetic determinants are not the only source of variation, despite recent technological advances they still remain largely outside our direct control (Lino *et al.* 2018; Liang *et al.* 2020; Zuccaro *et al.* 2020; Alanis-Lobato *et al.* 2021) By extension, environmental factors determine 75% of overall longevity. It is commonly believed that a fit and healthy lifestyle is beneficial to lifespan, and research provides evidence for this assumption. Decreasing the intake of harmful substances and increasing the amount of aerobic exercise have been proven to greatly increase longevity (Steppan *et al.* 2014; Staff and Maggs 2017; Mandsager *et al.* 2018).

Caloric restriction (CR), the reduction of total caloric intake without starvation, has long-since been the ‘gold standard’ in biogerontological pursuits since its initial discovery, extending lifespan up to 50% (McCay *et al.* 1935). However, caloric restriction is a relatively severe intervention with several detrimental effects (Lee and Longo 2016), such as a decrease in bone mineral density (Villareal *et al.* 2006). In addition, strict lifetime adherence is improbable (Wing and Phelan 2005). Dietary restriction (DR), which is the limitation of a specific component (often protein content), captures many of the same healthspan benefits provided by CR without a reduction in overall food intake and minimises adverse effects (Lee and Longo 2016). While a restriction of carbohydrates (A. Sanz *et al.* 2006) or lipids (Alberto Sanz *et al.* 2006) have no specific lifespan-extending effects, protein restriction increases lifespan by up to 20% (Pamplona and Barja 2006). Restriction of specific amino acids, in particular methionine, may also result in a similar lifespan extension (Zimmerman *et al.* 2003; Miller *et al.* 2005; Caro *et al.* 2009). DR has been demonstrated in a wide variety of species, e.g. monkeys (Kemnitz 2011), rodents (Simons *et al.* 2013), nematodes (Pontoizeau *et al.* 2014), flies (McCracken, Adams, *et al.* 2020) and most probably also including humans (L. Fontana *et al.* 2010; Trepanowski *et al.* 2011). Studies which have employed geometric models of nutrition have since shown that it is specific nutrients, not calories alone, that mediate the reduction in age-specific mortality (Tatar 2011; Solon-Biet, Mitchell, de Cabo, *et al.* 2015). Despite this, relatively little is known about the mechanisms via which DR slows ageing (Tatar 2011; Sohal and Forster 2014; Geach 2016).

Fundamentally, extending longevity is not the same as delaying ageing. If one were to live inside of a protective bubble, the inexorable consequences of ageing would persist. However, DR appears to not only extend the lifespan of an individual, but also their healthspan (Lee and Longo 2016), Thus, living longer would not simply equate to spending a greater amount of time infirmed in later life. However, it is important to consider the possibility of longevity manipulation as an artefact of experimentation on inbred model organisms (Piper and Partridge 2007). Determining the effects of ageing in the wild is difficult (Nussey *et al.* 2013; Roach and Carey 2014). Though recent research suggests that the effects of DR are also true of wild populations (albeit less consistently) and is not simply a result of homozygosity or adaptation to laboratory conditions (Harper *et al.* 2006; Metaxakis and Partridge 2013; Mautz *et al.* 2019). Therefore, the fundamental benefit of DR is not only conserved within animal models but is probably transferable to populations outside of an experimental environment.

The most commonly supported evolutionary theory of DR explains its selective benefit as the upregulation of somatic maintenance processes to survive periods of food scarcity and to maximise reproductive fitness upon a return to favourable conditions (Harrison and Archer 1989; Holliday 1989). Therefore, after a period of DR, organisms are expected to have a superior soma, resulting in fitness benefits when food conditions improve. We recently found however that in flies (*Drosophila melanogaster*) there appears to be an excess of mortality upon returning to a protein-rich diet after a period of DR that surpasses the mortality rate of flies fed a rich diet for their whole lives (Chapter 1; McCracken, Adams, *et al.* 2020). Furthermore, this mortality cost was not compensated by a larger reproductive output, as fecundity was also inferior compared to flies fed a regular diet continuously (McCracken, Adams, *et al.* 2020). Therefore, it remains unclear what the evolutionary explanation of DR is. Similar to the mechanisms of ageing, we therefore do not understand the mechanisms nor the basic biology of DR. When we understand the mechanisms of DR, we could possibly use them to treat ageing and to understand the evolution of DR, and with that, life-history evolution.

Mechanisms of DR

There are many highly interconnected proteins and pathways that are known to affect lifespan (Pan and Finkel 2017). Demographically, DR is shown to acutely counteract the causes of ageing within the fly (Mair *et al.* 2003). However, studies indicate that stress response pathways (and connected mediaries) are most highly upregulated as we get older (Haigis and Yankner 2010). More specifically, many of these stress response pathways involve overlapping elements of the nutrient-sensing response (Kanfi *et al.* 2008; Haigis and Yankner 2010; Sengupta *et al.* 2010), which is actively regulated by DR (Kenyon *et al.* 2010; Santos *et al.* 2016). Of these, sirtuins (Rogina and Helfand 2004), insulin/insulin-like growth factor (IGF-1) signalling (Holzenberger *et al.* 2003), the kinase target of rapamycin (TOR) signalling (Kapahi *et al.* 2004) and AMP-activated protein kinase (AMPK) (Greer *et al.* 2007) have all demonstrated significant pro-longevity effects. This collective change in regulation forms the basis of the adaptive response to changes in energy status and cellular damage observed under stress and ageing (Haigis and Yankner 2010). These adaptive responses involve the coordinated regulation of multiple cellular processes, including proteostasis, transcription, autophagy and mitochondrial function. This highly regulated network thus maintains the balance of cellular damage and repair in the face of stress and declining function as age advances.

No single DR mimetic or mimetic regime captures the longevity benefit in its entirety (Roth and Ingram 2016; Liang *et al.* 2018). Though rapamycin's pro-longevity modulation of mTOR is well documented (Harrison *et al.* 2009; Selvarani *et al.* 2021), the benefits of other DR mimetics such as resveratrol, metformin and NAD+ precursors are inconsistent (Miller *et al.* 2011; Schultz and Sinclair 2016; Blagosklonny 2018; Klimova *et al.* 2018; Harrison *et al.* 2021). It is possible that interspecies (Pitt and Kaeberlein 2015), or even genotype-dependent variation (McCracken, Buckle, *et al.* 2020) is

responsible for such effects, yet the benefit resulting from DR itself is highly conserved (Gresl *et al.* 2001; Pamplona and Barja 2006; Kapahi *et al.* 2017). Nevertheless, the mechanism of response to targeted, and well established pharmaceutical interventions, such as rapamycin, may not be shared with that of environmental perturbations such as DR (Hall 2017). Though both present a longevity benefit, TOR inhibition and DR application promote lifespan in overlapping, yet partially distinct pathways involving the mTOR1 and mTOR2 complexes (Laplante and Sabatini 2012). This shows that there remains considerable scope to fully understand the mechanistic responses to DR (even for mechanisms which are currently well-researched), and the need for novel candidates.

A dietary switch paradigm to understand DR

Most studies of DR apply DR throughout life. This has the potential downside of also triggering long-term compensatory mechanisms. These mechanisms might be related to starvation avoidance, for example, and may thus not be causally evolved in triggering the longevity benefits of DR (Mair *et al.* 2003). It is probable that there are a lot of such mechanisms and as such, physiological measurements to uncover the prolongevity mechanisms of DR can be considered clouded by compensatory mechanisms expected to occur during long-term DR. Therefore, it is reasonable to assume that there are two distinct regulatory phases in response to DR. The first is an adaptive phase, where energy expenditure rapidly adjusts to changes in energy availability, and the second, of long-term maintenance of these adjustments. Though the adaptive phase may also contain elements unique to this process, those which overlap with long-term maintenance may be responsible for the pro-longevity response underpinning DR. Therefore, comparing the short- and long-term responses to DR, it may be possible to distil the mechanisms by which the instantaneous reduction in age-specific mortality is achieved. Furthermore, this same approach may provide novel mechanistic insight into the excessive mortality faced when returning from DR. Such an approach becomes even more promising if there is a convincing phenotype to DR (Good and Tatar 2001; Mair *et al.* 2003; Mair *et al.* 2004; Partridge, Piper, *et al.* 2005). In the fly, there is a strong and rapid (within 2-4 days) lowering of mortality risk in response to DR (Chapter 1; McCracken, Adams, *et al.* 2020).

There are clear differences in the effect of dietary restriction between the sexes of multiple species such as mice (Kane *et. al* 2018), flies (Moatt *et al.* 2016; Magwere *et al.* 2004) and monkeys (Mattison *et al.* 2017). Previously the effects of sex have largely been ignored within the ageing field, and continue to be underappreciated (Chen *et al.* 2022; Garratt 2020). However, there is a renewed interest in uncovering the sex-specific mechanisms of DR (Kane *et. al* 2018; Simons and Dobson 2022). Pathways with known ties to longevity, such as growth hormone (Junnila *et al.* 2016; Sun *et al.* 2017), insulin (Ashpole *et al.* 2017; Mattison *et al.* 2017), sirtuins (Sharples *et al.* 2015) and even mTOR (Baar *et al.* 2015) display sexual dimorphisms (Garratt 2020). In *Drosophila*, females are thought to display a greater DR effect than that found in males (Simons and Dobson 2022; Magwere *et al.* 2004). The use of one sex removes any additional confounds due to sexual interaction such as sociality which may affect lifespan (Narayan *et al.* 2022). Once mated, females may lay eggs continuously. However, egg and reproductive costs do not underlie DR (O'Brien *et al.* 2008). Selecting for a strong DR effect, where possible, enhances the discovery potential for novel molecular mechanisms of DR which may otherwise be overlooked when the response is low. Though sex-specific differences may underlie the DR response in *Drosophila* it would be equally interesting to uncover these mechanisms. However, it is practically infeasible to conduct large-scale investigations into the DR effect using both sexes within our lab group; therefore, females were favoured. This, in combination with the dietary-switching paradigm maximises the chance to uncover novel mechanisms of DR.

I used metabolomic and proteomic approaches to investigate the whole female fly's response to DR within this paradigm to pinpoint candidate DR mechanisms. This approach further allowed me to answer questions more broadly, relating to the speed at which flies respond metabolically to DR, which mechanisms might underlie a recent overfeeding response we discovered (Chapter 2 & Chapter 4) and whether different *Drosophila* species respond similarly to DR on the metabolome level (Chapter 3).

The use of omics to understand complex biology

Central to this thesis is understanding the concept of a systems biological approach to understanding complex phenomena. The central dogma of molecular biology dictates that there is a flow of genetic information within a biological system (Crick 1958). Though originally used to describe the flow of information from the genome (DNA) to the transcriptome (RNA) and proteome (proteins), it has since been updated to include the metabolome (Costa Dos Santos *et al.* 2021). The phenotype is the net effect of these organisational layers and environmental factors. In isolation, the analysis of any one organisational layer, be it the genome (genomics), transcriptome (transcriptomics), proteome (proteomics) or metabolome (metabolomics) may provide key insights into phenotypic variation (Whitaker *et al.* 2014; Wang *et al.* 2006; Barzilai *et al.* 2012). However, when combined, a more complete picture of phenotypic variation may be achieved (Hasin *et al.* 2017, Promislow and McCormick 2018).

A common theory of ageing describes it as a failure of coordination of thousands of RNAs, proteins, and metabolites that form a biological network to modulate longevity across multiple tissues and cellular organelles (Dillin *et al.* 2014). It is important to note that the interaction between these organisational layers is complex, and whilst research into these interactions is relatively still within its infancy, they may be what is required to discover underlying biological processes that influence natural variation in ageing, age-related disease, and longevity (Hoffman *et al.* 2017). For example; A combination of transcriptomic and metabolomic techniques has proven useful in uncovering the subtle signals observed in nutritional intervention in humans (Burton-Pimentel *et al.* 2021). Though the network connections used in their analysis were generally specific to the metabolome or the transcriptome (rather than both), some connections were only found when both datasets were combined. An integrative proteomic and transcriptomic analysis of DR in *Drosophila* by Gao *et al.* (2021) identified 20 proteins that were consistently regulated during DR with known links to ageing (mTORC1, antioxidant, DNA damage repair and autophagy) and 15 genes that were stably regulated by DR at both transcriptional as well as translational levels. Lastly, Hoffman *et al.* (2017) reviewed a combination of metabolomic and proteomic profiling techniques in an attempt to help identify the underlying causal mechanisms that link genotype to phenotype.

Ultimately, it is proteins that make up the enzymes that catalyse different biological reactions, and metabolites that are the building blocks of structural elements and biochemical pathways. Therefore, these analyses have been prioritised (within this thesis) to determine any functional elements of the DR response in *Drosophila* which have yet to be uncovered. Figure 0.1 provides an overview of these interactions (within the context of this thesis), and discusses examples of interaction between organisational layers and the differences between them. What's most important to understand is that changes in one organisational layer may have far reaching consequences in another, and environmental factors such as DR may have profound effects upon the system as a whole.

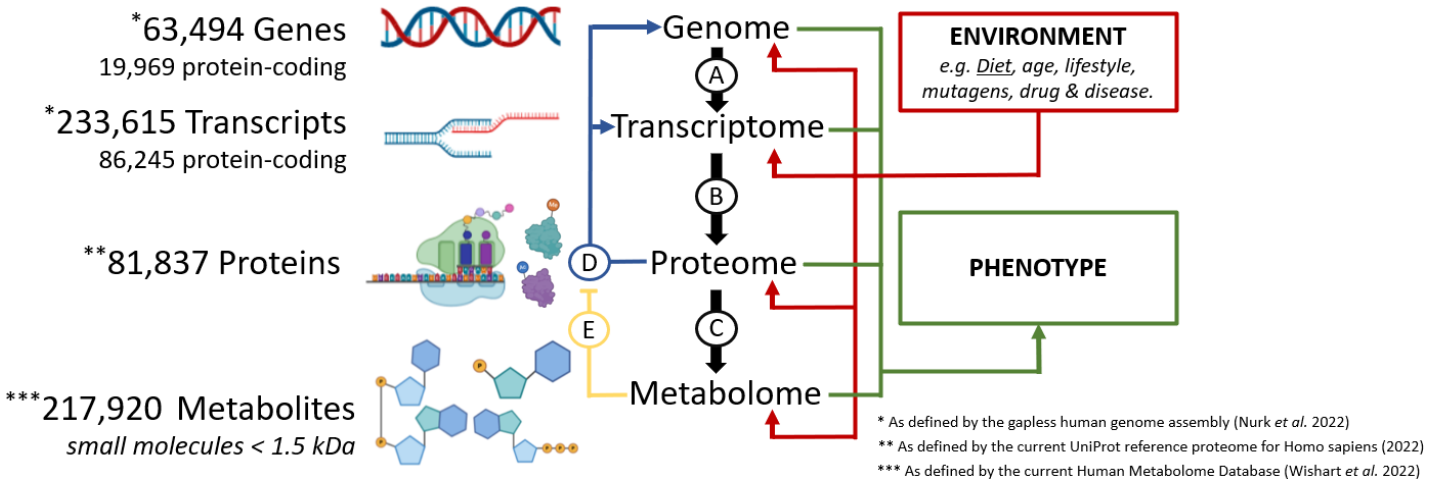

Figure 0.1 | The complex interactions of the genome, transcriptome, proteome and metabolome in biological systems.

The complex interaction of organisational layers that ultimately result in an organisms' phenotype. Environmental factors may directly affect organisational layers; Dietary adjustments can result in diverse transcriptional responses (Whitaker *et al.* 2014), notably at an early age (Cui *et al.* 2018). Ageing is known to result in the loss of protein homeostasis (Magalhaes *et al.* 2018) and mutagenesis may change the information contained in our genes (Chatterjee and Walker 2017). Pharmaceuticals may alter many of the protein-ligand interactions usually regulated by small molecules of the metabolome (Du *et al.* 2016). **(A)** DNA from the genome is predominantly transcribed into mRNA. Alternative splicing allows for multiple proteins, with distinct functions, to be translated from one gene (Black 2003). Many forms of RNA exist and are expressed to varying degrees, though are not present in all species (Caprara and Nilsen 2000). **(B)** mRNA is translated into a linear chain of amino acids at the ribosome with the help of tRNA. As this protein chain is formed, it undergoes three-dimensional folding which stabilises the structure and facilitates protein function; further post translational modifications (such as methylation and acetylation) may alter protein function (Stacey Arnold and Kaufman 2003). **(C)** The metabolome is the collection of all small molecules that facilitate function within the cell. The identities, concentrations and fluxes of these substances are the final product of interactions between gene expression, protein expression and the cellular environment (Kaddurah-Daouk and Krishnan 2009). However, it is worth noting that small-proteins < 1.5 kDa (which may not relate to metabolism) may be detected alongside metabolites due to the mass-spectroscopy techniques used. **(D)** Proteins are important to create and maintain cellular structure, but may also have roles in regulating other organisational layers. Transcription factors directly regulate gene expression (Latchman 1993), whilst ribosomal proteins (with ribosomal RNA) generate ribosomes which facilitates translation (Korobeinikova *et al.* 2012). **(E)** Metabolites may interact with other macromolecules through competitive and allosteric binding. Examples include modulation of enzymatic activity and changes to gene expression via transcription factors (Costa Dos Santos et al. 2021; Lempp *et al.* 2019).

High throughput omics have revolutionised the modern approach to biological research (Hasin *et al.* 2017). Much of this success has resulted from not only the development of these techniques, but also from the significant decrease in costs associated with next-generation sequencing (NGS) approaches, especially when compared to traditional techniques (Yu *et al.* 2018). Sequencing of whole genomes for clinical or research applications is now not only feasible but commonplace (Stranneheim *et al.* 2021), something which would not have been considered possible some decades ago (International Human Genome Sequencing Consortium 2004). Ageing research has also benefited from the development of these technologies. For example, whole genome sequencing (WGS) of semi-supercentenarians has revealed the elevated roles of DNA repair and clonal haematopoiesis as potential mediaries of healthy ageing and cardiovascular health (Garagnani *et al.* 2021). Transcriptomics has been used to characterise the human process of ageing and can be used to predict the biological age of an individual by differential gene expression profiles alone (Shokhirev and Johnson 2021). Similar approaches have been employed to study the effects of a range of healthspan-promoting dietary manipulations within humans, including protein restriction (Wahl and LaRocca 2021).

Studies of differential gene expression in model organisms have consistently identified hundreds of genes that respond to DR (Swindell 2009; Bauer *et al.* 2010; Wuttke *et al.* 2012). However, this in itself is a problem, as it becomes challenging to separate genes that are causative of the DR-longevity response from those simply reactive to the dietary intake or composition. Studying the DR response shortly after dietary change may help to reduce the number of candidate mechanisms. An elegant example of a switch-based transcriptomic investigation into the mechanisms of DR was conducted by Whitaker *et al*. (2014), who found 144 genes with an immediate response to DR. These were mostly associated with a downregulation of dietary-responsive pathways, possibly the direct result of decreased protein intake. However, they found a significant upregulation in the rate of folate biosynthesis under DR, deficiency of which is linked to cardiovascular disease, cancer and cognitive dysfunction (Scaglione and Panzavolta 2014). As these correspond to DR's onset at a point when an instantaneous reduction in mortality is also observed, these mechanisms probably convey at least some of the lifespan benefit that is observed. However, this paradigm has been utilised to only a limited degree (Chapter 2; Chapter 4; Wu *et al.* 2009; Whitaker *et al.* 2014; Hou *et al.* 2016).

With regards to dietary stimuli, the proteome represents the current capacity of an organism to respond to environmental change (which is adjusted by transcriptomic regulation), whilst the metabolome reflects the current cellular state in terms of resource allocation or cell signalling interactions. Both metabolomic and proteomic approaches hold great promise in advancing nutrition-based research (Wang *et al.* 2006; Tebani and Bekri 2019), and may help to deliver a more complete and robust understanding of DR. However, unlike sequence-based approaches, far fewer studies employ these methods, possibly due to their increased technical challenge and cost (Matsuda 2016; Snapkov *et al.* 2022). Furthermore, the lack of standardisation between methodological approaches makes inter-study comparisons difficult and highly labour-intensive (LaBarre *et al.* 2021). Despite this, there have been several investigations into the application of long-term DR which use mass-spectrometry based approaches. Some choose to investigate the broader effects of age and DR (Copes *et al.* 2015; Laye *et al.* 2015), whilst others focus on specific biomarker discovery (Hoffman *et al.* 2017; Walters *et al.* 2018). Though general and specific approaches are equally valid in further understanding the complexity of DR, they continue to be underutilised within the research community. This thesis aims to utilise these mass spectrometry techniques, proteomics (using orbitrap) and non-targeted metabolics (using Waters) combined with a dietary switch protocol in the fly, to uncover a core set of candidate longevity mediaries that underpin DR.

Benefits of using *Drosophila* as a study model

The first study to utilise the humble fruit fly as a model for ageing was conducted by Pearl and Parker (1921). More the result of a happy accident (as their original experimental design utilised a colony of mice which were 'completely destroyed'), the fly was ultimately used as the ageing model for the extensive program of experimental work which they had originally planned. Even for the time, the genetic behaviour and possibilities of the fly were more thoroughly understood than any other animal (Morgan 1920), which arguably is still as true today as it was back then (Mackay *et al.* 2012). The fly's low maintenance costs, rapid generation, ease and availability of complex genetic constructs (Bangham 2019), has made the fly indispensable for biological research (Tolwinski 2017). This is especially true in studies of ageing, where one day in the life of a fly is roughly equivalent to a year in the life of a human (Pearl and Parker 1921). Furthermore, the fly is uniquely suited for DR research as it shows a convincing and rapid adjustment of age-specific mortality (within 2-4 days) to DR (Chapter 1; Good and Tatar 2001; Mair *et al.* 2003; Mair *et al.* 2004; Partridge, Piper, *et al.* 2005; McCracken, Adams, *et al.* 2020).

The fly contains a relatively simple and compact genome (Hjelmen *et al.* 2019), containing some 14,000 protein-coding genes (Adams *et al.* 2000). Approximately 60% of its genome is homologous with humans (Mirzoyan *et al.* 2019), and between 65-75% of human disease-linked genes have

homologues in the fly (Ugur *et al.* 2016; Mirzoyan *et al.* 2019). Though flies and humans bear little resemblance in terms of gross morphology, many internal organ systems are functionally analogous, sharing a great number of molecular, cellular and physiological processes (Ugur *et al.* 2016; Mirzoyan *et al.* 2019). The fly's nutritional requirements are mainly associated with the needs of reproduction, movement and somatic maintenance (Piper *et al.* 2005). An increase in nutritional availability bolsters reproductive capacity at the cost of a reduced lifespan (Chippindale *et al.* 1993; Chapman and Partridge 1996), up until a point, where excessive nutritional 'richness' results in both a decreased lifespan and rate of oviposition (McCracken, Buckle, *et al.* 2020). Fruit flies are largely monophagous; not specialised to specific food sources, but to the yeast that ferments on them (Loeb and Northrop 1916). As such, the combination of yeast (be it dead, or alive) with a sugar-source such as table or cane sugar forms the basis of most laboratory food recipes. Flies are often housed within vials, or in our case for longevity experiments in purpose-built demography cages (Good and Tatar 2001) with the dietary media contained being the only source of nutrition. As a result, the use of hundreds, if not thousands of flies for experimental purposes is relatively simple and inexpensive compared to other model organisms and is often one of its greatest strengths in studies of demography and population genetics.

The dietary protein axis is the primary longevity determinant in flies (Lee *et al.* 2008; Jensen *et al.* 2015). Therefore, a reduction in the protein content (i.e. yeast) of the diet alone is sufficient to generate the DR effect (Chapter 1). It is possible that there are compensatory feeding effects using such an approach (Carvalho *et al.* 2005; Fanson *et al.* 2012), where there is an overconsumption of the DR media to obtain a desired protein intake. However, flies will only over consume to an extent, prioritising the ratio of yeast to sugar at the expense of other nutrients such as water (Fanson *et al.* 2012). Studies of nutritional geometry have since confirmed that it is the protein to carbohydrate ratio (P:C) which effects fitness, with greater rates of oviposition under richer diets (P:C of 1:2) compared to a maximised longevity observed under DR (P:C of 1:16) (Lee *et al.* 2008; Jensen *et al.* 2015). As the flies used here for experimentation could not regulate their macronutritional intake freely, the DR P:C was enforced at all times (where required).

Concluding remarks

Throughout my doctoral studies, I have continued to decipher the mechanisms which underpin the longevity benefit of DR using the latest technologies currently available. Each chapter contained herein shall focus on a specific aspect of DR using a switch-based (Chapters 1, 2 & 4) or interspecies (Chapter 3) methodological approach. Together my thesis uses novel techniques combined with unique dietary and interspecies approaches to understand DR to ultimately better understand the elusive physiology of DR.

Chapter 1: Diet switches as an experimental paradigm to study the mechanisms of dietary restriction using *Drosophila melanogaster*.

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Abstract
Dietary restriction (DR) is arguably one of the most potent and well-researched methods of extending lifespan and healthspan in many species. The translation of such benefits to human physiology is difficult, as studies of DR showing benefits on lifespan often use impractical life-long manipulations. For the fruit fly, *Drosophila melanogaster,* instant benefits of DR have been demonstrated, and understanding such instant benefits of DR could help us understand how we achieve such benefits in humans at risk of age-related pathology. We studied age-specific mortality to investigate the immediate effects of DR using large sample sizes in the fly (n = 4,696 across all treatment types using 40 individual demography cages). Mortality rapidly responded to nutritional availability, decreasing upon the application of DR. In addition, we applied a reverse switching paradigm, moving from a period of DR to a full diet, previously shown to exacerbate mortality compared to continuously rich fed flies. We also detect a similar mortality 'overshoot'. The rapid change in mortality within 48 hours in response to the diet provides a unique paradigm to study the mechanisms of DR. The physiology responsible for such rapid mortality changes might be less convoluted, with long-term adaptation known to occur under long-term dietary conditions. In the subsequent chapters, such physiology will be investigated using omics from flies sampled in these experiments.

Contributions:
AJC, TIG, and MJPS conceived and designed the experiment. MJPS, GA and LH conducted the experiment. AJC analysed the data and wrote the manuscript with input from all authors.

Acknowledgements:
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Introduction

Caloric restriction (CR) is the reduction of caloric intake without malnutrition, typically 60-70% of *ad libitum* (AL) consumption without compromising on micronutrient composition (Piper and Bartke 2008; Trepanowski *et al.* 2011). CR is considered a benchmark for biogerontological pursuits since it was found to extend lifespan in rodents (McCay *et al.* 1935) and has now been reported to extend lifespan in species ranging from single-celled organisms to primates (Koubova and Guarente 2003; Lawler *et al.* 2008; Redman and Ravussin 2011; Pifferi *et al.* 2018). In addition to extending lifespan, CR reduces the incidence of diabetes, cancer and several cardiovascular, neurodegenerative, renal, and respiratory diseases (de Lucia *et al.* 2018; Imai 2009; Vaquero and Reinberg 2009). Intriguingly, its pro-longevity effects are not wholly caused by reducing vulnerability to these age-related diseases.

The considered application of CR is known to be beneficial to human health. The CALERIE trial (a randomised phase II clinical trial) is the most prominent study of moderate CR in humans to date

(Martin *et al.* 2016). However, the level of long-term compliance required to attain this benefit remains a major criticism of such an approach. Study participants failed to reach the 25% (a relatively modest) CR target, only achieving an average 12% reduction over the two years. Although moderate CR achieves a pro-longevity benefit independently of weight loss in other vertebrates (Redman and Ravussin 2011), the benefit of concurrent weight loss cannot be discounted (Look AHEAD Research Group 2017). Although low to moderate levels of CR are beneficial, severe CR (without malnutrition) includes detrimental side effects such as extreme leanness, a loss of sex drive and increased sensitivity to cold, decreasing levels of social interaction as a result (Most *et al.* 2017).

A solution that would capture the longevity benefit of CR without its inherent risk is desired. However, the molecular changes which underpin the ageing process are not well understood (Ahadi *et al.* 2020). Current CR mimetics display varying degrees of success (Chiba *et al.* 2010; Schinaman *et al.* 2019; Irie *et al.* 2020) but fail to replicate the longevity benefit to the same extent and can present additional risks to health (Lee and Min 2013; Blagosklonny 2018; Blagosklonny 2019; Glossmann and Lutz 2019). Suppose we understand the mechanisms underlying the connections between restriction in diet and ageing in detail. In that case, we can start to translate the benefit of CR without its adverse effects on overall well-being. In addition, understanding the underlying mechanisms of diet restriction can help avoid unexpected side effects.

Recent insight into how CR might convey its health benefits was made by using a range of diets that provide a combination of macronutrient composition without compromising on calories (Mitchell *et al.* 2016). The advent of the geometric framework for nutrition (GFN) has since been used to provide detailed insight into the complex relationship between caloric and macronutrient content (Raubenheimer and Simpson 1997) in a range of species, including humans (Wali *et al.* 2020). The GFN is a top-down approach that tests all possible combinations of nutritional composition within defined experimental parameters (Simpson *et al.* 2017). In this instance, the GFN can dissect the contrasting impacts of macronutrient content on lifespan (Piper *et al.* 2011; Raubenheimer *et al.* 2012). The addition of dietary amino acids decreases lifespan in multiple species (Grandison *et al.* 2009; Edwards *et al.* 2015; Fontana *et al.* 2016). Methionine is of particular interest, and is thought to be associated with the regulation of ageing via several different mechanisms (Kitada *et al.* 2019). However, a comprehensive study of mice has since demonstrated that the critical factor driving the survival effect is the protein:carbohydrate ratio and not the caloric content alone (Solon-Biet *et al.* 2014), echoing findings previously found in the fly (Tatar 2011). As caloric intake is not reduced, it is increasingly likely that the long-term application of DR in humans could be successful if chosen as a pro-longevity behavioural modification (Wing and Phelan 2005; Fontana *et al.* 2016).

Studies on the fly have indicated that DR conveys an immediate reduction in mortality when applied in later life (Good and Tatar 2001; Mair *et al.* 2003). Diet is shown to have a rapid and repeatable effect on mortality and is possibly independent of prior nutritional status (Good and Tatar 2001; Mair *et al.* 2003; McCracken, Adams, *et al.* 2020). However, recent findings show that age-specific mortality can become exacerbated following periods of DR (when returning to nutritionally-rich conditions). The resulting mortality exceeds those who existed in a rich environment for their entire life (McCracken, Adams, *et al.* 2020). This phenomenon, referred to as a mortality overshoot, has implications for how DR is interpreted. DR may not be without cost if a rich diet is resumed. Moreover, flies may switch their mortality trajectory rapidly and repeatedly in response to manipulating their dietary state (McCracken, Adams, *et al.* 2020), suggesting that the detrimental overshoot of mortality results from a conflicted response to the prior metabolic framework. The central role of energy metabolism in longevity has long since been a consideration within the application of DR (Anderson and Weindruch 2007) and may be vital in understanding the harmful effects of a sudden nutritional influx following a period of DR.

We studied the demographic response to the onset of DR in the ywR strain of *Drosophila melanogaster*, the dietary dynamics of mortality resulting from the late-age application of DR and the mortality 'overshoot' phenomenon. This study will also provide a platform for further multi-omic investigation, such as Whitaker et al. (2014). The purpose of this chapter is to outline the demographic effect of DR in ywR *Drosophila*, used in the main experiments of my thesis (Chapter 2 and Chapter 4). As a result, a proportion of the flies used in this demographic investigation were sampled in subsequent experimentation to assess the concurrent proteomic responses to dietary change.

Methods

Fly stocks.

The yw (ywR) flies used in this experiment were obtained from the Marc Tatar laboratory. The line originated from the Rolifson laboratory and used the standard yw background. Flies were cultured using rich media consisting of 8% autolysed yeast, 13% table sugar, 6% cornmeal, 1% agar and nipagin at 0.225% (w/v). Restricted media contained 2% autolysed yeast, leaving all other components at the original concentration. Bottles were provided for expansion containing an additional 0.4% (v/v) propanoic acid and were sprinkled with additional water daily if they appeared dry until flies began to pupate. The F1 generation was transferred each day to a bottle containing age-matched cohorts of 10-15 females and 3-4 males, where they were kept for two days for mating. Flies were sorted under light anaesthesia (Flystuff Flowbuddy; < 5 litres/min). Flies that were externally damaged in any way were not used. Experimental flies were kept in 40 purpose-built demography cages [see Good and Tatar (2001)] each containing 100-120 females. All flies were maintained on a rich diet (days 1-2) before being split equally between rich and restricted diets. The dietary protein axis is the primary longevity determinant in flies (Lee *et al.* 2008). As such, caloric compensation (via an increased carbohydrate ratio) is not required in the study of DR (Jensen *et al.* 2015). Cooked media was kept at 4-6 °C for a maximum of 2 weeks. Media was allowed to acclimate (to approximately 25 °C) before use. Census was collected every other day, removing dead flies before replacing the dietary medium. Flies were right-censored if sampled, escaped or rendered immobile by the food. Flies were housed in a climate-controlled environment consisting of a 12h light/dark cycle, between 50-60% relative humidity at 25 °C.

Demography experimental design.

The experimental design is shown in Figure 1.1. The 'switch to rich' group refers to flies continuously fed a restricted diet before being switched to a rich diet. The 'Switch to DR' group refers to flies continuously fed a rich diet before being switched to DR. A total of 40 demography cages were included in this study (10 cages, approximately 1,200 flies per treatment), totalling 4,696 flies, of which 1,506 were right-censored (flies which had been removed for sampling, escaped or had gotten stuck within the dietary media). A total of 1,200 flies were sampled 48 hours after the dietary switch for use in additional experimentation. All flies were switched to new dietary media simultaneously regardless of their original eclosion date (time 'X', Figure 1.1). The dietary switch was performed at age 20 (days post-eclosion) for the first half of the demography cages and age 19 for the second half. The experiment ran until all flies had died.

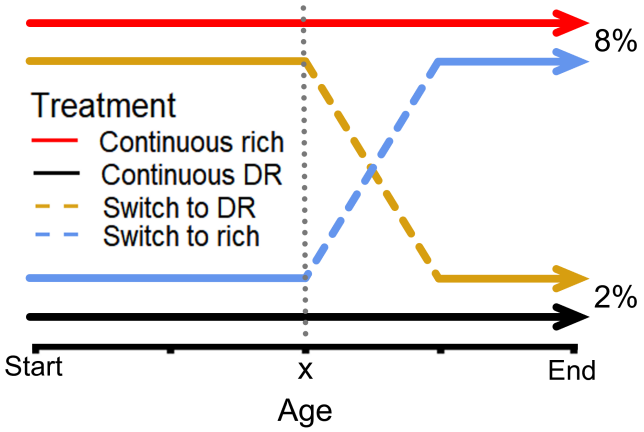

Figure 1.1 | Demographic design.
The experimental population of ywR drosophila were initially divided between rich and restricted media. When the rich cohort approached a survival of 50% (timepoint x), both rich and restricted populations were split; one half maintained their existing diet and the other half were switched to the opposing diet, resulting in 4 dietary groups. Flies were maintained under these dietary conditions until death. **Continuous rich** = flies fed a protein-rich (8% autolysed yeast) diet until death. **Continuous DR** = flies fed a protein-restricted (2% autolysed yeast) diet until death. **Switch to DR** = flies initially fed a protein-rich diet until late age when the diet was switched to DR. **Switch to rich** = flies initially fed a protein-restricted diet until late age when the diet was switched to protein-rich.

Data analysis.

R (R Core Team 2021) version 3.6.3 was used to conduct all analyses. The “coxme” (Therneau 2020a) and “Survival” (Therneau 2020b) packages were used to conduct demographic analysis. Summary statistics of the demographic data (Table 1.1) were generated from Kaplan-Meier analysis of survival, accounting for censored events. Age-specific mortality was calculated from the raw data. Mixed effect cox proportional hazard models were used as it is possible to include the experimental cage as a random term to correct for minor variation attributed to cage location (pseudo replicated effect) (Therneau *et al.* 2003). Interval-based models that used time-dependent covariates were used to further test mortality dynamics following a switch in diet. Each model uses the continuous rich treatment as the reference category, except if otherwise stated. Flies were censored according to their age relative to the switching event in all models.

Results

Dietary manipulation directly affects yw fly survivorship.

Both median and maximal lifespans increased under DR compared to the rich environment. Switching cohorts and continuously fed groups had similar maximal lifespans. There was, however, a noticeable reduction in the switch to DR median lifespan due to the adverse effect of early life mortality (Table 1.1). Such observations can be made from the Kaplan-Meier survival plot (Figure 1.2) and are further summarised below.

Model results show that DR significantly decreases mortality when applied continuously (Table S1.1; log hazard = -2.51± 0.12, z = -21.44, P < 0.001) even when applied in later life (Table S1.6; log hazard = -2.04± 0.36, z = -5.61, P < 0.001). These estimates are in agreement with other studies of DR with variation in genotype [see Table S7: McCracken, Adams, *et al.* (2020)]. However, the magnitude of the DR effect in this study far exceeds that previously observed, using both identical dietary media and housing conditions. Switch treatments prior to the switch were comparable to their respective continuous treatments. Comparison at DR: Table S1.2; log hazard = -0.03± 0.20, z = -0.16, P = 0.87, comparison of rich: Table S1.3; log hazard = 0.01± 0.25, z = 0.04, P = 0.97.

Table 1.1 | Summary statistics of experimental demography by treatment.
Lower and upper CI's refer to the lower and upper confidence intervals of median fly age, calculated from the Kaplan-Meier analysis of the demographic data using the survival package's 'survfit' function (Therneau 2020b). **Max** = maximum lifespan (days), calculated as the mean lifespan of the longest surviving 10% of each population. **n** = total number of flies per cohort. **Events** = total number of flies contributing to the survival calculation. Each fly is treated as an individual; therefore, the possible effects of each cage are not corrected.

Treatment	Median age	Lower CI	Upper CI	Max	n	Events
Continuous rich	22	22	23	36.16	1224	866
Continuous DR	55	53	57	72.64	1087	693
Switch to rich	22	22	22	32.15	1203	844
Switch to DR	34	24	40	67.98	1182	787

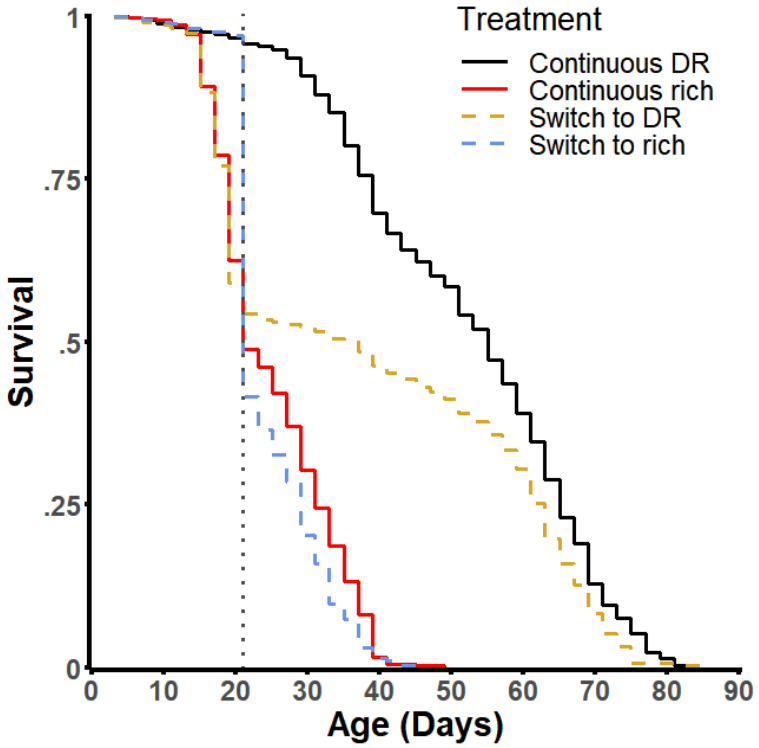

Figure 1.2 | DR provides a considerable benefit to longevity, even in later life.
Kaplan-Meier survival plot by treatment. Survival curves of each dietary cohort; **Continuous DR** n = 693, **Continuous rich** n = 866, **Switch to DR** n = 787, **Switch to rich** n = 844. Flies fed rich media following a period of DR resulted in mortality exceeding that of flies fed a rich diet for their whole life. The dietary switch was conducted on day 19. The vertical line (day 21) represents the initial record of demographic effect post-dietary switch (as flies were maintained every other day) and represents the instantaneous effect of dietary change on survival.

Age-specific mortality rapidly responds to the application of DR.

The calculation of age-specific mortality allows for a detailed comparison of risk across ages (Vaupel *et al.* 1998). Mortality rapidly responded to a change in diet (Figure 1.3). The switch to rich cohort was quick to adopt the mortality trajectory of the continuous rich group; DR to rich: Table S1.4 (day 4 after switch); log hazard = -0.10 ± 0.36, z = -0.27, P = 0.79. The switch to DR rapidly adopted the mortality trajectory of the continuous rich group; rich to DR: S5 (day 2 after switch); log hazard = 0.76± 0.50, z = 1.52, P = 0.13. These dynamics of the switch treatments are also visualised in detail by plotting interval-based cox model output (Figure 1.4). Such mortality amnesia has been observed previously (Good and Tatar 2001; Mair *et al.* 2003; McCracken, Adams, *et al.* 2020) and implies that diet determines the immediate risk of dying and that diet's historical effects are non-existent.

Interestingly, a prior DR diet resulted in a substantial short increase in mortality when switched to a rich diet, compared to continuously rich fed flies (Table S1.4: log hazard = 1.04± 0.16, z = 6.40, P < 0.001). This 'overshoot' of mortality has been described previously [see Figure 1.3: McCracken, Adams, *et al.* (2020)] and is shown to disproportionately affect group mortality. Returning to a non-DR state outweighs any benefit observed during the DR period and exacerbates future mortality risk (Figure 1.2 and Table S1.6).

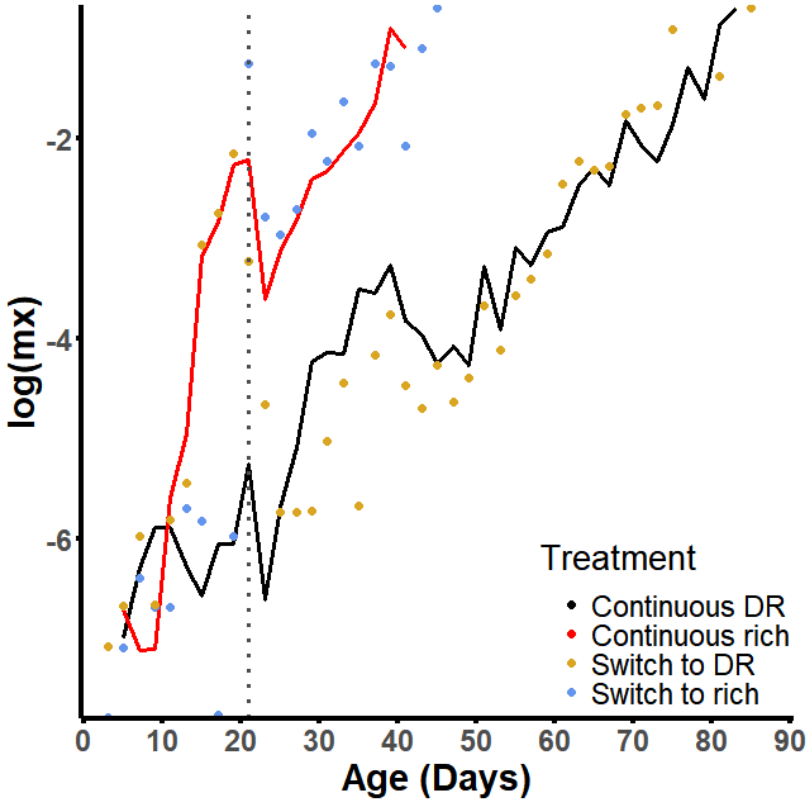

Figure 1.3 | Late age dietary intervention significantly affects age-specific mortality.
Age-specific mortality of the different dietary regimes of age-matched ywR drosophila used in this study. Mortality trajectories of each dietary cohort; **Continuous DR** n = 1087, **Continuous rich** n = 1224, **Switch to DR** n = 1182, **Switch to rich** n = 1203. Sample sizes include right-censored flies. Rich diets that followed a period of DR (i.e. Switch to rich) resulted in such an increase in mortality that the total survival of the cohort was lower than (or equal to) those fed a continuous rich diet for their whole life; highlighted by the significant difference in age-specific mortality post-switch. The vertical dotted line (day 21) represents the initial demographic effect post-dietary switch (conducted on day 19).

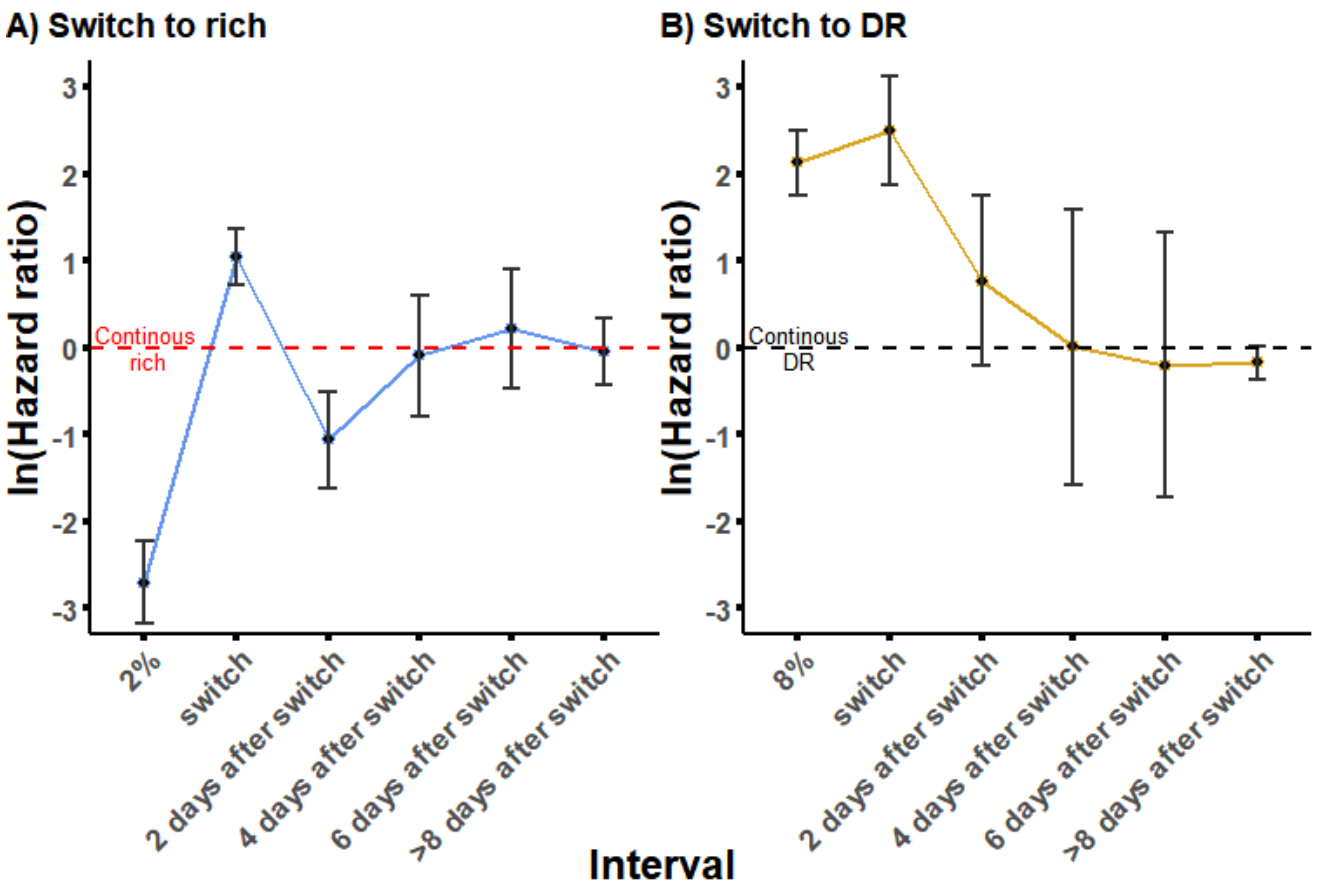

Figure 1.4 | Temporal dynamics of switching groups.
Interval-based survival estimates; switch treatments compared to continuous treatments at each time point. Figure axes represent time point (interval) against the natural log hazard ratio for each switch-continuous comparison. **(A)** Switching dynamic of flies previously facing DR rapidly approaches and surpasses the age-specific mortality of the continuous rich diet (dashed red line). The dietary effect on age-specific mortality takes four days to reach a state of equilibrium post-switch due to mortality exacerbation, followed by subsequent overcompensation before matching the trajectory of the continuous rich group. **(B)** Switching dynamic of flies previously facing a rich dietary environment as they become subject to DR. Age-specific mortality is shown to gradually decline (comparatively), reaching a state of equilibrium with the continuous DR cohort (dashed black line) two days after the switch. Error bars represent the 95% confidence intervals of the estimate of the log hazard ratio at each time point.

Discussion

DR's effect on age specific mortality is almost instantaneous (Figure 1.4). Therefore, measuring physiology shortly after a dietary switch (as conducted here) would provide the greatest opportunity to pinpoint the most significant prolongevity-generating mechanisms without possible confounds resulting from long-term physiological compensation to a restricted dietary state. Testing at an increased frequency (e.g. 6/12 hourly intervals) across multiple organisational layers (i.e. Transcriptome / Proteome / Metabolome) may provide a greater resolution of molecular temporal dynamics that most strongly correlate with the DR effect. Though changes in physiology which fall outside of this window may still have a known geroprotective effect, it is unlikely to be responsible for the instantaneous longevity benefit observed under DR.

Dietary restriction extends longevity and lowers late-age mortality.

Flies placed on DR reduced their mortality instantly, implying that it is not 'too late' to benefit from the effect of DR if still alive. In fact, the application of DR during late age (when mortality risk begins to increase exponentially) may be an optimal strategy; not only is the possible risk to human

development avoided (English and Uller 2016; Martins *et al.* 2011; Soliman *et al.* 2014; Forgie *et al.* 2020), but it is increasingly likely that such a diet will be voluntarily followed until death. Modulation of the diet, such as making vegetarian or vegan choices, is widespread in society and is easier to maintain than a permanent diet. A reduction of dietary protein may simply serve as a choice that could be made to attain a decreased mortality risk regardless of their ethical or environmental standing. However, aged individuals face several morbidities where a dietary protein is beneficial. So the benefits of reducing protein intake (DR) might not always outweigh its adverse effects in those cases (Houston *et al.* 2008; Baum *et al.* 2016; Draganidis *et al.* 2016; Landi *et al.* 2016). Such inter-species comparisons of DR benefits must be considered; the results, as presented here, may otherwise seem to contradict others, such as that shown in humans (Levine *et al.* 2014), which describe a benefit of increased dietary protein intake in later life. This was not the case here, as flies maintained in a protein-rich environment declined rapidly and consistently, showing no improvement in mortality rate at very late life (Figure 1.3). However, such contrasts do not invalidate the use of such models (Mair *et al.* 2003; Whitaker *et al.* 2014; 2020), vertebrate or otherwise [see Mirzaei *et al*. (2016) for review], from the study of the possible conserved mechanisms which underpin the DR effect.

Mechanisms explaining DR and the diet switch.

Two distinct groups of hypotheses concerning the mechanisms of DR can be distinguished: the damage-based hypotheses and risk-based hypotheses [see Mair *et al., (2005)*]. Under the damage hypothesis, if DR were to reduce the rate of age-related damage accrual, the age-specific mortality would continue to rise, but at a reduced rate compared to that previously observed. However, under the risk hypothesis, nutritional availability could instantly modulate the risk of death. Therefore, the age-specific mortality rate should decrease following the application of DR, as the inherent risk of death is reduced due to reduced exposure to harm, as we observed (Figure 1.4). This suggests that it is, in fact, the nutritional availability, which increases the risk of death and the longevity benefit observed under DR is an escape from exposure to harm (Mair *et al.* 2003).

In light of the Mair hypothesis, a framework of the hallmarks of ageing has been defined, which guides the mechanistic interpretation of ageing [see López-Otín et al. (2013)]. Three main categories define the ageing process [see Figure 2.6, López-Otín et al. (2013)]; namely, the primary, antagonistic and integrative hallmarks of ageing. The primary hallmarks of ageing, such as genomic instability, telomere attrition, epigenetic alteration and loss of proteostasis, result in cellular damage that worsens over time and affects the host's longevity (Koga *et al.* 2011; Calado and Dumitriu 2013; Morris *et al.* 2019). Although such factors can be ameliorated by either experimental or environmental means (Levine *et al.* 2014; Passarino *et al.* 2016; Athanasopoulou *et al.* 2018), they remain unlikely candidates to explain the immediate and significant benefit to the mortality observed under DR (Figure 1.3).

The modulation of mortality demonstrated under DR is likely to be the culmination of damage responses generated by antagonistic hallmarks in the cell, each of which is tightly linked to metabolic alterations (López-Otín *et al.* 2016). For example, excessive nutritional intake is known to drive mitochondrial dysfunction (Kusminski and Scherer 2012), increase the rate of cellular senescence (Fontana *et al.* 2018) and deregulate nutrient-sensing pathways, which results in a cost to lifespan (Kenyon *et al.* 2010; Koga *et al.* 2011; Barzilai *et al.* 2012). However, such findings are tempered by the additive nature of the ageing hallmarks (López-Otín *et al.* 2013). Therefore, experimental amelioration of nutrient sensing by DR may impinge on other factors. This interconnectedness may explain the delay in mortality normalisation between the switched and continuous groups (Figure 1.4). In addition, effects of integrative hallmarks, such as stem cell exhaustion (Ruzankina and Brown 2007) and alterations to intercellular communication (Folgueras *et al.* 2018) may have a more pronounced effect in later life, magnifying the apparent benefit of DR. Though such effects would be independent of other factors, such as sex and genotype.

The fitness cost of refeeding following a period of DR.

Nutrient scarcity is a selective pressure that has driven the evolution of most cellular processes (Efeyan *et al.* 2015). The most common evolutionary explanation for the basis of life-extension by DR is that when organisms experience DR, they allocate resources to survival and postpone reproduction until conditions improve (Kirkwood *et al.* 1979; Harrison and Archer 1989; Holliday 1989; Masoro and Austad 1996; Shanley and Kirkwood 2000; Kirkwood and Shanley 2005). However, what is not expected is that the return to a nutritionally rich state might further exacerbate mortality. Such an effect could indicate a clinical condition known as refeeding syndrome (in humans), where mortality is compounded by the rapid influx of nutrition following a period of starvation (Mehanna *et al.* 2008). Nevertheless, as DR benefits longevity in the absence of caloric reduction, such a detrimental impact to longevity (in response to a return of dietary protein) could signify a state of dietary maladaptation after subjugation to DR. Our results (Table S1.4, Table S1.5) show that flies subjected to DR in later life more readily adopt the mortality trajectory of the continuous DR cohort, compared to those which return to a rich diet after a period of DR (2 days versus 4), which is in agreement with others (Mair *et al.* 2003; Mair *et al.* 2004; Whitaker *et al.* 2014). Both demographics reflect a rapid shift in mortality due to dietary change; however, as the switch from DR to a rich diet oscillates around the baseline (Figure 1.4A), compared to a steady transition in DR (Figure 1.4B), the late-age reversal of DR could signify systemic maladaptation that bears a hidden cost to the host. Such intricacies will be subject to further investigation for the 1,200 flies collected alongside this study.

Evolutionary considerations of DR.

Current evolutionary theory dictates that as resource availability becomes more restricted, flies reduce their reproductive capacity for a relative increase in somatic maintenance to survive intermittent periods of famine (Shanley and Kirkwood 2000; Adler and Bonduriansky 2014). However, this hypothesis has recently been questioned (Moatt *et al.* 2020). Specifically, in the fly, the overshoot in mortality when flies return from a DR to a rich diet is not consistent with this theory (McCracken, Adams, *et al.* 2020). We observe these costs again here in the ywR strain, opening up an opportunity to study the effects of overfeeding using a different experimental manipulation.

Diet switching: a paradigm to study the mechanisms of DR.

As there is a sudden switch induced in the fly's physiology when moving from the DR diet to the rich diet or vice versa, this provides a relevant paradigm to study the mechanisms of DR (Smith *et al.* 2008; Whitaker *et al.* 2014; Hou *et al.* 2016). When animals are exposed to diets continuously, it is expected that additional compensatory physiology might diverge between treatments. As the mortality effects are so prominent and rapid after a switch of diet, such compensatory physiology might therefore not cloud the physiology that underlies the mechanisms of DR. This paradigm has been employed previously to study mechanistic aspects of DR (Whitaker *et al.* 2014), but only when moving from rich to DR, and only by whole transcriptome measures (via microarray). We will now use the complete paradigm as presented here across the proteome and metabolome to provide mechanistic insight into the DR response to further its potential translation to humans.

Supplementary

Coefficients are reported as logged hazards unless otherwise stated with significance based on z tests. Right censoring was included.

Table S1.1 | The continuous effect of DR in ywR flies.

		Base treatment: Continuous rich		
coefficient	**estimate**	**Exp (estimate)**	**SE**	**P**
Continuous DR	-2.5153	0.0808	0.1173	**< 0.001**

Table S1.2 | Before the switch, flies in the switch to DR cohort are comparable to flies maintained on a continuous rich diet.
The comparison is made before the switching point to confirm treatment parity before the dietary change.

	Base treatment: Continuous rich			
coefficient	**estimate**	**Exp (estimate)**	**SE**	**P**
Switch to DR	-0.0325	0.9680	0.1988	0.8703

Table S1.3 | Before the switch, flies in the switch to rich cohort are comparable to flies maintained on a continuous DR.
The comparison is made before the switching point to confirm treatment parity before the dietary change.

	Base treatment: Continuous DR			
coefficient	**estimate**	**Exp (estimate)**	**SE**	**P**
Switch to rich	0.0110	1.0110	0.2530	0.9651

Table S1.4 | Changes in age-specific mortality intervals of the switch to rich flies.
As flies adapt to the new dietary environment, mortality estimates normalise to the base treatment category.

	Base treatment: Continuous rich			
coefficient	**estimate**	**Exp (estimate)**	**SE**	**P**
2% (prior to switch)	-2.7103	0.0665	0.2405	**< 0.001**
switch	1.0404	2.8304	0.1626	**< 0.001**
2 days after switch	-1.0633	0.3453	0.2848	**< 0.001**
4 days after switch	-0.0962	0.9083	0.3555	0.7867
6 days after switch	0.2110	1.2349	0.3468	0.5430
>8 days after switch	-0.0541	0.9473	0.1962	0.7827

Table S1.5 | Changes in age-specific mortality intervals of the switch to DR flies.
As flies adapt to the new dietary environment, mortality estimates normalise to the base treatment category.

	Base treatment: Continuous DR			
coefficient	**estimate**	**Exp (estimate)**	**SE**	**P**
8% (prior to switch)	2.1282	8.3996	0.1918	**< 0.001**
switch	2.4966	12.1409	0.3231	**< 0.001**
2 days after switch	0.7604	2.1391	0.5008	0.1290
4 days after switch	0.0054	1.0054	0.8090	0.9947
6 days after switch	-0.2025	0.8167	0.7780	0.7946
>8 days after switch	-0.1758	0.8387	0.0965	0.0685

Table S1.6 | The mortality overshoot phenomena.
Both the continuous and 8% variables were set as the base comparators of the interactive model. Both continuous and late-age DR is beneficial, whilst switching to a rich environment at late-age exacerbates age-specific mortality, exceeding that of continuous rich.

coefficient	**estimate**	**Exp (estimate)**	**SE**	**P**
TreatmentSWITCH	1.0297	2.8002	0.3065	**< 0.001**
Diet2%	-2.5132	0.0810	0.3564	**< 0.001**
TreatmentSWITCH:Diet2%	-2.0380	0.1303	0.3633	**< 0.001**

Chapter 2: Metabolomics after diet switches in *Drosophila melanogaster* identifies candidate mechanisms of dietary restriction

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Abstract
Dietary restriction (DR) is a powerful and well-studied manipulation of ageing, increasing lifespan and health across many species. The translation of DR's benefits to human medicine is difficult, as studies of DR showing benefits on lifespan often use unrealistic and impractical life-long manipulations. Our understanding of DR's mechanisms is far from complete. The fruit fly, *Drosophila melanogaster*, shows instant reductions in mortality risk under DR, providing a promising paradigm for understanding its mechanisms. Here we use this paradigm, measuring the metabolome of whole female flies, using liquid chromatography mass-spectrometry (LC-MS) non-targeted metabolomics, on rich and DR diets and immediately after (1 and 2 days) switching to these diets. A number of metabolites were found to change significantly between steady-state dietary conditions, and the metabolomic response was immediate to a dietary switch and most strong after 48 hours. Importantly, there was enrichment of shared differential metabolite content across the dietary groups, which largely agreed in direction. Therefore, the dietary treatment design we employed was successful at distilling at least part of the physiology that underlies the DR response. In addition, we identified a greater proportion of metabolites shared with DR physiology that shift under refeeding (DR to rich food) than the onset of DR (rich to DR). The response was also more rapid in this refeeding group, possibly identifying the separate physiology underlying previously reported exacerbated mortality under this refeeding paradigm. Pathway enrichment was not possible as a limited amount of metabolites were detected. However, we used literature research of individual metabolites we identified that changed significantly across the treatment categories to identify several possible mechanisms underlying DR. We identified ceramide, NAD+, xylitol, palmitate, uridine, vitamin B7, ecdysone and protoporphyrin mechanisms as potential candidates involved in DR. The most interesting of these candidates consist of the novel implication of Xylitol in the DR-pro-longevity response. Specifically for the DR-associated refeeding response, we implicate palmitate in this syndrome. Our study further provides further proof-of-principle for the use of short-term dietary switches to dissect dietary responses into their most promising candidate mechanisms.

Contributions:
AJC, TIG, and MJPS conceived and designed the experiment. AJC, MJPS, and LH conducted the experiment. HW assisted with LC-MS operation. AJC analysed the data and wrote the manuscript with input from all authors.

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Introduction

The diet is an important environmental component of all animals known to modulate lifespan (Senior *et al.* 2015). Caloric restriction (CR) was one of the first experimental manipulations known to extend the lifespan of model organisms (McCay *et al.* 1935). Dietary restriction (DR) reduces a specific dietary component, usually total protein content (Mair *et al.* 2005; Trepanowski *et al.* 2011). Both dietary regimes, CR and DR, have a marked effect on the systemic regulation of animals (Culwell *et al.* 2008; Avanesov *et al.* 2014; Blekhman *et al.* 2014; Hoffman *et al.* 2014; Rollins *et al.* 2019). However, the mechanisms by which DR benefits health are complex and far from fully elucidated (Green *et al.* 2022). There is currently no single mimetic or genetic manipulation that fully replicates the health and lifespan benefits of DR across model organisms (Liang *et al.* 2018). Furthermore, the genetic background of models subjected to DR modulates the DR's longevity effect on lifespan (Liao *et al.* 2010; Swindell 2012; McCracken, Buckle, *et al.* 2020). It has also been suggested that DR extends lifespan simply by preventing diseases caused by overfeeding and obesity as a restrictive environment most accurately represents nutritional conditions in the wild (Hayflick 2010). Moreover, it has been suggested that the CR benefit is simply an artefact of fasting and not inherently a result of decreased caloric content (Pak *et al.* 2021). Though such conclusions have been contested (Luigi Fontana *et al.* 2010; Solon-Biet, Mitchell, Coogan, *et al.* 2015), there is continued discordance within the biogerontological community (Baur *et al.* 2010). However, it is unanimously agreed that the multifaceted connection between DR and basic ageing mechanisms is complex. Measuring components of the organisms that provide insights into physiology on a holistic level may provide ways to better understand the underlying mechanisms of DR. Transcriptomics (measuring 'all' RNA expression) [e.g. (Ding *et al.* 2014; Whitaker *et al.* 2014)] and proteomics (measuring 'all' protein expression) [e.g. (Gao *et al.* 2020)] have been used in this field. Here we focus on the measurement of 'all' small molecules, metabolites, that are present in an animal, a technical approach that has been relatively underused, especially within the study of DR.

The metabolome, the total number of compounds formed in or necessary for metabolism present in an organism, cell, or tissue, can be considered the final interface between regulatory control and phenotypic effect (Laye *et al.* 2015). Metabolomics, the study of the metabolome, is the latest addition to the systems understanding of biology (Alseekh and Fernie 2018). When the biology of interest is linked to metabolism, key progress can be made. It has been primarily used in ageing for biomarker discovery (Srivastava 2019; Tolstikov *et al.* 2020) and as a tool to provide a mechanistic understanding and improve the diagnostic capabilities of diseases such as type 2 diabetes (Pallares-Méndez *et al.* 2016), Alzheimer's (Xie *et al.* 2021), Parkinson's (LeWitt *et al.* 2017) and cancer (McDunn *et al.* 2015). A potential downside of metabolomics is the ambiguity around metabolite identification when untargeted (and to a far lesser degree targeted) metabolomics is used (Beger *et al.* 2016). Targeted metabolomics only provides data on a known limited set of metabolites and is therefore arguably less suitable for the discovery of novel mechanisms.

The application of metabolomics has been used to study DR across a range of model organisms such as worms (Pontoizeau *et al.* 2014), flies (Laye *et al.* 2015; Jin *et al.* 2020), rodents (Jové *et al.* 2014; Walters *et al.* 2018) and primates (Collet *et al.* 2017; Rhoads *et al.* 2018; Walters *et al.* 2018). However, the differences between technological approaches and the varied results captured from the metabolomic analyses make it difficult to find common ground between DR and its mechanisms. The metabolic fingerprinting approach used by Pontoizeau *et al*. (2014) revealed significant changes in metabolite profiles between the aged and the DR state, showing low phosphocholine abundance was correlated with a greater life expectancy. The targeted metabolomics approach (105 selected compounds of interest) used by Jin *et al.* (2020) identified a novel role of α-KG/glutamine synthesis pathways in the DR response. The nontargeted metabolomics approach used by Laye *et al.* also revealed the effects of DR in ageing (e.g., amino acid and NAD metabolism), and used differential

coexpression analysis to identify an increased network correlation of DR flies at older ages. Walters *et al.* (2018) utilised a metabolomic screen to reveal novel functions of circulating sarcosine, with sarcosine shown to wane with age, showing that sarcosine contributed directly to age-related alterations in the metabolome and proteostasis. These metabolomic investigations into the effects of DR all identified novel mechanisms or biomarkers of longevity, improving our current understanding of DR as a whole. Although important, care must be taken in the interpretation of these findings as they are not directly comparable due to their differences in approach and compound detected. Furthermore, these studies all used long-term DR.

When DR is applied for a sustained period, physiological adjustments are probable (e.g. energy source preference or differences in carbohydrate or fat utilisation) that need not underlie the longevity response. Following this rationale, short term dietary manipulations have been used to better understand the DR response (Wu *et al.* 2009; Whitaker *et al.* 2014; Hou *et al.* 2016), as there is a sudden switch induced in the fly's physiology when moving from the DR diet to the rich diet or *vice versa*, this provides a relevant paradigm to study the mechanisms of DR (Smith *et al.* 2008; Whitaker *et al.* 2014; Hou *et al.* 2016). Mechanisms that are triggered in both long-term and short-term DR responses are more probable to underlie DR's health benefits, especially when health benefits of DR are observed during short-term treatment. The fly (*Drosophila melanogaster*) is uniquely suited for this DR study paradigm as it shows a rapid response in mortality rate to DR (Chapter 1; Mair *et al.* 2003; McCracken, Adams, *et al.* 2020). Moreover, we recently identified costs of refeeding following long-term DR (Chapter 1; McCracken, Adams, *et al.* 2020) that are yet to be investigated mechanistically.

We have observed these phenotypes again in the ywR strain of *Drosophila melanogaster* used in this study. The onset of DR results in a rapid and significant decrease in mortality, approximating the mortality trajectory of flies subjected to DR continuously within 4 days (Chapter 1, Figure 1.4). Whether this effect of DR is due to a removal of sustained physiological costs of a high-protein diet or a short-term specific mechanistic response is unknown (McCracken, Adams, *et al.* 2020). The refeeding syndrome or mortality overshoot, observed as exacerbated mortality in flies that return from a DR to a rich diet, may result from metabolic toxicity that may or may not be related to the longevity effects of DR. When flies are sampled shortly after a switch to DR or rich conditions, compensatory physiology expected to occur under long-term diets might be of a lesser effect, thus more clearly revealing the mechanisms of interest.

This paradigm is used here, and we measure the whole fly's metabolome. We compared two dietary switching groups to long-term DR and fully fed conditions at 24 hours and 48 hours after dietary change, allowing us to dissect the associated changes in the metabolome and, more importantly, identify shared responses (Figure 2.1). Of main interest are metabolites that show concordant changes in the shared metabolic response between dietary switching conditions and continuous dietary treatments (Figure 2.1, overlapping sections, dashed lines), as it is these metabolites that would be most probable to underlie the health benefits of DR and the costs of refeeding.

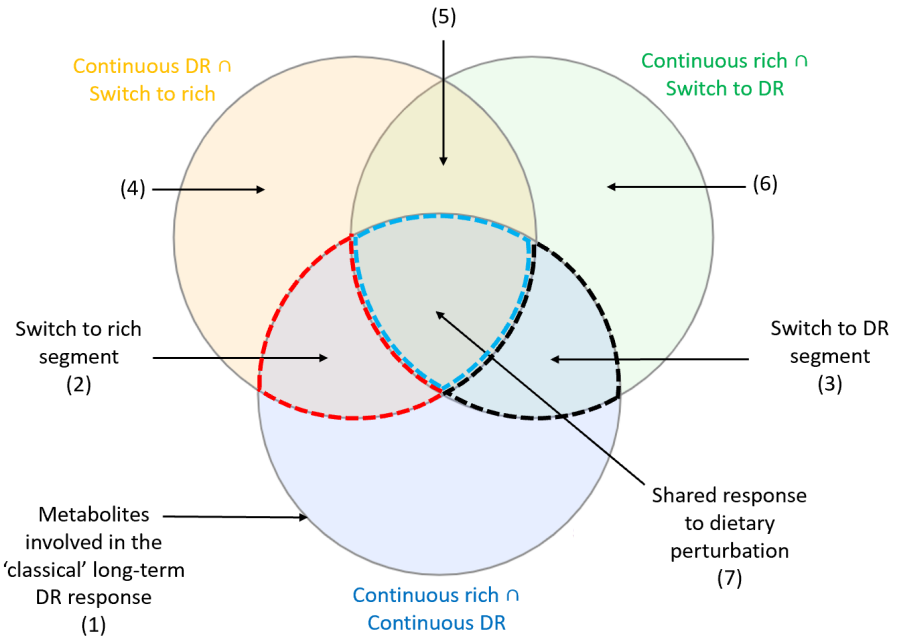

Figure 2.1 | Overview of the experimental design and analysis paradigm.
The interaction (denoted by the symbol '∩') of significant metabolites identified between the continuous rich (high protein) and continuous DR (low protein) diets represent results analogous to the 'classical' long-term application of DR. Venn diagrams of overlapping dietary comparisons of significant metabolites as identified by standard t-tests ($P < 0.05$, FDR $\alpha < 0.05$) will be used to illustrate the relationships amongst dietary conditions. (1) = Metabolites involved with the 'classical' long-term application of DR are represented in the bottom blue circle. (2) = Metabolite overlap between the Continuous DR ∩ Continuous rich and Continuous DR ∩ Switch to rich dietary comparisons (dashed-red segment). This segment represents the metabolites that uniquely respond to a rapid influx of dietary protein. (3) = Metabolite overlap between the Continuous rich ∩ Continuous DR and Continuous rich ∩ Switch to DR dietary comparisons (dashed-black segment). This segment represents the metabolites important to the initial onset of DR. The segmented sections (2,3) are key to distilling the experimental phenotype of DR onset and the mortality overshoot phenomenon. Additional elements responsive to dietary perturbation (4,5,6,7). (4) = Metabolites unique to the switching mechanism of dietary protein influx. (5) = Metabolites shared between dietary switching mechanisms. (6) = Metabolites unique to the switching mechanism of dietary protein restriction. (7) = Metabolites with a shared response to dietary perturbation. Metabolites with a directional agreement between low and high protein conditions are of additional importance in the role of metabolomic regulation.

Methods

DR experiment.

Flies were sampled from a small-scale replicate experiment as described in Chapter 1. In short, age-matched cohorts of ywR flies were grown in bottles containing rich dietary media (8% autolysed yeast, 13% table sugar, 6% cornmeal, 1% agar and nipagin at 0.225% [w/v]). All flies were allowed to mate for 48 hours before females were transferred to purpose-built demography cages [see Good and Tatar (2001)] containing 100-120 individuals. Flies were sorted under light anaesthesia and any visibly damaged flies were discarded. Flies were equally split between a rich and restricted protein diet (restricted: 2% autolysed yeast with all other components equal) as done previously. All flies were then maintained on these diets until age 19 (days post-eclosion), when each diet group (high and low protein) was split and switched to the opposing dietary condition, resulting in four dietary groups (continuous DR, continuous rich, switch to DR and switch to rich). Flies were housed in a climate-controlled environment consisting of a 12:12h light/dark cycle, between 50-60% relative humidity at 25 °C. The dietary switching event took place 3 hours into the light/dark cycle.

Sample collection and processing.

Individual whole flies were collected 24 and 48 hours after the diet was switched in sterile safe-lock eppendorfs and immediately snap-frozen in liquid nitrogen and stored at -80 ℃. During sampling handling, samples were placed on ice during metabolite extraction and mass spectrometry analysis. Samples were stored at -20 ℃ between processing steps. The reagents used were HPLC grade and chilled (2-8 ℃) before use. All equipment and additional storage containers were chilled (2-8 ℃) unless otherwise stated.

Metabolite extraction.

Metabolites were extracted using a methanol chloroform procedure. Whole female flies were lysed using a Fast-prep-24 (Fisher Scientific) in a methanol chloroform water solution (140 µl methanol, 55 µl chloroform, 55 µl water) for 40 seconds. Samples were then vortexed for 10 seconds and iced for 5 minutes before centrifugation (14,000 rpm, 4 ℃, 2 minutes). The supernatants of each sample were collected into new centrifuge tubes and temporarily stored on ice. The resulting tissue pellet was re-lysed in a methanol chloroform solution (65 µl methanol, 65 µl chloroform) for 40 seconds. Samples were then vortexed for 10 seconds and iced for 10 minutes before centrifugation (14,000 rpm, 4 ℃, 2 minutes). The supernatant of each sample was again separated and added to the first part of the extraction that was stored on ice earlier. From these combined supernatants, the chloroform (organic) phase was separated from the aqueous phase with additional water (87.5 µl) and further centrifugation (14,000 rpm, 4 ℃, 15 minutes). The organic phase (bottom) was collected and used in this study.

Ultra-performance liquid-chromatography and mass spectrometry.

Samples were prepared in LC-MS vials following a 1:10 dilution with methanol and water (1:1, v/v) containing 0.1% Formic Acid (FA). Mass spectrometry (MS) analysis was conducted using a Waters Synapt G2 mass spectrometer using ultra-performance liquid-chromatography as a sample introduction method (Column: Waters ACQUITY BEH C18. Solvent A: Water +0.1% FA. Solvent C: Acetonitrile +0.1% FA. Purge: 95:5 Water/Acetonitrile. Wash: 50:25:25 Isopropanol/Water/Methanol +0.1%FA). Each biological sample was run in triplicate. Each technical replicate was run for 480 seconds, equating to 24 minutes per biological sample (vial). Two LC-MS plates were used, each containing 48 samples and were separated by time point. Samples were run sequentially (12 samples per condition, in the order of continuous DR, switch to rich, switch to DR and continuous rich) to minimise cross-sample column effects. Preliminary principal component analysis (PCA) revealed that all outlier samples were located within the leading samples when the treatment condition of the samples changed. We chose to remove these outliers as they could have resulted from cross-over contamination between treatment groups. The main discriminating factor of these outliers was an overall higher intensity measured, as the outliers separated within principal component (PC) 1 and were also evident from the sample by feature intensity and total intensity plots.

Data processing.

R (R Core Team 2021) version 4.1.0 was used to conduct all analyses unless otherwise stated. The raw data files generated by MassLynx were converted to .mzXML, an open mass spectrometry format, with Proteowizard (Chambers *et al.* 2012). The captured profile spectra were also centroided at this stage to facilitate further analysis. From here, each sampling time point was processed and analysed separately. Peak deconvolution parameters were optimised with isotopologue parameter optimisation (IPO) (Libiseller *et al.* 2015) using a training set of 12 randomly selected raw data files (three files per dietary group). XCMS (Smith *et al.* 2006; Tautenhahn *et al.* 2008) was used to conduct peak deconvolution and correct mass calibration gaps (Benton *et al.* 2010) using the optimised

parameters generated from IPO. Peak deconvolution identified 2,517 peaks (i.e. features) in the 24 hour time point and 1,504 peaks in the 48 hour time point.

Samples will not include intensity information for every identified peak across samples if the criteria for peak picking (defined by IPO) are not met within a sample. However, if the peak was detected in other samples within the same dietary group, intensity information was generated by integrating the signal in the same chromatographic domain (i.e. peak filling). Peaks filled accounted for 1.18% of the total data following the high-quality filtering and replicate merging steps (see below; 0.26% in 24h, 3.00% at 48h). Note, zero values can still result from this method if intensity within the peak's chromatographic domain is absent (zero values post-peak filling: 24h < 0.01%, 48h = 0.01%).

Estimated ion chromatograms (EICs) for all picked peaks following XCMS processing were generated using the 'featureChromatograms' function and manually categorised as 'High-quality' or 'Low-quality' to minimise technical noise without compromising the underlying biological signal (Schiffman *et al.* 2019). High-quality peaks display a good peak morphology (bell-shaped, although not strictly required if other conditions are satisfied), have a tight and overlapping retention time alignment between samples, and integrate the correct chromatographic region across all samples. In addition, peaks were only retained with a signal intensity ≧ 500 counts per second (CPS) (baseline noise was estimated to be ≦ 200 CPS). We acknowledge that it is possible for a small number of high-quality but low-intensity features to be omitted when this approach is followed. EIC peaks that had yet to crest were removed as it would not be possible to accurately determine retention time estimates. Only high-quality peaks were retained for subsequent analysis (High-quality peaks: 24h = 1,422, 48h = 723. Low-quality peaks: 24h = 1,095, 48h = 781).

Quality filtering reduced the proportion of peak-filled intensities and eliminated missing intensity values from the data. Median retention time (RT) and mass to charge (m/z) values were significantly reduced across both time points. The effects of quality filtering have been visualised in Figures S2.1 and S3.2. Technical replicates were merged by taking the median feature intensity within technical replicates. Median normalisation was first performed to account for systematic differences among samples, i.e. individual feature intensity values were divided by a feature's median intensity across samples. All samples then underwent generalised logarithm transformation (Durbin *et al.* 2002), to correct for highly-skewed intensity distribution: $\log_{10}(x + sqrt(x^2+a^2)/2)$, where x = original intensity value and a = one-tenth of the minimal positive values in the data. Finally, the data underwent autoscaling, where each feature was divided by the feature's standard deviation to compare metabolites based on correlations (van den Berg *et al.* 2006). Sample normalisation, transformation and scaling were performed using the MetaboAnalyst workflow (Pang *et al.* 2021).

Data analysis.

Several steps were taken to characterise the intensity and the distribution of the dataset post-processing (Figure S2.3). The between-sample intensity was comparable and normally distributed, and there was an observable separation between dietary conditions (for both time points) using both supervised and unsupervised methods of multivariate analysis (Figure S2.3E-H). Feature annotation and pathway assignment were performed using mummichog (Li *et al.* 2013), using the mass to charge, retention time and experimental parameters (Ionisation mode: positive. Mass tolerance as determined by IPO: 24h = 47.75 ppm, 48h = 43.25 ppm) to match known metabolomic compounds in the *Drosophila melanogaster* KEGG database (Kanehisa *et al.* 2021). The inclusion of retention time (RT) can improve feature annotation as a single metabolite is often detected as multiple metabolic features (alternate matched forms), each having the same RT but with a slight difference in m/z value (Dunn *et al.* 2013). In total, 229 of the 2,145 total features were annotated across both time points (24h: 124, 48h: 105). A total of 19.5% of annotated features were found across both time points

(Figure S2.4). Note, a single feature could be annotated as multiple KEGG entries if a feature matches more than one reference compound in the drosophila KEGG compounds database. Therefore, each row in Tables 2.1-2.4 represents a single feature but can include multiple annotations.

Significance testing between dietary groups was conducted using unpaired unequal variance t-tests using Welch approximation. P values were adjusted for multiple comparisons using the Benjamini & Hochberg (1995) method. Only significant ($\alpha = 0.05$) features following this false discovery rate (FDR) correction were retained. To test for under and over-representation (depletion/enrichment) between Venn diagram overlaps of dietary comparisons (Figure 2.1), hypergeometric testing was conducted using the 'phyper' function from the stats package in R. This test is equivalent to a one-tailed Fisher's exact test. Enrichment testing of the central region (overlap in all three sets) was performed via probability simulation using 100,000 iterations. Significance testing of dietary correlations was conducted using Mann-Whitney tests using the correlation matrices of each timepoint used to generate the clustered heat maps (Figure 2.2), with self-correlated values (equal to 1) excluded.

Results

The rate of systemic metabolomic change was varied in response to protein concentration.

Similar to the studies conducted by Laye *et al.* (2015) and Pontoizou *et al.* (2014), it was possible to separate the metabolome of the fly according to dietary state (Figure 2.2, Figure S2.3). As two sampling points (24h and 48h) were investigated in this study, it was also possible to investigate the relative speed at which the metabolome responded to the switched diet. Such a change in the whole metabolome can be examined by the correlation between feature intensities across the whole metabolome at sequential time points. The prediction being that the variance in the metabolomic regulation of the dietary switch group will eventually convert to that of the newly imposed diet.

At 24 hours, switching diets remained clustered with their original dietary conditions (Figure 2.2A), indicating little to no systemic metabolic response to dietary change. At 48h (Figure 2.2B), there was some rearrangement of clustering, with switching groups now co-clustering, perhaps indicating a signature of response to dietary change in general. Notably, all samples switched to a rich diet now clustered more closely to continuous rich conditions and separated from the continuous DR cluster. One sample even became a member of the terminal dendrogram branch, indicating a high degree of metabolic similarity to the rich diet condition it was now grouped with. In contrast, samples from flies switched to DR did not cluster within the branch corresponding to flies subjected to continuous DR, even after 48 hours (Figure 2.2B).

The mortality response to the dietary switches is induced within 2 to 4 days (Chapter 1; McCracken, Adams, *et al.* 2020). Our data indicates that a complete conversion of the metabolome to reflect the continuous state would unlikely be reached within the timeframe that this mortality phenotype can be observed. Perhaps indicating that a full metabolic shift towards the metabolic state observed under the continuous diets was not required for the DR and mortality overshoot responses observed. Therefore as hypothesised (Figure 2.1), a smaller set of metabolic pathways than those observed under the continuous diets could underlie the physiology of interest responsible for DR and the refeeding syndrome. Indeed, at the individual feature level, differences between switching diets and their original continuous groups were found across both time points, with over 1 in 10 features found to be significantly different (Figure 2.3). Relatively more individual features were affected at 48h as well (39.4% versus 47.7%), although fewer features were identified and annotated at this time point.

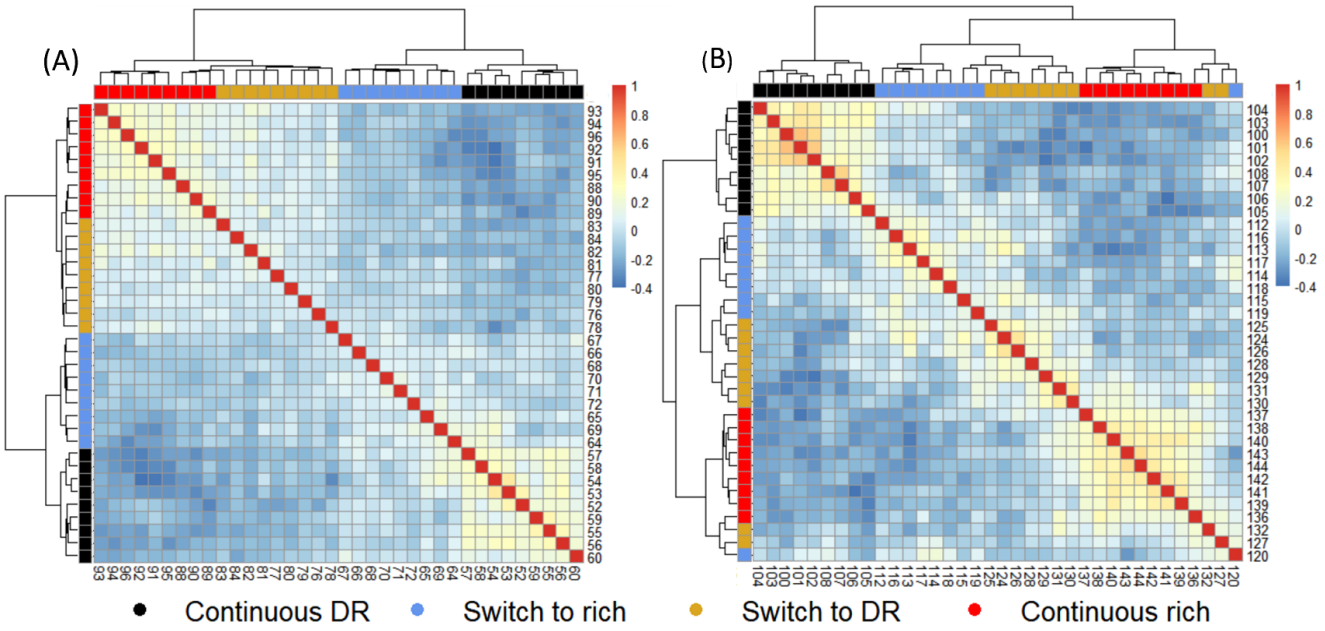

Figure 2.2 | Systemic metabolome change across experimental time points.
Clustered heat maps were generated using the correlation between samples (individual flies) and all detected metabolic feature intensities. Sample numbering is reflected along the X and Y axis. **A:** 24h (number of metabolites = 1,422), **B:** 48h (number of metabolites = 723). Clustering method: Ward. Correlation coefficient: Pearson. Distance measures: Euclidean. Optimal leaf ordering (Bar-Joseph *et al.* 2001) was used to reorder dendrogram leaves to improve visual clarity without adjusting clustering results. Sample correlation was performed using the intensity information at the feature level to capture the maximal metabolomic variance resulting from dietary perturbation and to avoid bias from database dependencies.

Mann-Whitney tests comparing the correlation of metabolic feature intensity across samples between low- and high protein diets were not significantly different in both 24 hour ($W = 2748$, $P = 0.5344$) and 48 hour ($W = 2524$, $P = 0.7874$) timepoints (Figure 2.2). This indicates that the metabolome is tightly regulated to continuous dietary conditions. However, both switching conditions showed a significant difference when compared to their original dietary states; **Switch to rich**: [24h] $W = 4972$, $P < 0.0001$. [48h] $W = 4456$, $P < 0.0001$. **Switch to DR**: [24h] $W = 5064$, $P < 0.0001$. [48h] $W = 4336$, $P < 0.0001$. This indicates that the metabolome is highly responsive to the effects of dietary change, in both directions.

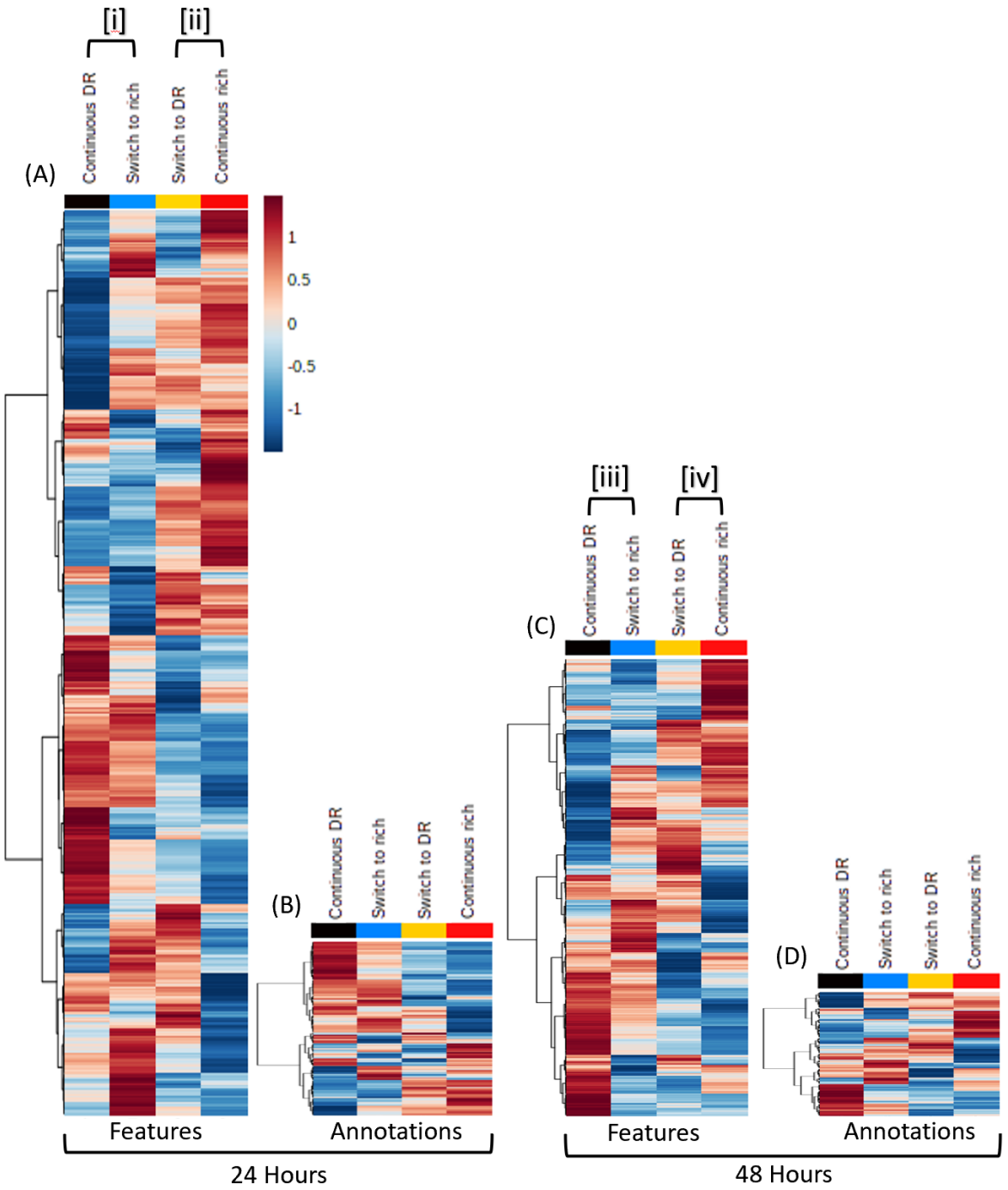

Figure 2.3 | Averaged metabolomic difference between dietary conditions.
Hierarchical clustering heatmaps (method: complete, distance measure: euclidean) of all identified metabolomic features (**A**: n = 1,422**, C**: n = 723) and those with KEGG annotations (**B**: n = 223**, D**: n = 133) across both time points. Heatmaps provide an intuitive visualisation of the concentration of averaged individual metabolite intensities (rows) by dietary condition (columns). Heatmaps are scaled by the total number of features (where A = 100%). Metabolomic divergence between switching and continuous diets was greater in the 48h time period: **[i]** = At 24h , 12.73% of features were significantly different between the continuous DR and switch to rich dietary conditions (n = 181), which rose to 22.96% (n = 166) at 48h **[iii]**. **[ii]** = At 24h, 4.22% of features were significantly different between the continuous rich and switch to DR dietary conditions (n = 60), which rose to 18.81% (n = 136) at 48h **[iv]**. Results are also reported as a percentage to facilitate comparison between time points.

Dietary switching separates out metabolites related to DR.

Individual metabolites that differed between dietary conditions were compared to distil overlapping and contrasting responses (Figure 2.1). We found significant enrichment of metabolites that differed between continuous DR and rich-fed conditions and were also observed to change in the dietary

switching conditions (Figure 2.4 A-D, Table S2.2). Such overrepresentation was found irrespective of whether data was analysed at a feature or annotated level, or 24h or 48h after the dietary switches (Table S2.2). Therefore, we were able to distil a key set of metabolites that underlie long-term DR that also change rapidly upon dietary change, and the physiology related to these metabolites could thus underlie the observed mortality responses to DR.

We previously observed that the mortality risk reduction when switching to DR was relatively more rapid than the induction of exacerbated mortality on the switch to a rich diet (McCracken, Adams, *et al.* 2020). Interestingly, this was not reflected in the metabolome, with nearly three times the number of significant features responding to an increase in dietary protein (Figure 2.4A: orange circle, n = 181) compared to its reduction (Figure 2.4A: green circle, n = 60) at 24 hours. It therefore appears that a dietary switch from DR to rich-fed conditions induces a stronger, more rapid or broader metabolic response than a switch to DR (Figure S2.6). The group of metabolites responsible for this difference also largely overlap with the long-term DR response suggesting they relate to physiology related to DR (switch to rich: n = 111, compared to the switch to DR: n = 24). At 48h, the number of metabolites that significantly differed in the switching conditions was more similar (Figure 2.4C, Figure S2.7). The initial more rapid response observed at 24h may suggest a more rapid switch in the metabolome when switching from DR to fully fed conditions than the induction of DR.

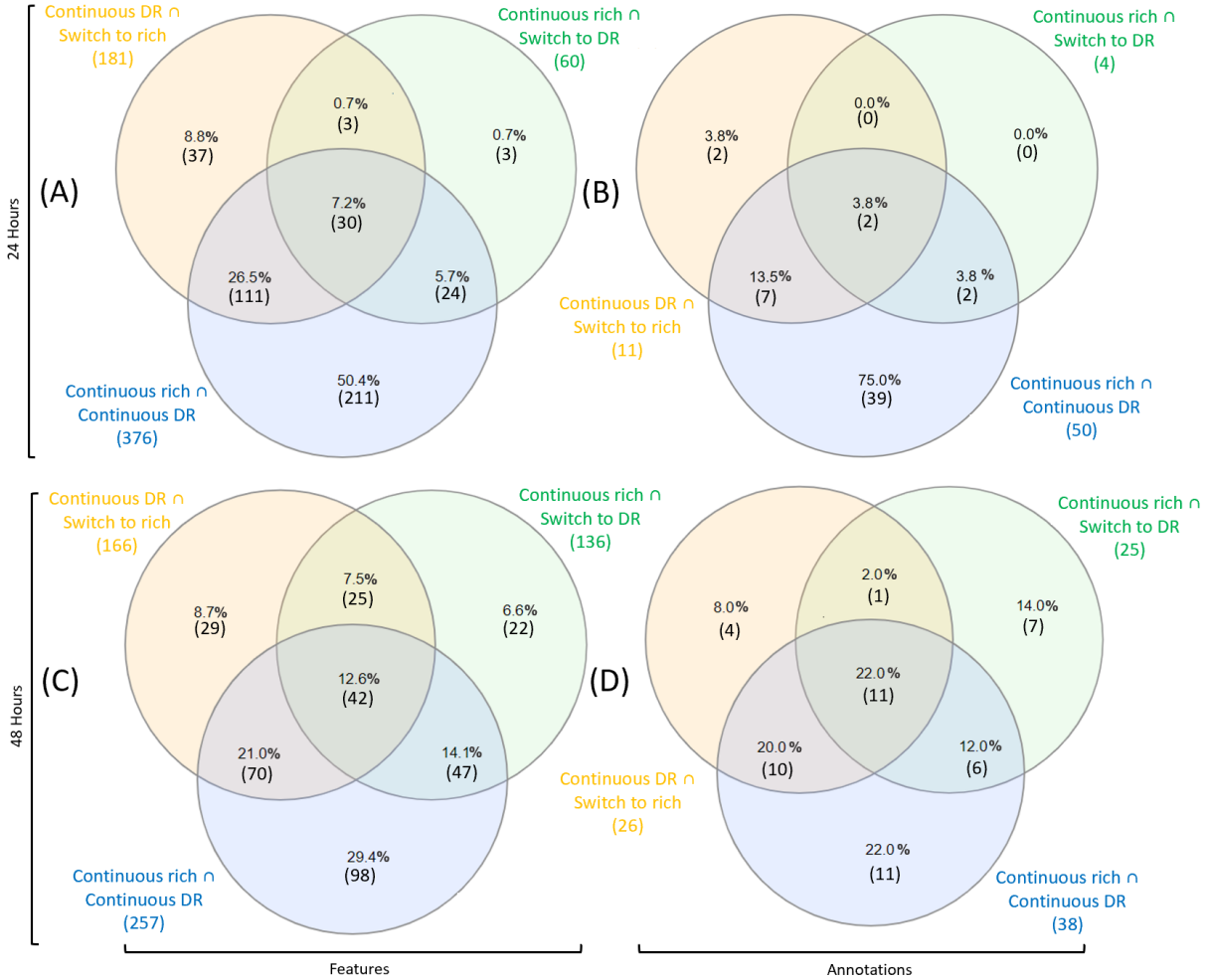

Figure 2.4 | Interaction of significant diet-related metabolites across time points.
Venn diagrams represent the number of significantly changing metabolites between diet conditions. (**A**,**B**) represents the 24h time period whilst the bottom row. (**C**,**D**) represents the 48h time period. (**A**,**C**) represents the metabolome at the feature level, whilst (**B**,**D**) represents the metabolome at the annotated metabolite level. Each Venn diagram (**A**-**D**) is reported as a percentage to aid comparisons across time points and conditions. Counts are listed in brackets. Number of features: 24h (**A**) n = 1,422, 48h (**C**) n = 723. Number of annotated metabolites: 24h (**B**) n = 223, 48h (**D**) n = 133.

To further understand DR and the response to refeeding, we examined the annotated metabolites that changed in response to dietary treatment in detail. For this, we focussed on the 48h time point only, as the 48h was closest to the observed mortality phenotype, showed some divergence in clustering of the whole metabolome (Figure 2.2) and showed a higher proportion of significant differences between dietary conditions on the individual feature level (Figure 2.3). We list these metabolites, noting multiple possible annotations (as we used untargeted metabolomics) per feature, and investigate fold change direction in terms of concordance or discordance between dietary conditions. In the discussion, we combine these findings with the literature to identify candidate mechanisms of DR.

The shared metabolic response to short- and long-term DR.

A total of 47 features (14.1%), or 6 annotated metabolites (Figure 2.4C, 2.4D, left middle section), overlap between the short-term application of DR and continuous DR. This overlap identifies putative metabolites underlying the DR mortality response (Table 2.1) as they include metabolites that respond both to short- and long-term DR. Interestingly the six metabolites identified as such respond similarly in direction in both dietary conditions, further increasing our confidence that these metabolites could underlie the DR mortality response.

Table 2.1 | Metabolites responding concordantly to the onset of DR and long-term DR.
Dietary comparison (48h time point): **(A)** Continuous rich ∩ Continuous DR, **(B)** Continuous rich ∩ switch to DR. The continuous rich dietary condition was used as the base for each dietary comparison. Positive log_2(FC) values represent a larger metabolite intensity in the continuous rich dietary group, whilst negative values represent a larger metabolite intensity in the continuous DR or switch to DR dietary group.

Pathway(s)	Compound(s)	KEGG ID(s)	(A) log_2(FC)	(B) log_2(FC)
Pentose and glucuronate interconversions	D-Xylitol, L-Arabitol	C00379, C00532	-0.3618	-0.3278
Purine metabolism, Metabolism of xenobiotics by cytochrome P450	Adenosine, Deoxyguanosine, Benzo[a]pyrene-4,5-oxide, Benzo[a]pyrene-7,8-diol	C00212, C00330, C14851, C14852	0.2379	0.1623
Purine metabolism, Metabolism of xenobiotics by cytochrome P450	Adenosine, Deoxyguanosine, Benzo[a]pyrene-4,5-oxide	C00212, C00330, C14851	0.1275	0.0888
Sphingolipid metabolism	Sphinganine	C00836	-0.3637	-0.4111
Nicotinate and nicotinamide metabolism	Nicotinamide mononucleotide (NMN)	C00455	-0.3708	-0.4510
Insect hormone biosynthesis	2,22-Dideoxy-3-dehydroecdysone	C16498	2.5160	1.5944

Overlap in metabolic response between long-term DR and refeeding.

A total of 70 features (21%), or 10 annotated metabolites (Figure 2.4C, 2.4D, right central section), significantly responded both to a switch to rich food conditions and long-term DR (Table 2.2). Note that the change in abundance of the metabolites identified is expressed towards rich fed conditions, and concordance in direction (sign) was expected if the metabolic response to the dietary treatment was similar. Two of the ten metabolites, Crustecdysone and 3-Hydroxyecdysone, associated with insect hormone biosynthesis), display significant discordance in directional agreement between dietary comparisons (t-tests between log-fold changes, $P < 0.0001$).

Table 2.2 | Metabolites with a shared response to refeeding and long-term DR.

Dietary comparison (48h time point): **(A)** Continuous DR ∩ Continuous rich, **(B)** Continuous DR ∩ switch to rich. The continuous DR dietary condition was used as the base of each dietary comparison. Positive log_2(FC) values represent a larger metabolite intensity in the continuous DR dietary group, whilst negative values represent a larger metabolite intensity in the continuous rich, or switch to rich dietary group.

Pathway(s)	Compound(s)	KEGG ID(s)	(A) log_2(FC)	(B) log_2(FC)
Tyrosine Metabolism, Phenylalanine, Tyrosine, Tryptophan, Ubiquinone and other terpenoid-quinone biosynthesis	L-Dopa, 4-Hydroxyphenylpyruvic acid, Vanillylmandelic acid	C00355, C01179, C05584	1.3508	0.6199
Fatty acid elongation, degradation & biosynthesis	Palmitic acid	C00249	0.8526	0.7135
Retinol metabolism, Insect hormone biosynthesis	Retinal, (10S)-Juvenile hormone III diol, 9-cis-Retinal	C00376, C16505, C16681	-0.3624	-0.2771
Biotin metabolism	Biocytin	C05552	-0.2129	-0.2551
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	(GlcN)1 (Ino(acyl)-P)1 (Man)1	G00146	1.7025	0.8238
Insect hormone biosynthesis	Crustecdysone	C02633	-0.3700	0.5265
Insect hormone biosynthesis	3-Dehydroecdysone, Crustecdysone	C02513, C02633	-0.5149	0.5098
Terpenoid backbone biosynthesis	Dehydrodolichol diphosphate	C05859	-0.6382	-0.5093
Purine metabolism	Guanosine triphosphate	C00044	1.3830	0.9547
Porphyrin metabolism	Protoporphyrin IX	C02191	-0.2448	-0.3185

The overlap in shared metabolic response across both switching conditions and long-term DR.

A total of 42 features (12.6%), or 11 annotated metabolites (Figure 2.4C, 2.4D, central section) significantly responded to both a diminished and sudden influx of dietary protein (both switching conditions), and long-term DR (Table 2.3). The metabolites listed below (10/11 annotations) agree in direction across all three dietary conditions. Therefore, if a metabolite was elevated under protein-rich conditions, it was depleted under DR, or vice versa. These metabolites thus share a central role in response to DR, and their shared direction suggests they represent the shared metabolites related to DR based physiology.

Table 2.3 | Metabolites with a shared response to dietary protein flux.

Dietary comparison (48h time point): **(A)** Continuous rich ∩ Continuous DR, **(B)** Continuous DR ∩ Switch to rich, **(C)** Continuous rich ∩ Switch to DR. The continuous dietary conditions (continuous rich when comparing continuous diets) are used as the baseline of each dietary comparison. Positive log_2(FC) values represent a larger metabolite intensity in the continuous dietary group, whilst negative values represent a larger metabolite intensity in the switching groups (or continuous DR). Directional agreement: **A**,**B**,**C** = -ve,+ve,-ve (metabolites which are elevated under DR) / **A**,**B**,**C** = +ve,-ve,+ve (metabolites which are elevated under protein-rich conditions).

Pathway(s)	Compound(s)	KEGG ID(s)	(A) log_2(FC)	(B) log_2(FC)	(C) log_2(FC)
Tyrosine and Phenylalanine metabolism	p-Hydroxyphenylacetic acid, 3,4-Dihydroxyphenylacetaldehyde, 3,4-Dihydroxyphenylglycol, Ortho-Hydroxyphenylacetic acid	C00642, C04043, C05576, C05852	-0.5414	0.1330	-0.2795
Purine metabolism	Adenosine, Deoxyguanosine	C00212, C00330	0.4302	-0.3731	0.2291
Fatty acid degradation	L-Palmitoylcarnitine	C02990	0.8161	-0.1377	0.3611
Arachidonic acid metabolism	Leukotriene A4	C00909	1.8583	-0.2700	1.8469
Pyrimidine metabolism, Drug metabolism - other enzymes	Uridine 5'-monophosphate, Pseudouridine 5'-phosphate, Thioinosinic acid	C00105, C01168, C04646	-1.8255	1.0552	-0.3699
Drug metabolism - other enzymes	5-Fluorodeoxyuridine monophosphate	C04242	-1.5680	1.0777	-0.2331
Pyrimidine metabolism	Orotidine-5'-monophosphate	C01103	-1.3311	0.8690	-0.3055
Terpenoid backbone biosynthesis	Dehydrodolichol diphosphate	C05859	0.6382	-0.5093	0.1951
Terpenoid backbone biosynthesis, Arachidonic acid metabolism	Dehydrodolichol diphosphate, Leukotriene D4	C05859, C05951	0.8465	-0.6008	0.2298
Galactose, Ascorbate, Aldarate, Starch, Sucrose, Amino sugar and nucleotide sugar metabolism, Pentose and glucuronate interconversions	Uridine diphosphate glucose, Uridine diphosphategalactose	C00029, C00052	-1.4989	1.1202	-0.1719

Discussion

The metabolic response to DR was shared and can be dissected as hypothesised by comparing continuous dietary conditions with switching conditions. Not only did we find an overlap of the DR responses of both individual switching conditions (Tables S2.1 and S2.2), but importantly there were also significantly more shared metabolites across these three dietary comparisons (Table S2.3). The physiology governing the mortality responses observed across these different dietary switches could thus be shared. These metabolites related to DR physiology have been identified using this unique and now proven fruitful paradigm (Figure 2.1). Perhaps most convincing of the merits of this approach was the large concordance in directional metabolite responses shared across dietary conditions.

Although the hypothesis-generating, untargeted approach that we used provides an unbiased insight into the mechanics of DR, it is less replicable than targeted metabolomics (Fiehn 2002). This

challenge was evident in the comparison between the 24 hour and 48 hour time points where overlap was low (Figure S2.4), even within the continuous dietary treatments (Figure S2.5). The two reasons most probably responsible for this are that first on the feature level slight differences in technical or biological noise results in different features being detected and are thus hard to compare across experiments and mass spectrometry runs, and second that not all features are annotated at high certainty and can thus not be compared across studies. However, those that did overlap were concordant in direction (Table S2.1). One other caveat in this study was that whole flies were used. While it is possible to look at tissue or single-cell level metabolomics (Lee *et al.* 2020), it is far more technically and resource demanding. Widespread systemic effects should underlie DR responses and are thus arguably present in all tissues. In addition, metabolic responses need not appear in the tissues where the physiology exerts itself, e.g. downstream metabolism could operate in other tissues. Whole fly metabolomics is therefore arguably a good starting point to identify the physiology responsible for a phenotype of interest. Despite the overall limited view on the whole metabolome achieved in any study of the metabolome, a large range of individual metabolites was identified and annotated and these are discussed below in light of the literature.

Metabolites with a shared response to the short- and long-term application of DR

Sphingolipid metabolism as a possible mechanism of lifespan extension by DR.

Sphinganine, a sphingolipid and precursor of ceramide, was found to be significantly elevated under both continuous and short-term DR (Table 2.1). The role of sphingolipids in ageing and age-related disease has been studied in a range of organisms and cell types (Trayssac *et al.* 2018). The bioactive sphingolipid, ceramide, is an adaptive stress effector induced in response to various stimuli and increases during ageing (Hannun and Obeid 2002) and is implicated in various human diseases (Niccoli and Partridge 2012). Ceramide has further been shown to mediate cellular senescence and mitophagy (Vaena *et al.* 2021). As senescence results in proliferative arrest, elevated ceramide abundance serves as a biomarker of cellular age (Trayssac *et al.* 2018). Ceramide production is highly conserved across many species (Pewzner-Jung *et al.* 2006) and is generated by either the degradation of sphingomyelin or via *de novo* synthesis using the precursor sphinganine (Hannun and Obeid 2002). Both pathways may be activated separately or in parallel, depending on the kind of stress and cell type (Liao *et al.* 1999). Similar to our study, Green *et al.* (2017) found that the abundance of ceramide (and related factors) becomes elevated in response to CR. As prior literature indicates, elevated ceramide production induces cellular senescence and increases with age, it is puzzling how we and Green *et al.* find ceramide increases under pro-longevity DR conditions. One possible explanation is that acute senescence is protective (Childs *et al.* 2014), although, in a largely post-mitotic animal like the fly, these mechanisms are probably less relevant. Another explanation is that the elevated abundance of ceramide is not simply the result of accelerated ageing, but rather it is upregulated to provide its beneficial role as an adaptive stress effector under nutritionally restrictive conditions. Inactivation of *Drosophila* alkaline ceramidase (ceramidases catalyse the hydrolysis of ceramides to generate sphingosine and fatty acids) has been shown to elevate levels of most ceramide species yet increases lifespan by 50% and increases resistance to oxidative stress (Yang *et al.* 2010). Possibly, the application of DR (and CR, as shown by Green *et al.*) may have similar effects.

A possible geroprotective effect of elevated NAD+ metabolism due to DR.

Nicotinamide mononucleotide (NMN) was significantly elevated under short- and long-term DR (Table 2.1). NMN is the immediate precursor of nicotinamide adenine nucleotide (NAD+) (Gossmann *et al.* 2012). It can be produced from nicotinamide riboside (NR) which is used as a dietary supplement to

elevate endogenous NAD+ levels (Mehmel *et al.* 2020). NAD+ is an essential coenzyme in various metabolic pathways (such as glycolysis and the TCA cycle), an important substrate for sirtuins (which facilitate DNA repair) and declines with age in a wide range of species (Schultz and Sinclair 2016). Elevating NAD+ abundance has been suggested to be a fruitful strategy for treating various conditions and extending lifespan (Mills *et al.* 2016; Das *et al.* 2018; Irie *et al.* 2020; Green *et al.* 2022), and DR has been shown to directly modulate NAD+ dependent mechanisms (Moroz *et al.* 2014). However, the possible benefits of NAD+ pathway modulation have been overestimated (Williams *et al.* 2022) and it is possible that the interest in possible health benefits resulting from elevation of NAD+ metabolism via supplementation is overstated due to competing pharmaceutical interests (Trammell *et al.* 2016; Dellinger *et al.* 2017).

A good geroprotective candidate will have several lines of evidence pointing to its efficacy, including human studies showing improvements in outcomes related to quality of life and even mortality [such as the use of metformin in cancer patients, see: (Saraei *et al.* 2019; Yu *et al.* 2019)] and animal studies of lifespan extension [such as rapamycin across multiple species, see: (Blagosklonny 2019; Selvarani *et al.* 2021)]. The interventions testing program (ITP), is an initiative of the national institute on ageing (NIA) with the sole purpose of evaluating agents that are considered plausible candidates for delaying rates of ageing (Miller *et al.* 2007). The testing criteria is highly rigorous as potential candidates are evaluated using hundreds of heterogeneous mice in parallel studies across multiple institutions. They confirm the benefits of known longevity mediators, such as rapamycin treatment (Harrison *et al.* 2009; Miller *et al.* 2011), which has since progressed to phase II clinical trials. However, a recent study into the possible benefit of NR supplementation shows that upregulation of NAD+ metabolism fails to increase lifespan in either males or females (Harrison *et al.* 2021). We observe an elevation of NMN (indicating increasing NAD+ production) within 48h of a dietary switch, which indicates a quick and actively regulated response to a decrease in dietary protein content. Though NAD+ supplementation may not convey a direct geroprotective effect, endogenous modulation through dietary control may still provide a benefit.

DR upregulates polyols to convey a possible protective effect.

Xylitol was significantly elevated under short- and long-term DR (Table 2.1). Although commonly known as a sugar-free sweetener, xylitol is produced endogenously in small quantities as an intermediary product of carbohydrate metabolism in many species (Ur-Rehman *et al.* 2015). It is a non-digestible carbohydrate with a number of possible health benefits (Salli *et al.* 2019), and may be passed to either the citrate cycle or the pentose phosphate pathway to ultimately generate ATP. There is a higher relative abundance of sucrose in the DR diet resulting from the reduced yeast content. Sucrose may be converted into several polyolic compounds, including xylitol (Cheng *et al.* 2014). It is possible that a stronger reliance on sucrose metabolism under DR could thus be why xylitol was found at higher levels under DR (Colinet and Renault 2014). However, a study by Campbell *et al.* (2019) suggests that adult flies actively upregulate the production of polyols such as xylitol to prevent damage under anoxic conditions. We observe elevated xylitol abundance under both short- and long-term DR across both time points (Table S2.1), which indicates that it is actively produced and sustained under DR.

Metabolites with a shared response in refeeding following a period of DR

Palmitate as a biomarker for mortality overshoot potential.

Palmitic acid (PA) was significantly decreased under refeeding and a continuous high-protein diet (Table 2.2). PA is the most abundant and widespread natural saturated acid, present in plants,

animals and microorganisms (Gunstone *et al.* 2007; Carta *et al.* 2017) and can be provided in the diet or synthesised endogenously via *de novo* lipogenesis from carbohydrates. The advantages of storing lipids over carbohydrates as metabolic fuel include a higher caloric content per unit weight of substrate. Thus, the use of lipids as a primary metabolic substrate permits the accumulation of a large reservoir of energy which may be used not only during periods of prolonged energy demand but also during periods of nutritional scarcity (Biolchini *et al.* 2017). Elevated lipid content under DR has been previously observed in the fly (Piper *et al.* 2005; Laye *et al.* 2015). Although proteins may be catabolised to yield energy, they are neither a readily available form of energy nor the preferred form of energy storage, such as fat (Chatterjee and Perrimon 2021). It is possible that the accrual of PA is a side effect of compensatory feeding (Carvalho *et al.* 2005; Fanson *et al.* 2012), where overconsumption of the DR media to obtain sufficient protein content also results in an increase of sugar-based energy resources which are stored for future use. It is also possible that this storage is preferred as supplemental water is not given and must be obtained through the same dietary medium. Condensation of sugar monomers releases water, which in itself, may be in an increased demand in specific situations (McCracken, Buckle, *et al.* 2020). However, this does not explain the significant decrease of stored PA when switched to rich conditions, especially if desiccation stress was a concern. Therefore, it is possible that the increase in anabolic activity (resulting from an increased dietary protein intake) exceeds energy availability from the rich diet, which PA degradation possibly abates. Consequently, the elevated rate in protein turnover bears an excessive mortality cost under such conditions (Basisty *et al.* 2018).

The dynamics of another pathway member support this interpretation, L-palmitoylcarnitine (Table 2.3), which is elevated under all protein-rich conditions and depleted under DR (Central overlap, Figure 2.4D). β-oxidation (energy release through the TCA cycle) occurs primarily within mitochondria. However, the mitochondrial membrane prevents the direct entry of long chain fatty acids such as PA, so prior conversion to smaller-chain compounds, such as L-palmitoylcarnitine, is required to generate energy from energy-dense stores (Tein 2015). L-palmitoylcarnitine is then shuttled from the cytosol to the mitochondrial matrix via the action of carnitine palmitoyltransferase to undergo oxidation (Angelini *et al.* 2019). This suggests that the accumulation of PA (serving as an energy store) is preferred under DR or accumulates as overall energy turnover is lower. We previously show that the additional mortality costs observable when flies are switched back to a rich diet are contingent upon the prior duration of DR (McCracken, Adams, *et al.* 2020). This further supports this hypothesis that PA accrual under DR results in a mortality cost under refeeding. PA distribution and metabolism is strictly controlled (Carta *et al.* 2017). However, should it be possible to maintain or depress the rate at which PA becomes depleted in response to a protein-rich environment, the excessive costs to mortality may become subverted. The general benefits of DR are unlikely due to an accumulation of PA. However, the switch in metabolism that occurs from DR to fully fed conditions could itself be hazardous, and PA store usage itself could have a causal role or could be a biomarker of the refeeding syndrome.

GPI-anchor becomes depleted under a protein-rich environment.

The precursor compound (GlcN)1 (Ino(acyl)-P)1 (Man)1 is used in the generation of Glycosylphosphatidylinositol (GPI) and was shown to be significantly depleted in both protein-rich environments (Table 2.2). GPI is a lipid anchor for the membrane attachment of a wide variety of essential signalling, hydrolytic and cell adhesion proteins (Schuck and Simons 2006). GPI anchoring allows cells to control the localisation of proteins by modulating their trafficking behaviour (Schuck and Simons 2006), and recent research has revealed several diseases resulting from insufficient GPI-anchor abundance within the cell (Katagiri *et al.* 2011; Rosenbaum *et al.* 2012; Kinoshita 2016; Manea 2018). Little is known about the specific role of GPI concerning DR. If protein-rich conditions reduce the level of GPI-anchor availability, then it may be possible for a protein-rich diet to induce a partial pathology, i.e. a metabolic cost resulting from dietary choice.

Dolichol: a biomarker of cellular age.

Dehydrodolichol diphosphate, the immediate precursor to dolichol, was significantly elevated under protein-rich conditions (Table 2.2). Dolichol is a polyprenolic compound broadly distributed in almost all organelle membranes and is linked with the process of N-glycosylation, the synthesis and processing of glycoproteins, the transmembrane movement of sugars, and the response to oxidative membrane stress (Rip and Carroll 1982; Bergamini *et al.* 2004; Buczkowska *et al.* 2015; Cavallini *et al.* 2016). It is biosynthesised by the general isoprenoid pathway from acetate via mevalonate and farnesyl pyrophosphate and is considered to be a biomarker of biological ageing (Bergamini *et al.* 2004; Parentini *et al.* 2005). Studies indicate a 6 to 30 fold increase during ageing in the tissues of flies, rodents, and humans (Marino *et al.* 1998). Interestingly, it has been found that CR retards the age-associated accumulation of dolichol (Bergamini *et al.* 2004), perhaps caused by a reduction in the rate of non-enzymatic dolichol degradation with age (Parentini *et al.* 2003; Sgarbossa *et al.* 2003). The increase of dehydrodolichol diphosphate (two alternate forms of dehydrodolichol diphosphate were identified, Table 2.3) we observed when flies are fed rich diets could thus suggest accelerated ageing. Not all biomarkers are causally involved in the ageing process. Dolichol supplementation has proven to be effective in restoring liver elasticity (thus reducing cirrhosis risk) (Golovanova *et al.* 2010; Golovanova *et al.* 2016) and has demonstrated improvement in treating the anxiety-like behaviour of Alzheimer's disease where atypical isoprenoid composition is evident (Fedotova *et al.* 2016).

Ecdysone metabolism - an unexpected metabolic response to dietary change.

Three ecdysteroid compounds (2,22-Dideoxy-3-dehydroecdysone [Table 2.1], 3-Dehydroecdysone and Crustecdysone [Table 2.2]) were elevated under protein-rich conditions. However, refeeding following a period of DR was found to unexpectedly decrease ecdysteroid abundance when compared to the continuous-fed group (Table 2.2). The study of ecdysone signalling within Drosophila has classically focused on its key role in metamorphosis (Baehrecke 1996). However, recent studies have found ecdysone also affects post-developmental processes such as reproduction, behaviour, stress resistance, and lifespan (Schwedes and Carney 2012). The response to environmental conditions, such as starvation, also increases ecdysone levels (Terashima *et al.* 2005). Low ecdysteroid concentrations are essential for normal oogenesis. However, nutritional shortage enables the ovaries to retain the ecdysteroid they produce. If this exceeds the tolerated ecdysteroid threshold in the fly, it causes individual egg chambers to undergo apoptosis, consequently limiting the number of eggs produced in relation to food intake (Terashima *et al.* 2005). However, we find that ecdysone was at a higher concentration under protein-rich conditions. As the flies are not subjected to starvation conditions, and the oviposition rate is greatest under a protein-rich diet (McCracken, Adams, *et al.* 2020), we suggest that the relative abundance of ecdysone does not exceed these tolerated thresholds. Therefore, we suggest that the detected elevation in ectosteroids corresponds to the increased demands of yolk protein required for egg maturation (Terashima *et al.* 2005). This hypothesis is supported by the concordant increase of juvenile hormone (also detected under protein-rich conditions) required to facilitate yolk protein synthesis. DR's effects on longevity are achieved in virgin and germline ablated flies (Partridge, Gems, *et al.* 2005; Barnes *et al.* 2006), and could thus be considered independent of its direct effects on reproduction. Therefore, the changes in ecdysone metabolism are unlikely to underlie DR's longevity effect.

Elevated biocytin resulting from an increase in dietary yeast.

Biocytin is a conjugate of D-biotin and L-lysine, where the carboxylate of D-biotin is coupled with the ϵ-amine of L-lysine via a secondary amide bond (Mishra *et al.* 2010) and was found to be significantly elevated under protein-rich conditions (Table 2.2). As an intermediate in the metabolism of biotin, it is cleaved by biotinidase to form biotin (vitamin B7), which may be used in several different processes which mostly result in the regeneration of biocytin. Biotin is involved in several metabolic pathways

such as gluconeogenesis, fatty acid synthesis, and amino acid catabolism by acting as a prosthetic group for pyruvate carboxylase and propionyl-CoA carboxylase, beta-methylcrotinyl-CoA carboxylase, and acetyl-CoA carboxylase (Rodríguez Meléndez 2000). An increase in the level of biocytin under protein-rich conditions could indicate an increased requirement for biotin. However, the elevated levels of biocytin observed within a protein-rich diet are likely congruent with dietary composition, as biotin is obtained from the yeast included in the diet (Tolaymat and Mock 1989). As biotin cannot be synthesised endogenously (Zempleni *et al.* 2009), is non-toxic and freely excreted (Zempleni and Mock 1999), the accrual of biocytin during a period of excess availability may simply serve as a protection against future carboxylative demands and is unlikely to come at a mortality cost.

The dichotomous role of protoporphyrin in longevity.

Protoporphyrin-IX (PP-IX) is the final substrate used in the biosynthesis of heme which, in turn, is used in the production of haemoglobin (Sachar *et al.* 2016) and was found to be significantly elevated under protein-rich conditions (Table 2.2). Little is known about the endogenous role of PP-IX within the fly, as insects lack erythropoiesis (Mandilaras *et al.* 2013). However, *D. melanogaster* has a limited number of porphyrin metabolism pathway elements relating to the production and metabolism of heme-based compounds to which PP-IX may be converted. A study conducted by Pimental *et al.* (2013) showed that the external supplementation of PP-IX was harmless and even increased lifespan, so long as the antioxidant response (superoxide dismutase activity) was left intact. However, it is hard to compare their supplementation dosing to our measured concentration. The reduction in lifespan under PP-IX supplementation observed in the *sod* mutant flies could also suggest that PP-IX is harmful under heightened oxidative stress, not inconceivable during refeeding.

Metabolites with a shared directional response to dietary protein modulation across all dietary comparisons

DR may reduce inflammation through leukotriene modulation.

Leukotriene A4 (LTA4) was significantly elevated across all protein-rich conditions (Table 2.3). Leukotrienes (LTs) are eicosanoid lipid mediators derived from phospholipase-released arachidonic acids that are involved in a range of physiological, homeostatic and pathophysiological processes (Funk 2001; Harizi *et al.* 2008; Greene *et al.* 2011; Bertin *et al.* 2012; Lone and Taskén 2013). LTs are particularly potent mediators of inflammation (Brain and Williams 1990). LTA4 is a chemically and biologically unstable substance (Pace-Asciak and Asotra 1989) whose primary function is to serve as a substrate for the biosynthesis of subsequent LTs (LTC4 and LTD4). Although its mere existence within insects has been questioned, the synthesis of eicosanoid or eicosanoid-like biolipids is, in fact, possible (Scarpati *et al.* 2019), and LTA4 function (LTA4 hydrolase presence) has been identified in the fly (Gong *et al.* 2004). LTA4 was shown to be significantly depleted under DR conditions, thus potentially reducing levels of ageing-associated chronic inflammation (Morgan *et al.* 2007; González *et al.* 2012).

Uridine as a conserved pro-longevity mediator of DR.

Uridine-5'-monophosphate (UMP) is a nucleotide used as a monomer in RNA synthesis, which is generated from orotidine-5'-monophosphate (OMP) by uridine monophosphate synthetase (Eisenberg *et al.* 1990; Gugliucci *et al.* 2019). Both compounds were elevated in response to DR (Table 2.3). DR leads to a mild but widespread inhibition of transcriptional and translational activity with a concordant depression of protein synthesis (Ding *et al.* 2014). Therefore, the elevated abundance of the nucleotide precursors OMP and UMP observed under DR likely resulted from the accumulation of this

precursor caused by the inhibition of downstream RNA synthesis. UMP elevation may therefore be a side effect rather than a causal player in the longevity effect of DR.

However, external supplementation of UMP has been shown to be potentially beneficial, changing fatty acid composition and lipid metabolism (Zhang *et al.* 2019) and providing neuronal and memory benefits (Wang *et al.* 2005; Holguin *et al.* 2008). Furthermore, a recent study conducted by Liu *et al.* (2022) has identified uridine as a potent regeneration factor across multiple species. All uridine-containing compounds identified in this study (a total of four features, Table 2.3 and Table S2.3) are congruent with DR, being more highly expressed in a restrictive environment in all cases. Though the notion of improved somatic maintenance under DR as an adaptive strategy to maximise long-term fitness has been contested (Harrison and Archer 1989; Holliday 1989; Adler and Bonduriansky 2014; McCracken, Adams, *et al.* 2020), it is possible that otherwise unused resources are diverted towards lifespan extension. Such processes are likely to cease upon returning to nutritionally favourable environments (Kirkwood 2005), however this short-term benefit, no matter how limited, may signify an optimal allocation of available resources.

DR results in increased utilisation of sugar-based energy sources.

Uridine diphosphate glucose/galactose levels were elevated in protein-restricted conditions (Table 2.3), indicating the increased dependence on sugar in the low-protein dietary medium. Although lactose was absent from the diet provided, UDP-glucose may be freely converted to UDP-galactose via dGALE to satisfy developmental requirements (Daenzer *et al.* 2012). Both compounds are used extensively in nucleotide sugar metabolism or as precursors to produce polysaccharides such as glycogen, the primary glucose store in the fly (Yamada *et al.* 2018). Similar to the findings of elevated PA, the accrual of UDP sugars might indicate an increased rate of polysaccharide synthesis and storage, maximising energy availability for when anabolically favourable conditions return.

Conclusion

Long-term application of DR is likely to result in a physiological compensation that need not underlie the pro-longevity response (Table S2.5). As in the fly, DR's effect on mortality is almost instant, measuring physiology shortly after a switch to a new diet as we used in our study may prove useful in pinpointing the elusive mechanisms of DR. It has been suggested that the sudden decrease in mortality risk under DR is not necessarily the result of metabolic compensation, but could be an escape from costs associated with a high-protein diet (McCracken, Adams, *et al.* 2020). However, depending on how such costs are interpreted, there are indications from the metabolomics study conducted herein of regulation under DR rather than a passive escape of costs alone. For example, there appears to be a positive modulation of known mediaries of longevity, namely ceramide, uridine, NAD+, LT, and possible novel mediaries, namely xylitol under DR. Furthermore, there are no avoided metabolic 'costs' unique to the onset of DR (Table S2.4: positive $\log_2$(FC) values), as metabolites decrease in abundance from the application of DR are reactive to the change in diet and not regulated by it. Both amino acids (L-leucine, L-isoleucine) and pantetheine are obtained from dietary yeast; therefore, its relative depletion under DR reflects the decrease in the dietary yeast source. Phenylacetaldehyde is an odour present within the food source (Ziegler *et al.* 2013), therefore is also likely to be at a reduced concentration within the DR diet. Further work should be conducted to manipulate these molecules within demography-based *Drosophila* screens (such as that used in Chapter 1) to determine possible prolongevity effects, if any, across a range of conditions. Manipulations to increase (e.g. by dietary supplementation) or decrease (e.g. via drug inducible overexpression of the enzyme specific to the substrate) metabolite abundance may be targeted to specific periods, e.g. immediately following the dietary switch, or during the mortality overshoot period. In addition, functional assays (e.g. walking/climbing) may be performed to determine any non-mortality effects such manipulations may result in.

The excess mortality observed when flies return from DR was a more complex issue to investigate as the phenotype is dependent on the prior metabolic state. We observe a continually higher rate of mortality under protein-rich conditions when compared to DR (Chapter 1, Figure 1.2). However, this was exceeded by those re-fed following DR (Chapter 1; McCracken, Adams, *et al.* 2020). This effect can be interpreted as a 'refeeding syndrome', clinically defined as refeeding after starvation (Mehanna *et al.* 2008). We could therefore expect toxicity effects, with metabolites either with a known toxic nature produced under metabolic imbalance, or expect metabolites to increase upon refeeding beyond the levels seen in continuously rich-fed flies. Many of the metabolites responded rapidly to a sudden influx of dietary protein (Table 2.2). However, metabolite concentrations never significantly exceeded the levels found under steady-state conditions, indicating possibly that metabolite abundance is tightly regulated even under dynamic metabolic flux and not providing any direct metabolic indications of toxicity through refeeding. In addition, there were few metabolites identified that are unique to the influx of dietary protein (that do not share DR-based physiology) with an excessive abundance, or known toxicity effects (Table S2.3) which could have explained the 'refeeding syndrome' effect. However, PA abundance was found to indicate the potential of mortality overshoot and possibly acts as a short-term energy boost which correlates with the mortality response observed in our study of demography (Chapter 1). It is possible that preventing the depletion of energy-stores (such as PA), or at least limiting the rate of depletion under nutritionally favourable conditions (i.e. a rich diet) may prove to be effective in controlling the mortality overshoot effect. This hypothesis draws parallels with the treatment of refeeding syndrome patients, where excessive immediate nutrition can prove to be fatal as anabolic demands exceed micronutrient availability (Mehanna *et al.* 2008; Doig *et al.* 2015). It would be of interest to directly measure known indicators of the refeeding syndrome (depleted potassium, phosphate and magnesium) across the mortality overshoot phase using a targeted metabolomic approach, to establish whether a gradual increase in protein concentration would avoid the excessive mortality costs returning from DR.

The mechanisms that underpin dietary effects on longevity are yet to be fully understood. The use of metabolomics to measure the underlying regulation of specific compounds has already proven fruitful (Laye *et al.* 2015; Jin *et al.* 2020) and we now apply these techniques using dietary switches. We replicate known candidate mechanisms of DR, ie. ceramide, uridine, NAD+ and LTs. Importantly we identify a novel candidate mechanism through xylitol and associated metabolism. For the refeeding effects we implicate palmitate as a shared metabolic response with DR which warrants future mechanistic study. In general, there is promise in delineating dietary responses, using dietary switches, especially when they are known to induce a phenotype (as in the fly) and this paradigm deserves to be expanded within metabolomics (using targeted approaches) and other omics, such as transcriptomics and proteomics.

Supplementary

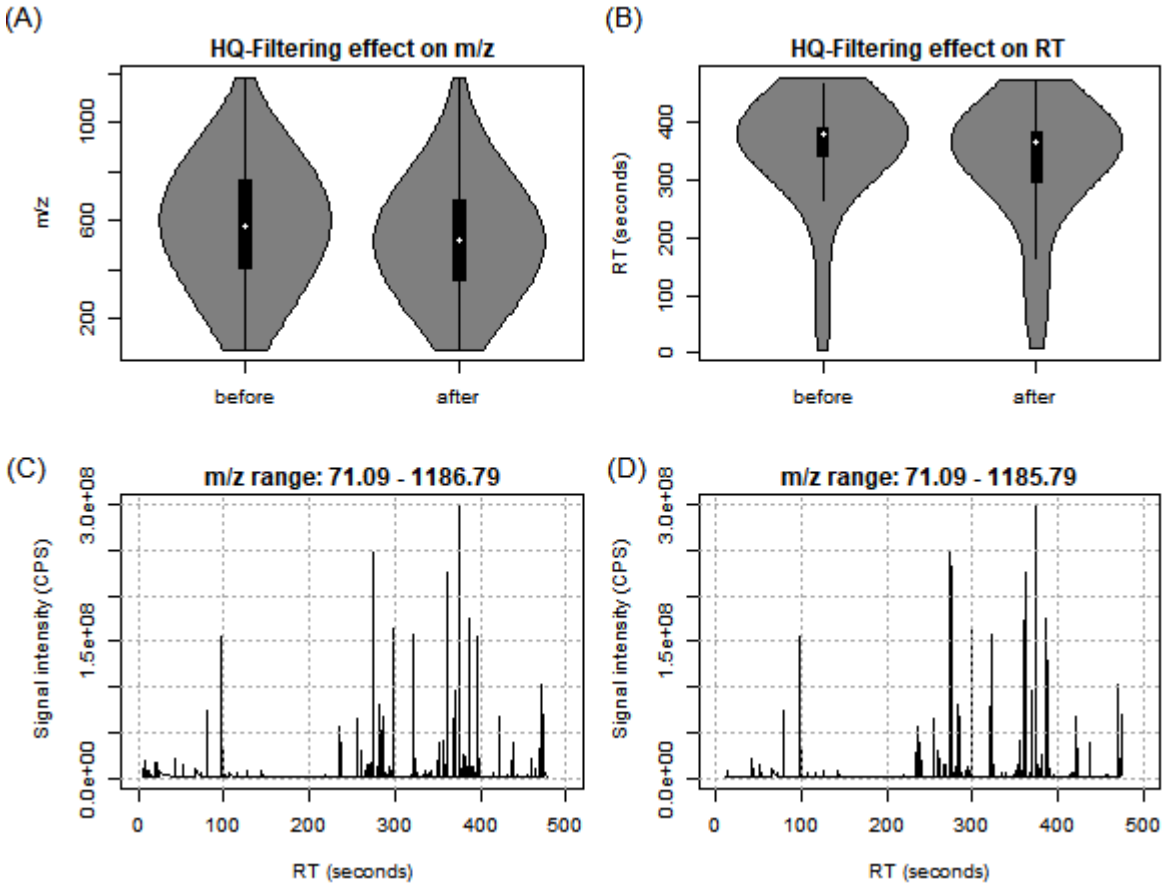

Figure S2.1 | The effect of high-quality filtering on the 24 hour time point.

Violin plots represent the changes to both m/z (**A**) and RT (**B**) as high-quality filtering was applied. The median m/z value was significantly reduced (581.4876 to 522.3495) following high-quality filtering (Two-sample Mann-Whitney test: V = 366,839, P < 0.0001, 95% confidence intervals: 509.7798-534.9151). The median RT value was also significantly reduced (379.3448 seconds to 351.3546 seconds) following high-quality filtering (Two-sample Mann-Whitney test: V = 264,569, P < 0.0001, 95% confidence intervals: 346.7974-355.4988 seconds). Base peak chromatogram (BPC) plots represent the summed intensity of the most intense peak at every point in the analysis. BPCs before (**C**) and after (**D**) the application of high-quality filtering visualise the effect on overall intensity.

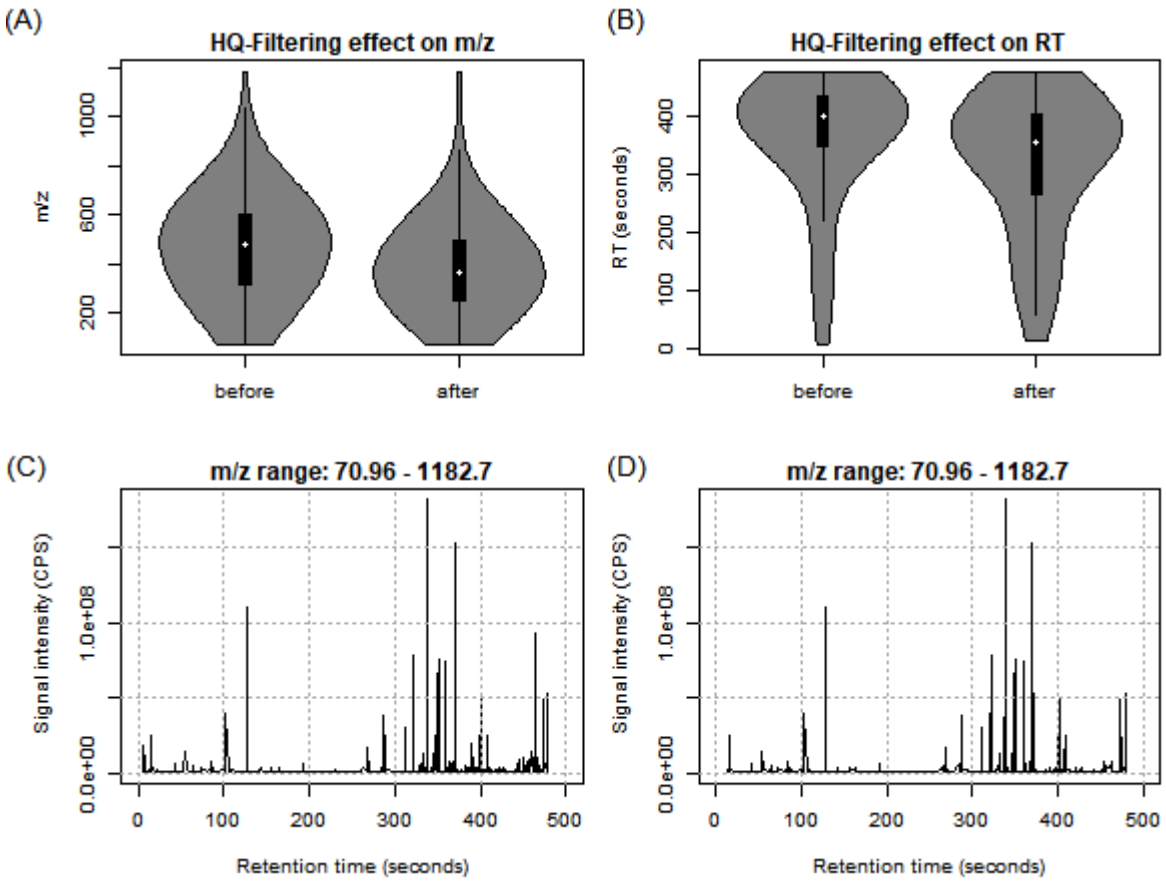

Figure S2.2 | The effect of high-quality filtering on the 48 hour time point.

Violin plots represent the changes to both m/z (**A**) and RT (**B**) as high-quality filtering was applied. The median m/z value was significantly reduced (479.7448 to 375.7127) following high-quality filtering (Two-sample Mann-Whitney test; V = 51,744, P < 0.0001, 95% confidence intervals: 363.6308-387.9959). The median RT value was also significantly reduced (399.6495 seconds to 339.0999 seconds) following high-quality filtering (Two-sample Mann-Whitney test: V = 49,459, P < 0.0001, 95% confidence intervals: 327.9227-348.3807 seconds). BPC plots represent the summed intensity of the most intense peak at every point in the analysis. BPCs before (**C**) and after (**D**) the application of high-quality filtering visualise the effect on overall intensity.

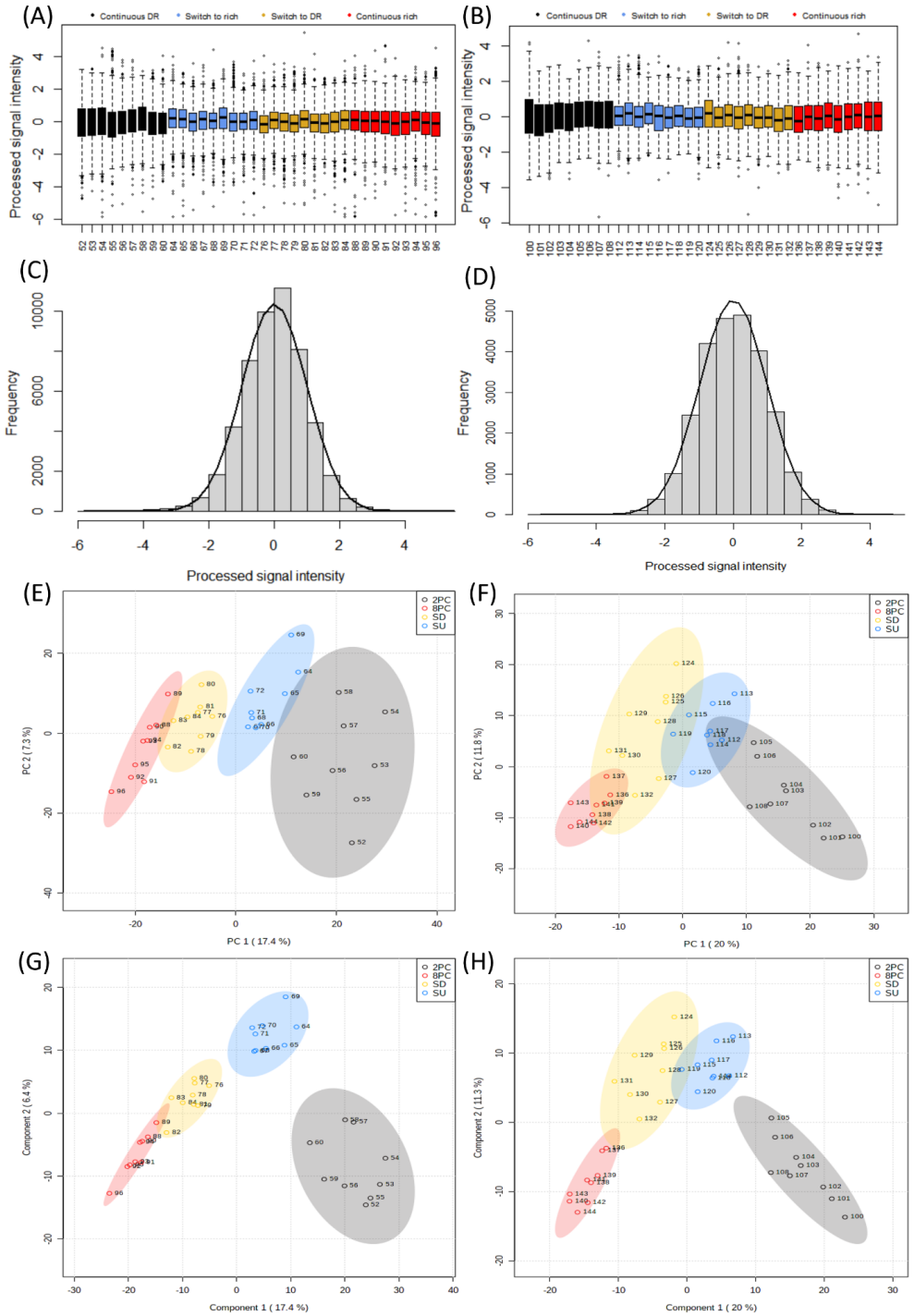

Figure S2.3 | Post-processing characterisation of both time points.

(**A**,**C**,**E**,**G**) Plots corresponding to the 24h time period. (**B**,**D**,**F**,**H**) Plots corresponding to the 48h time period. Total sample ion intensities (**A**,**B**) were plotted to visualise possible differences between both samples and groups following the data processing. Histograms of processed feature intensities (**C**,**D**) approximate a normal distribution (curved line). Initial principal component analysis (**E**,**F**) separated samples according to class (clear separation between continuous diets), so partial least squares-discriminant analysis (**G**,**H**) was used to maximise this separation and identify differences in metabolites (chemical shifts) that are the basis of this class separation. Characterisation was performed at the feature level (non-annotated data) to capture the maximal metabolomic variance resulting from dietary perturbation (24h: n = 1,422, 48h: n = 723).

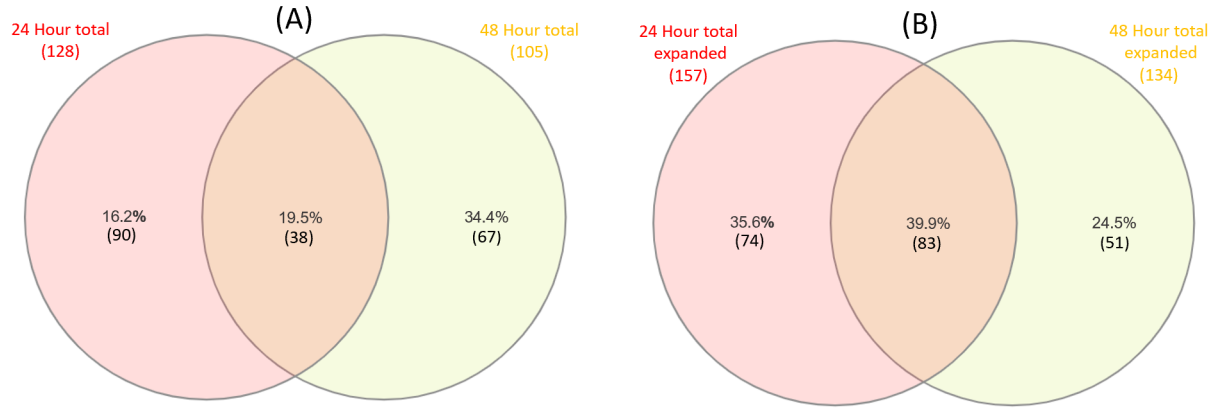

Figure S2.4 | Overall annotation overlap between time points.

Venn diagrams represent the total metabolic overlap between both time points. (**A**) = Comparison between unique KEGG terms (as used in figure 2.4). (**B**) = Comparison between the same terms, however, annotations containing multiple KEGG entries have been pooled and are treated individually. In total 38 metabolites were shared between both 24 and 48 hour time points. This increased to 83 possible KEGG entries when individual queries that match multiple entries were pooled together.

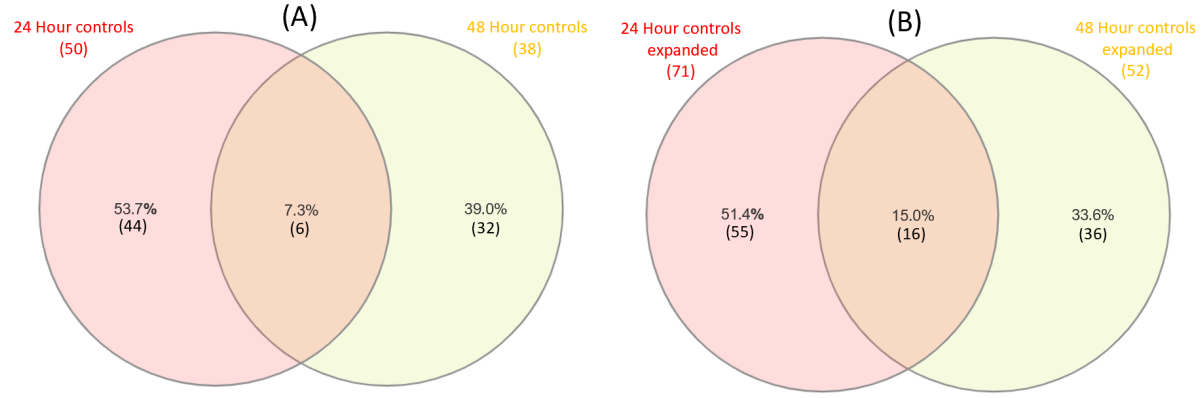

Figure S2.5 | Annotation overlap between continuous DR and continuous rich dietary conditions across both time points.

Venn diagrams represent the metabolic overlap between the continuous rich and continuous DR dietary comparisons (segment (1) as shown in figure 2.1). (**A**) Comparison between annotated individual metabolomic features (as used in figure 2.4). (**B**) Comparison between the same features, however, terms matching multiple annotations (KEGG entries) have been pooled and are treated individually.

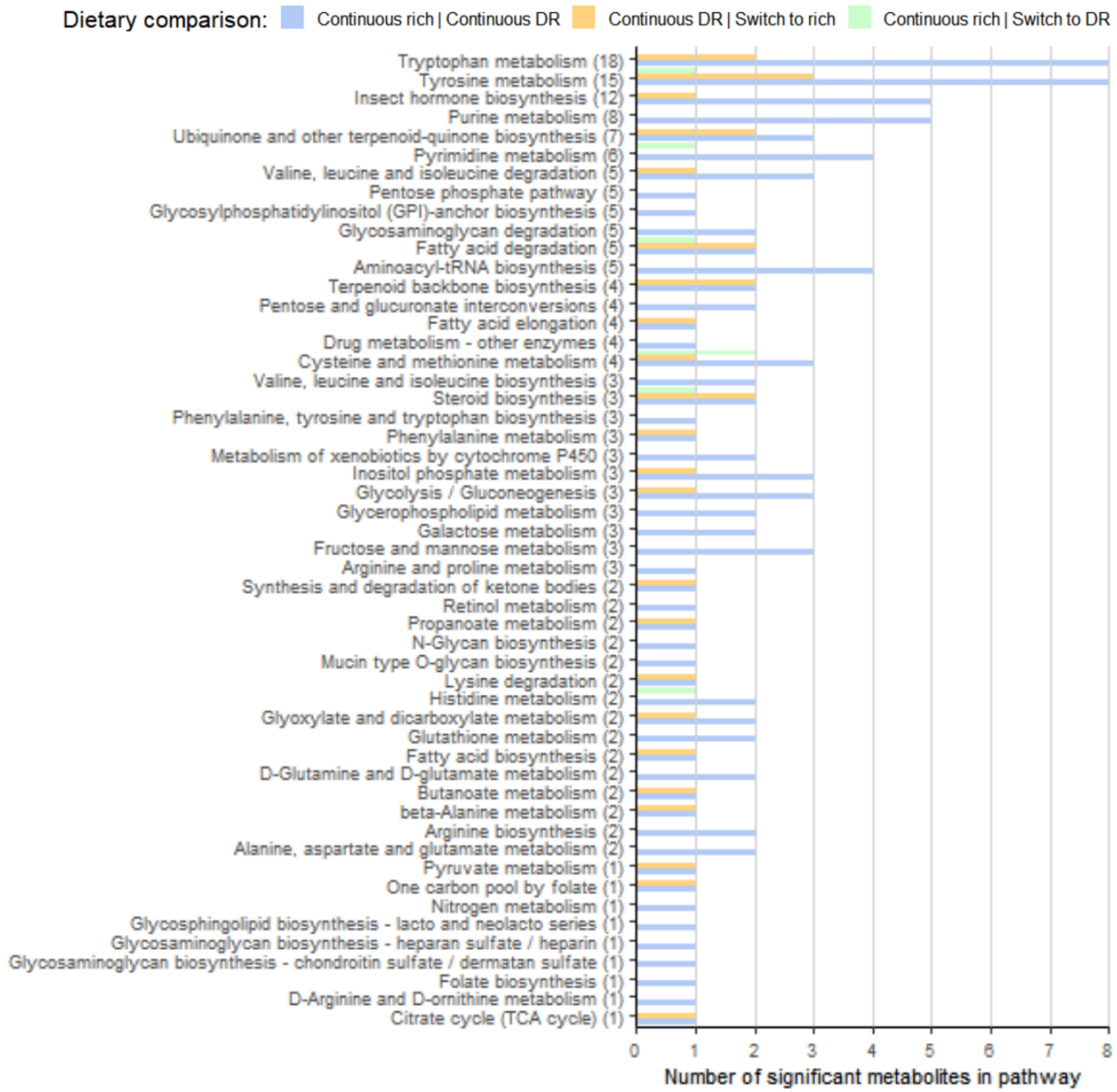

Figure S2.6 | Metabolic pathway use differs between dietary comparisons after 24 hours.

Barplot of each dietary comparison represents the number of significant metabolites (FDR $\alpha < 0.05$) associated with each pathway between dietary comparisons. Significance was calculated with standard t.tests, and pathway annotation was performed using the KEGG drosophila melanogaster reference database using both m/z and RT queries. A custom reference metabolome consisting of all detected metabolites was used as the background for pathway analysis. The total pathway coverage of all detected metabolites is listed in brackets following the pathway name. The comparison between continuous dietary groups is shown to have the largest effect after 24h. However, the switch to rich comparison does show a marked increase in pathway perturbation when compared to the switch to DR indicating a more rapid response to dietary change.

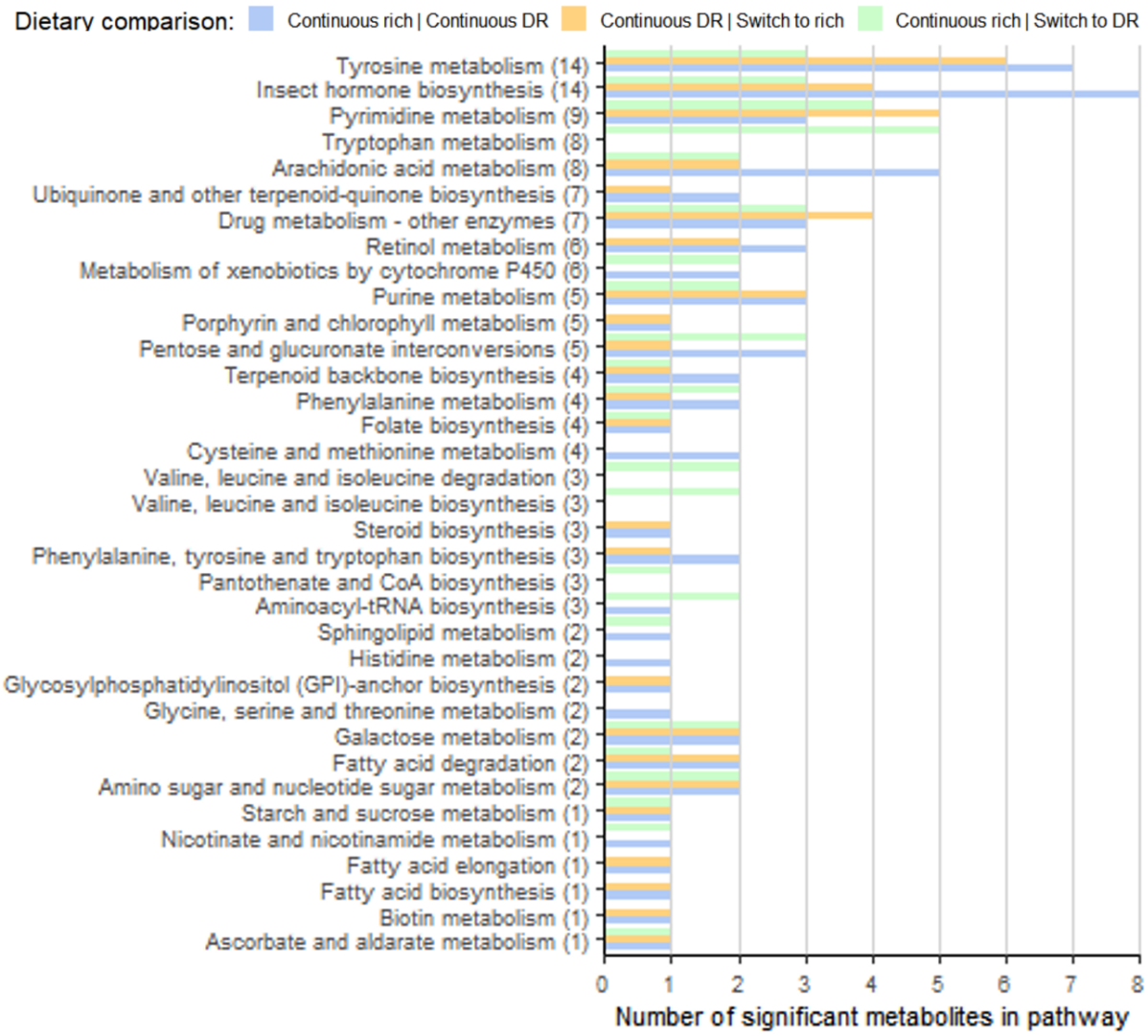

Figure S2.7 | Metabolic pathway use differs between dietary comparisons after 48 hours.
Barplot of each dietary comparison represents the number of significant metabolites (FDR $\alpha < 0.05$) associated with each pathway between dietary comparisons. Significance was calculated with standard t.tests, and pathway annotation was performed using the KEGG drosophila melanogaster reference database using both m/z and RT queries. A custom reference metabolome consisting of all detected metabolites was used as the background for pathway analysis. The total pathway coverage of all detected metabolites is listed in brackets following the pathway name. The comparison between continuous dietary groups is still shown to have the largest effect after 48h. However, the switch to DR comparison now shows an increase in pathway perturbation comparable to the switch to rich indicating a relative metabolic delay in response to DR.

Table S2.1 | Concordant metabolites involved in the 'classical' long-term DR response.

Significant metabolites identified across 24 and 48 hour time points with a directional agreement between time points. Directional agreement: **A**,**B** = +ve,+ve (metabolites which are elevated under protein-rich conditions) / **A**,**B** = -ve,-ve (metabolites which are elevated under DR). Dietary comparison: **(A)** Continuous rich ∩ Continuous DR at 24h, **(B)** Continuous rich ∩ Continuous DR at 48h.

Pathway(s)	Compound(s)	KEGG ID(s)	(A) log_2(FC)	(B) log_2(FC)
Cysteine, Methionine, Tyrosine and Histidine metabolism. Phenylalanine, Tyrosine, Tryptophan, Ubiquinone and other terpenoid-quinone biosynthesis.	4-Hydroxyphenylpyruvic acid, Methylimidazoleacetic acid, 1,2-Dihydroxy-3-keto-5-methylthiopentene	C01179, C05828, C15606	-0.4007	-0.3113
Pentose and glucuronate interconversions	D-Xylitol, L-Arabitol	C00379, C00532	-0.1960	-0.3618
Purine metabolism	Adenosine, Deoxyguanosine	C00212, C00330	0.2244	0.4302
Insect hormone biosynthesis	2,22-Dideoxy-3-dehydroecdysone	C16498	1.0773	2.5160
Insect hormone biosynthesis	3beta,5beta-Ketotriol	C16494	0.6579	0.5285

Table S2.2 | Hypergeometric test to model enrichment between continuous and switching diets.

Hypergeometric testing of venn diagram overlaps to determine enrichment between dietary conditions**. P** = probability of over-representation (enrichment). All sections tested were found to be significantly enriched (at both the feature and annotated level) across both time points.

	24h time point		48h time point	
	Feature level (P)	Annotated level (P)	Feature level (P)	Annotated level (P)
Switch to rich segment	< 0.0001	0.0018	< 0.0001	< 0.0001
Switch to DR segment	< 0.0001	0.0331	< 0.0001	0.0018
Central overlap	< 0.0001	0.0006	< 0.0001	< 0.0001

Table S2.3 | Metabolites unique to dietary protein influx.

Dietary comparison (48h time point). Metabolites listed are uniquely regulated in response to the intake of dietary protein, but do not share long-term DR physiology [Figure 2.1(4)]. **(A)** = Continuous DR ∩ Switch to rich. Positive log_2(FC) values represent a larger metabolite intensity under DR, whilst negative values represent a larger metabolite intensity in the switch to rich.

Pathway(s)	Compound(s)	KEGG ID(s)	(A) log_2(FC)
Pyrimidine metabolism	C00526	Deoxyuridine	0.1870
Fatty acid degradation, Steroid biosynthesis	C02990, C05103	L-Palmitoylcarnitine, 4a-Methylzymosterol	1.4196
Insect hormone biosynthesis	C02513	3-Dehydroecdysone	0.6826
Pyrimidine metabolism, Drug metabolism - other enzymes	C00460, C21751	Deoxyuridine triphosphate, 5-Fluorodeoxyuridine triphosphate	-0.2081

Table S2.4 | Metabolites unique to the onset of DR.

Dietary comparison (48h time point). Metabolites listed are uniquely regulated in response to the onset of DR, but do not share long-term DR physiology [Figure 2.1(6)]. **(A)** = Continuous rich ∩ Switch to DR. Positive log_2(FC) values represent a larger metabolite intensity under a high-protein diet, whilst negative values represent a larger metabolite intensity in the switch to DR.

Pathway(s)	Compound(s)	KEGG ID(s)	(A) log_2(FC)
Phenylalanine metabolism	C00601	Phenylacetaldehyde	0.2861
Valine, leucine and isoleucine biosynthesis & degradation, Aminoacyl-tRNA biosynthesis	C00123, C00407	L-Leucine, L-Isoleucine	0.1535
Tryptophan metabolism	C00643, C05636, C05638	5-Hydroxy-L-tryptophan, 3-Hydroxykynurenamine, 5-Hydroxykynurenamine	-0.2565
Folate biosynthesis	C20239	6-Carboxy-5,6,7,8-tetrahydropterin	-0.4100
Pyrimidine metabolism, Tryptophan metabolism	C00214, C03227, C05651	Thymidine, L-3-Hydroxykynurenine, 5-Hydroxykynurenine	-0.3297
Insect hormone biosynthesis	C16503	Methyl farnesoate	-0.3423
Pantothenate and CoA biosynthesis	C01134	Pantetheine 4'-phosphate	0.2861

Table S2.5 | Metabolites unique to long-term DR.

Dietary comparison (48h time point). Metabolites listed are uniquely regulated in long-term DR physiology [Figure 2.1(1)]. **(A)** = Continuous rich ∩ ContinuousDR. Positive log_2(FC) values represent a larger metabolite intensity under a high-protein diet, whilst negative values represent a larger metabolite intensity under DR.

Pathway(s)	Compound(s)	KEGG ID(s)	(A) log_2(FC)
Phenylalanine, tyrosine, tryptophan, Ubiquinone and other terpenoid-quinone biosynthesis. Tyrosine, Histidine Cysteine and methionine metabolism.	4-Hydroxyphenylpyruvic acid, Methylimidazoleacetic acid, 1,2-Dihydroxy-3-keto-5-methylthiopentene	C01179, C05828, C15606	-0.3113
Aminoacyl-tRNA, Phenylalanine, tyrosine, tryptophan, Ubiquinone and other terpenoid-quinone biosynthesis. Tyrosine and Phenylalanine metabolism.	L-Tyrosine	C00082	-0.4290
Cysteine and methionine metabolism, Glycine, serine and threonine metabolism	L-Cystathionine	C02291	-0.1393
Cysteine and methionine metabolism, Drug metabolism - other enzymes	5-Fluorouridine	C02291, C16633	0.1977
Insect hormone biosynthesis	(10S)-Juvenile hormone III acid diol	C16506	0.2738
Arachidonic acid metabolism	Prostaglandin H2, Prostaglandin E2, Prostaglandin D2	C00427, C00584, C00696	-0.2517
Terpenoid backbone biosynthesis	(S)-5-Diphosphomevalonic acid	C01143	-0.2086
Insect hormone biosynthesis	7-Dehydrocholesterol	C01164	-0.1905
Steroid biosynthesis	4alpha-Carboxy-5alpha-cholesta-8,24-dien-3beta-ol	C22112	0.1987
Insect hormone biosynthesis	20,26-Dihydroxyecdysone	C16500	-0.2031
Retinol metabolism	Retinoyl b-glucuronide	C11061	0.3921

Chapter 3: Shared metabolic responses to dietary restriction across *Drosophila* species

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Abstract

Dietary restriction (DR) results in a marked improvement to the life- and health-span of many species. However, the mechanisms which explain this response are far from fully understood. We investigate the conserved response to dietary restriction within the metabolome using a comparative panel of three *Drosophila* species (*D. ananassae*, *D. biarmipes* and *D. virilis*). We observe a separation reflected in the metabolome to dietary perturbation. However, there is also variation in the relative level of metabolic response to diet between species, which correlates with ecological dietary niche. On the individual metabolite level, we detected a small but significant fraction of metabolites that responded similarly across species. This indicates an at least partially conserved metabolic response to DR within *Drosophila* species. These specific metabolites were related to the reduction in the metabolism of amino acids and upregulation of DNA repair. We also again find that palmitic acid (PA) is depleted under rich diet conditions which supports our possible prior conclusions that when refeeding after DR, PA is rapidly spent and imposes a metabolic cost that could explain the excessive mortality observed. More generally, the shared metabolic responses we find across species, including possible shared candidate mechanisms of DR, provide increased credibility of evolutionary conserved mechanisms of DR and ageing which further demonstrates the utility of comparative approaches in our field.

Contributions:

AJC, SG, TIG, and MJPS conceived and designed the experiment. AJC, SG, MJPS, GA and LH conducted the experiment. HW assisted with LC-MS operation. AJC analysed the data and wrote the manuscript with input from all authors.

Acknowledgements:

We would like to thank Riki Kawaguchi for the code shared online to generate the probability simulations for the central Venn diagram overlap.

Introduction

A complex network of genetic and biochemical factors is involved in the ageing process (López-Otín *et al.* 2013). Genetic and environmental factors, such as diet, strongly modulate this network and its outcomes (Schwingshackl *et al.* 2017). Dietary restriction (DR), the reduction of dietary components without malnutrition, is one of the most powerful and well-researched methods of lifespan extension. The DR longevity response is conserved across many species, e.g. monkeys (Kemnitz 2011), rodents (Simons *et al.* 2013), nematodes (Pontoizeau *et al.* 2014), flies (McCracken, Adams, *et al.* 2020) and most probably also including humans (L. Fontana *et al.* 2010; Trepanowski *et al.* 2011). Despite this, relatively little is known about the mechanisms via which DR slows ageing (Piper *et al.* 2011; Tatar 2011; Trepanowski *et al.* 2011).

The response to a shared stimulus can generate a multitude of effects both within and between species. In pharmaceutical development, animal models are routinely used to test drug candidates and require careful consideration of interspecific differences to extrapolate possible effectiveness and

dosing in humans (Collins 2001). Such interspecies effects are also true of dietary composition, which results in changes to systemic regulation affecting growth, reproduction and longevity (Nazario-Yepiz *et al.* 2017; Watanabe *et al.* 2019). Even when the phenotypic response to DR is conserved across species, the mechanisms that lead to lifespan extension could be different or of differential importance across species. Therefore, studies that utilise interspecies comparisons may help to understand DR and ageing more effectively.

Within the field of biogerentology, comparative biology has been utilised in different ways. For example, Ricklefs (2010) compares the associations of ageing rate to body size between birds and mammals, discussing the evolutionary and mechanistic aspects of ageing within each species. Wuttke *et al.* (2012) utilise a gene network of more than 100 known DR mediaries across multiple model organisms (such as mice, yeast, worms, and flies) to show how they are both increasingly conserved at the molecular level and interact more than would be expected by chance. Plank *et al.* (2012) used transcriptomic meta-analysis to identify a conserved role of sterol metabolism within 61 gene expression profiles obtained from mammalian (rodent and pig) microarrays of caloric restriction (CR). Though not focusing on the effects of CR, Palmer *et al.* (2019) expanded this transcriptomic meta-analysis approach, using 127 mammalian (rodent and human) microarray and RNA-Seq datasets to identify genes that change in expression with age. They found that immune and stress response genes are upregulated in later life, depressing metabolic and developmental pathways. Additionally, they found that these age-related genes are expressed broadly across all tissue types, highlighting the effectiveness of whole-organism or multi-tissue analyses to study systemic effects such as those used in this study.

Fruit flies, specifically *Drosophila*, are well suited to better understand the DR effect across species in more detail. *D. melanogaster* has been used extensively to study the effect of diet on ageing (Piper *et al.* 2011; Kapahi *et al.* 2017). As it is possible to house and manipulate multiple *Drosophila* species in parallel under the same conditions, interspecies comparison within the same study is both realistic and achievable (Ma *et al.* 2018). Furthermore, *Drosophila* has served as a model species group for molecular genomics and evolution (Stark *et al.* 2007). We recently found that decreasing the yeast concentration using standard lab diets resulted in DR responses across multiple species (Gautrey *et al.* 2022). As such *Drosophila* provides an ideal model system to conduct comparative biology of DR as we can use the same housing conditions and dietary composition increasing validity and comparability of results across species. A sub-selection of these species with observed DR responses were made, targeting diverse ecological niches and are considered here to determine any potential variation in cross-species metabolic response.

As the biology of interest, DR and ageing, is inextricably linked to metabolism, the use of metabolomics (the study of all metabolites present within a tissue or organism) can prove highly effective in distinguishing between phenotypic effect and mechanistic response (Avanesov *et al.* 2014; Walters *et al.* 2018; Jin *et al.* 2020; Liu *et al.* 2021; Maslov *et al.* 2021). It has also proven to be effective in studies of different species (Blekhman *et al.* 2014; Maslov *et al.* 2021). Combining these experimental paradigms may present a unique insight into the conserved mechanisms of DR. The metabolome is the final regulatory interface, and it integrates information from both genomic and environmental sources (Hoffman *et al.* 2014; Laye *et al.* 2015). As all metabolites are directly involved in metabolism, studies that incorporate a metabolomic approach may explain a greater proportion of phenotypic variation than what has been achieved using genomic approaches alone (Manolio *et al.* 2009). Furthermore, the use of nontargeted metabolomics such as that used in this study to identify possible interspecies effects of DR provides a novel and unbiased view of possible longevity effects as it does not require *a priori* knowledge of specific mechanisms or regulatory elements. However, when comparing species, it is probably critical that similar diets are fed, especially when a metabolic response is measured. Different diets across highly divergent species could lead to differential

responses that need not be related to the DR longevity response. Identifying the conserved interspecific response to DR might help dissect a key set of regulators that mediate longevity across *Drosophila* and arguably other species.

To identify conserved mechanisms of the DR effect, enrichment analysis of significant dietary overlap between species will be conducted. If found to be significant, this would indicate a possibly conserved response to diet-induced metabolic regulation. Each result will be discussed within the context of the literature, highlighting known longevity effects and discussing possible mechanisms of action. In addition, the knowledge gained from this interspecies comparison of *Drosophila* (*D. ananassae, D. biarmipes* and *D. virilis*) will be contrasted with the findings from our previous work (Chapter 2), which investigated the effect of short- and long-term applications of DR on *D. melanogaster*.

Methods

Fly stocks.

Fly stocks from the species used were obtained from the Species Stock Centre at Cornell University, USA (*D. virilis* 15010-1051.88; *D. ananassae* 14024-0371.34; *D. biarmipes* 14023-0361.09). All flies were reared on rich media (8% autolysed yeast, 13% table sugar, 6% cornmeal, 1% agar and nipagin at 0.225% [w/v]). Freshly eclosed (<1 day old) age-matched cohorts were transferred for two days of mating in bottles. Female flies were then transferred to demography cages at a density of ~25 flies per cage. Different life history traits between *Drosophila* species may necessitate tailored adjustments to experimental protocols, as for example, potential differences in age of sexual maturity may prohibit successful mating if conducted too early (Pitnick *et al.* 1995). However, the DR longevity response is also present in virgin and germline ablated flies (Partridge, Gems, et al. 2005; Barnes et al. 2006), therefore, it is not a requirement for the flies to be successfully mated once the demography cages are constructed. Dietary restriction was imposed on the optimal pro-longevity diet as previously determined using a range of diets (Gautrey *et al.* 2022; McCracken, Buckle, *et al.* 2020), varying only yeast concentration (8% for rich, 4% for restricted). Flies were kept on these diets for one week, after which individual flies, 12 per species and dietary condition combination (72 total), were sampled using snap freezing in eppendorfs.

Metabolome extraction and acquisition.

Flies were handled, processed and analysed as described in chapter 2. In short, metabolites were extracted using a methanol chloroform procedure. All reagents, containers and transfer stages were chilled and kept on ice where possible. Whole flies were lysed in a methanol chloroform water solution. Samples were vortexed and centrifuged, with the supernatants of each being temporarily separated before undergoing a further round of lysis. Supernatants from each extraction were pooled. An excess of water was then added to each pooled supernatant and centrifuged to separate the aqueous and organic layers. The organic layer was collected and used in this study. Samples were then transferred to LC-MS vials following a 1:10 dilution with methanol and water (1:1, v/v) containing 0.1% Formic Acid (FA). Mass spectrometry (MS) analysis was conducted using a Waters Synapt G2 mass spectrometer using ultra-performance liquid-chromatography as a sample introduction method. Each sample was run in triplicate. Two LC-MS plates containing 72 samples separated by species were used (Plate 1: *D. Ananassae, D. Biarmipes*. Plate 2: *D. Virilis*). Batch effects between LC-MS plates were corrected with ComBat (Johnson *et al.* 2007). Outliers identified via preliminary principal component analysis (PCA) were removed from subsequent analysis.

Data processing and statistical analysis.

Data processing and statistical analysis were conducted using R (R Core Team 2021) version 4.1.0. Raw data was converted and centroided with Proteowizard (Chambers *et al.* 2012) and processed with XCMS (Smith *et al.* 2006; Tautenhahn *et al.* 2008). Optimised peak deconvolution parameters were calculated using IPO using all samples (Libiseller *et al.* 2015). Peak deconvolution identified 4,891 features across all species. Estimated ion chromatograms (EICs) for all picked peaks following XCMS processing were generated using the 'featureChromatograms' function and manually categorised as 'High-quality' or 'Low-quality' to minimise technical noise without compromising the underlying biological signal (Schiffman *et al.* 2019). Only high-quality peaks, 1,017 in total, were retained for subsequent analysis. Median retention time (RT) and mass to charge (m/z) values were significantly reduced as a result of quality filtering, the effects of which have been visualised (and described) in Figure S3.1. Technical replicates were merged by taking the median feature intensity within technical replicates. Peak filling accounted for 16.85% of the processed data.

Samples underwent median normalisation, generalised logarithm transformation (Durbin *et al.* 2002) and autoscaling (van den Berg *et al.* 2006) following the MetaboAnalyst workflow (Pang *et al.* 2021) as done previously. The intensity and distribution of metabolomic features were characterised (Figure S3.2) and annotated using mummichog (Li *et al.* 2013), using the mass to charge, retention time and experimental parameters (Ionisation mode: positive. Mass tolerance as determined by IPO: 57.65 ppm) to match known metabolomic compounds in the *Drosophila melanogaster* KEGG database (Kanehisa *et al.* 2021). *D. melanogaster* was used as a reference for annotation as it is the only drosophila-based metabolomics database available. Annotation coverage can be reduced due to small differences between fly species. Note, a single feature could be annotated as multiple KEGG entries if it matches more than one reference compound. Therefore, each row in Table 3.2 represents a single feature but can include multiple annotations. Significance testing of features between dietary groups was conducted using unpaired unequal variance t-tests using Welch approximation, and P values were not adjusted for multiple comparisons as the metabolic overlap between species was low. An unadjusted probability value of < 0.05 was used to establish dietary effects (significant features) within species. Significance testing of dietary correlations was conducted using Mann-Whitney tests using the correlation matrices of each species used to generate the clustered heat maps (Figure 3.1), with self-correlated values (equal to 1) excluded. Enrichment analysis was performed using hypergeometric testing and probability simulation using 100,000 iterations.

Results

The degree of dietary-induced change in the metabolome varies across species.

Species-level response to DR can be examined by the correlation between feature intensities across the whole metabolome. The prediction is that if the metabolomic response to DR is both widespread and systemic, correlations should be strongest between samples taken from flies that we fed the same diet. Such relationships can also be visualised using clustering, and samples from the same dietary condition are expected to cluster together (Figure 3.1). However, only within two of the three species studied, *D. ananassae* (Figure 3.1A) and *D. virilis* (Figure 3.1C), was there clustering observed within dietary conditions. There was no discernable separation between dietary conditions *D. biarmipes* (Figure 3.1B), and all samples share a similar metabolomic correlation coefficient. Mann-Whitney tests comparing the correlation of feature intensity across samples between low- and high protein diets were indeed significantly different in both *D. ananassae* ($W = 5444$, $P < 0.0001$) and *D. virilis* ($W = 3228$, $P = 0.0353$), but not significantly different in *D. biarmipes* ($W = 8572$, $P = 0.8221$). This indicates that the metabolome is responsive to DR in both *D. ananassae* and *D. virilis*, but less so, and potentially more tightly regulated in *D. biarmipes*. Similar conclusions can be drawn from multivariate analysis on these data (Figure S3.2 C-D).

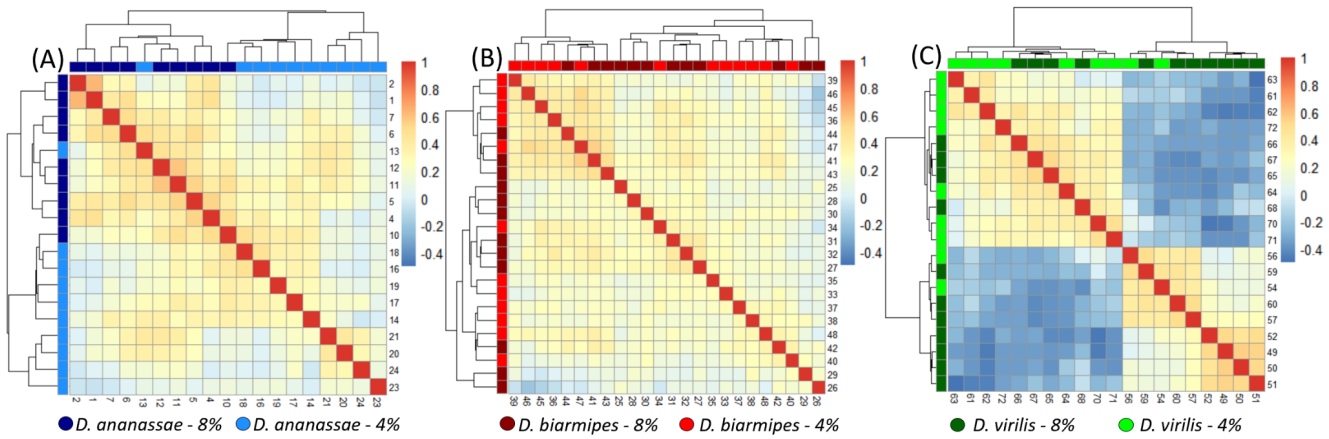

Figure 3.1 | Metabolomic response to DR is varied between species.

Clustered heatmaps were generated using the correlation between samples and all detected feature intensities (**A:** *D.* ananassae, **B:** *D. biarmipes*, **C:** *D. virilis*. Number of features = 1,017. Sample numbering is reflected along the X and Y axis. Clustering method: Ward. Correlation coefficient: Pearson. Distance measures: Euclidean). The scale was defined by the greatest species correlation range (*D. virilis*). Optimal leaf ordering (Bar-Joseph *et al.* 2001) was used to reorder dendrogram leaves to improve visual clarity without adjusting clustering results. Sample correlation was performed using the intensity information at the feature level to capture the maximal metabolomic variance resulting from dietary perturbation and to avoid bias from database dependencies.

Conserved metabolic response to DR.

When the metabolome is considered as a whole, the response to diet varied between species (Figure 3.1), whilst on the individual metabolite level, many significant changes in response to DR were identified across species (Figure 3.2). Crucially we can ask whether the same metabolites changed across species and whether this was more frequent than expected by chance. Overall there was a tendency for shared metabolic responses across species (Table 3.1). However, this was only detectable for annotated metabolites, but the number of annotated metabolites found to be significantly different between diets was relatively low. Across all three species (central overlap, Figure 3.2), there was a trend of a higher frequency with 2 shared metabolites ($p < 0.1$). Similarly, in dyads between species, a significantly higher proportion of shared metabolites responded to diet relative to chance (Table 3.1).

Table 3.1 | Hypergeometric test to model the association between species.

P = probability of over-representation (enrichment). Enrichment between species was varied. However, there are more shared annotated metabolites than expected in many dietary overlaps tested (significance at $P < 0.1$). Although all comparisons were not significant, there is a trend towards a conserved metabolic response to DR between the three *Drosophilidae* species investigated.

	Feature level (P)	**Annotated level** (P)
D. ananassae ∩ *D. biarmipes* ∩ *D. virilis*	0.3495	0.0993
D. ananassae ∩ *D. biarmipes*	0.0694	0.0238
D. biarmipes ∩ *D. virilis*	0.0680	0.0059
D. virilis ∩ *D. ananassae*	0.1232	0.0737

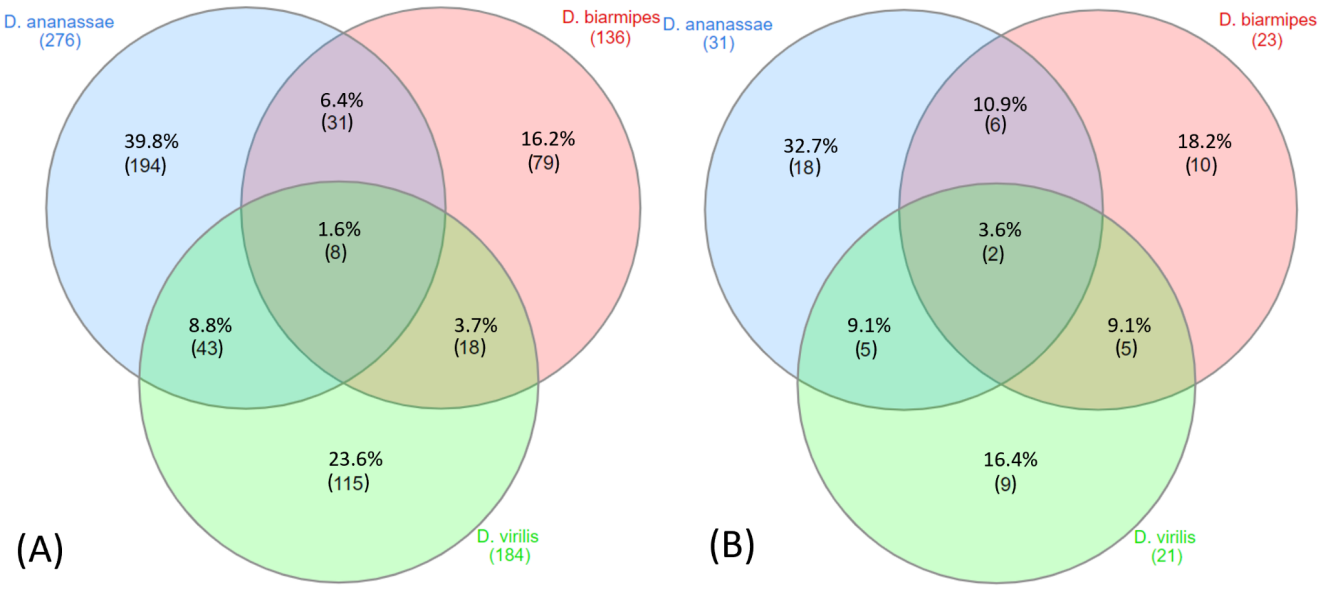

Figure 3.2 | Interspecies metabolic overlap in response to DR.
Venn diagrams represent the number of significantly changing metabolites between high- and low-protein diet conditions. (**A**) Represents the metabolome at the feature level. (**B**) represents the metabolome at the annotated metabolite level. Each Venn diagram is reported in a percentage to aid comparison, and counts are listed in brackets. Number of features: (**A**) n = 1,017. Number of annotated metabolites: (**B**) n = 165.

To further understand the mechanistic response to DR across species, we examined the annotated metabolites that changed in response to DR in detail (Table 3.2). A total of 100 features (Figure 3.2A overlaps, 20.5% of feature total) or 18 annotated metabolites (Figure 3.2B overlaps, 32.7% of annotation total) were identified across all dietary overlaps between species, six of which display directional concordance. To better understand the possible physiology underlying DR across species, each of these six metabolites was researched in the literature and are put into context in the discussion.

Table 3.2 | Interspecies metabolic overlap in response to DR.
DR significant metabolites shared across species. Metabolites are included if it is found to be significant in at least one other species. Metabolite intensities that are not in bold are provided for comparison only and are not found to be significant in the respective species. Metabolites with directional concordance (between significant overlaps) are denoted by underlined intensity values. The high protein (8%) diet is used as the baseline for each dietary comparison. Positive fold change values represent a higher metabolite intensity in the high protein condition, and negative fold change values represent a higher metabolite intensity at DR. **(An)** = *D. ananassae*, **(Bi)** = *D. biarmipes*, **(Vi)** = *D. virilis*.

Pathway(s)	**Compound(s)**	**KEGG ID(s)**	**(An)** $\log_2$**(FC)**	**(Bi)** $\log_2$**(FC)**	**(Vi)** $\log_2$**(FC)**
Tryptophan, Amino sugar and nucleotide sugar metabolism	N-Acetyl-D-Glucosamine 6-Phosphate, N-Acetyl-D-mannosamine 6-phosphate, N-Acetyl-glucosamine 1-phosphate, Cinnavalininate	C00357, C04257, C04501, C05640	**-0.2560**	**-0.1869**	**0.2181**
Drug metabolism - cytochrome P450	2-Hydroxynevirapine	C16608	**-0.6561**	**0.4260**	**0.6373**

Pathway(s)	Compound(s)	KEGG ID(s)	(An) log_2(FC)	(Bi) log_2(FC)	(Vi) log_2(FC)
Purine metabolism	AICAR	C04677	**-0.5345**	**-0.3940**	0.1367
Arginine and proline metabolism, Lysine biosynthesis and degradation	4-Acetamidobutanoic acid, Allysine	C02946, C04076	**0.2794**	**-0.1933**	0.1945
One carbon pool by folate, Glycine, serine and threonine metabolism	(6R)-5,10-Methylene- THF	C00143	**-0.6447**	**-0.0543**	0.2235
Drug metabolism - cytochrome P450	Morphine-6-glucuronide, Morphine-3-glucuronide	C16578, C16643	**-0.3355**	**0.4253**	0.3135
Drug metabolism - cytochrome P450	Codeine-6-glucuronide	C16577	**-0.4563**	**0.3923**	0.5455
Retinol metabolism	11-cis-Retinyl palmitate	C03455	**-0.2118**	**-0.1095**	-0.0703
Fatty acid elongation and biosynthesis	Long-chain acyl-CoA	C02843	0.1916	**-0.3904**	**0.3572**
Propanoate and beta-Alanine metabolism	Acrylyl-CoA	C00894	0.1553	**0.4414**	**0.4568**
Fatty acid, Ketone body, Valine, Lysine, leucine and isoleucine degradation. Terpenoid backbone and Ketone body biosynthesis.	Propionyl-CoA, Acetoacetyl-CoA	C00100, C00332	0.0496	**-0.2753**	**0.2644**
Butanoate, Propanoate, Pyruvate, Tryptophan, Glyoxylate and dicarboxylate metabolism. Fatty acid, Ketone body, Lysine, Valine, leucine and isoleucine degradation. Terpenoid backbone and ketone body biosynthesis.	Acetoacetyl-CoA	C00332	0.1815	**-0.3844**	**0.2644**
Butanoate, Propanoate, Pyruvate, Tryptophan, Glyoxylate and dicarboxylate metabolism. Ketone body, Lysine, Valine, leucine and isoleucine degradation. Terpenoid backbone and ketone body biosynthesis. Fatty acid elongation, degradation and biosynthesis.	Acetoacetyl-CoA, Long-chain acyl-CoA	C00332, C02843	0.2740	**-0.2531**	**0.3555**
Glutathione, Arginine and proline metabolism	Spermidine	C00315	**-0.9440**	-0.1000	**0.4289**
Insect hormone biosynthesis	2,22-Dideoxy-3-dehydroecdysone	C16498	**0.1971**	0.0836	**0.3116**
Glycosaminoglycan degradation	(GlcA)1 (GlcNAc)2	G00711	**-0.6031**	-0.1572	**0.5366**
Fructose, Mannose, Amino sugar and nucleotide sugar metabolism	GDP-4-Dehydro-6-deoxy-D-mannose	C01222	**0.4929**	-0.0601	**0.6326**
Glycosaminoglycan degradation	(GlcA)2 (GlcN)1 (GlcNAc)1 (S)3	G13036	**0.4932**	-0.0827	**-0.3953**

Discussion

Ecological differences in diet use correspond to global metabolome responsiveness across species.

DR results in a varied metabolomic and transcriptomic response between species, which may signify species-level ecological adaptation (Nazario-Yepiz *et al.* 2017; Watanabe *et al.* 2019). *D. ananassae* is adaptable in response to dietary change, regulating its stress response and fecundity similarly to *D. melanogaster* (Sisodia and Singh 2012). Although *D. virilis* is considered a dietary specialist as it feeds primarily on tree sap in the wild (Shultzaberger *et al.* 2019), it can adapt to other dietary conditions similar to *D. melanogaster* (Rodrigues 2014). However, *D. biarmipes* is considered a true dietary specialist, with a small dietary niche consisting of soft foods with high protein concentrations (Silva-Soares *et al.* 2017). Adaptation to dietary changes is likely to require widespread metabolic remodelling, for example using different nutrients to produce cellular energy or in different proportions (Watanabe *et al.* 2019). We observed variation between the three species of Drosophila investigated (Figure 3.1) in the systemic metabolic regulation in response to DR, and that the relative metabolic responsiveness of a species corresponds to the level of nutritional flexibility expected based on a species' nutritional ecology. There is a significant metabolic separation of flies by diet across the whole metabolome in both adaptable and semi-adaptable species (*D. ananassae* & *D. virilis*). Yet, in contrast, within the true dietary specialist *D. biarmipes* no such effect was observed. It remains to be investigated whether the degree of metabolic flexibility in response to DR or nutritional ecology in general is also related to the degree of lifespan extension achieved by DR. Metabolic flexibility could allow a larger protection of
maximal reproductive performance without direct costs on optimal longevity (McCracken, Buckle, *et al.* 2020).

Conserved interspecies metabolomic response to DR.

Although we found that the level of systemic regulation in response to diet is varied significantly between species (Figure 3.1), the longevity benefit resulting from DR is found across many species (Piper *et al.* 2011), including the *Drosophilidae* presented here (Gautrey *et al.* 2022). Any conserved mechanisms of DR could therefore help understand how DR slows ageing (Figure 3.2). We found a weak, but statistically significant, enrichment of annotated DR-responsive metabolites that were shared between species (Table 3.1). Candidate mechanisms related to these shared metabolites are thus predicted to underlie conserved mechanisms of DR with possible implications for how we could extend our own longevity. Metabolites with a unique annotation match and that display directional concordance in response to DR (between significant comparisons) are of particular interest and we discuss these below.

Palmitate is significantly elevated under DR and is conserved across species.

The concentration of 11-cis-Retinyl palmitate increased across all three species under DR (Table 3.2). 11-cis-Retinyl palmitate is a retinyl ester composed of retinol (vitamin A) and palmitic acid (PA) and is the most abundant form of vitamin A storage in animals (O'Byrne and Blaner 2013). Insufficient vitamin A abundance is the cause of a variety of disease states in mammals. However, in *Drosophila,* a significant reduction in dietary vitamin A is not lethal and does not cause developmental defects (Dewett *et al.* 2021). Although perhaps counter-intuitive supplemental vitamin A does show a small longevity benefit in flies with a diminished antioxidant capability (Bahadorani *et al.* 2008). The relative increase in vitamin A storage might therefore not be as important physiologically in terms of vitamin A storage but rather be a reflection of the increased PA content under DR. This is further supported by our previous observation of decreased bioactive vitamin A (retinal) and increased PA content under DR in *D. melanogaster* (Chapter 2, Table 2.2). As this and our previous study both used non-targeted

metabolomics, it is not possible to confirm that the exact same metabolites are identified across studies. However, the shared pathway and directional concordance of these metabolites implicates PA stores in the DR response.

PA is the most abundant and widespread natural saturated acid in many species (Gunstone et al. 2007; Carta et al. 2017) and can be obtained from the diet or synthesised *de novo* via lipogenesis from carbohydrates. The advantages of storing lipids over carbohydrates as metabolic fuel include a higher caloric content per unit weight of substrate, and they may also be used as a storage medium for fat-soluble vitamins such as retinol (Albahrani and Greaves 2016). Thus, the use of lipids as a primary metabolic substrate permits the accumulation of a large reservoir of energy and essential vitamins, which may be used not only during periods of prolonged energy demand but also during periods of nutritional scarcity (Biolchini et al. 2017). Elevated lipid abundance, such as PA, has been previously observed in studies of DR in the fly (Chapter 2; Piper *et al.* 2005; Laye *et al.* 2015; Plank *et al.* 2012). This suggests that the accumulation of PA (serving as an energy store) is preferred under DR. However, we previously found PA rapidly depletes following a period of refeeding after DR (Chapter 2) which may indicate that it is being used as a short-term energy store to bolster anabolic activity and we now identify this effect across four species of *Drosophila* (*D. melanogaster, D. ananassae, D. biarmipes and D. virilis*).

Repair and regeneration is upregulated under DR.

The concentration of (6R)-5,10-Methylene-THF (MTHF) was found to be significantly increased and directionally concordant in two of the three species investigated (*D. ananassae* and *D. biarmipes*) in response to DR (Table 3.2). MTHF is derived from the essential micronutrient folate, which is involved in several biochemical reactions and deficiency is linked to cardiovascular disease and cancer (Scaglione and Panzavolta 2014; Kubo *et al.* 2020). MTHF serves as a one-carbon donor and key co-substrate in the methylation of 2′-deoxy-uridine-5′-monophosphate (dUMP) to 2′-deoxy-thymidine-5′-monophosphate (dTMP) (Danenberg *et al.* 2016). Uridine has recently been discovered as a potent limb-regeneration factor conserved across multiple species (Chapter 2; Liu *et al.* 2022), and dTMP is one of the necessary nucleotide substrates for DNA synthesis and repair (Danenberg *et al.* 2016). An elevated rate of DNA repair under DR has been observed previously (Haley-Zitlin and Richardson 1993).

In the most commonly supported current evolutionary theory, the upregulation of somatic maintenance processes (such as DNA repair) during periods of food scarcity may form part of an adaptive strategy to maximise fitness upon a return to favourable conditions (Harrison and Archer 1989; Holliday 1989). However, we recently argued against this as flies face increased mortality upon returning to a protein-rich diet after a period of DR, at also to the detriment of overall fecundity and thus reducing overall predicted fitness returns (McCracken, Adams, *et al.* 2020). Whether the possible suggestion of MTHF's association is evidence for the hypothesis that there is indeed more investment into somatic maintenance at DR remains to be determined. In addition, the direction of these effects is difficult to determine. Increased somatic damage can lead to more DNA repair, and upregulated DNA repair in itself is therefore not necessarily a reflection of more investment in the soma.

Purine production is elevated in response to DR.

The concentration of 5-Aminoimidazole-4-carboxamide ribotide (AICAR) was found to be significantly increased and directionally concordant in two of the three species investigated (*D. ananassae* and *D. biarmipes*) in response to DR (Table 3.2). AICAR is an intermediate in the generation of inosine monophosphate (IMP) (Greenberg 1956) and forms part of the extensive purine metabolism pathway (Pedley and Benkovic 2017). However, as AICAR is distally removed from the possible metabolic termini (adenosine, hypoxanthine and guanosine), it is difficult to infer the effect of an increased

AICAR abundance resulting from DR. However, as all potential compounds result in a purine-compound, DNA synthesis and repair is likely elevated under DR as possibly indicated by the MTHF results.

Increased amino acid catabolism is detrimental to longevity.

The concentration of acrylyl-CoA was found to be increased across all three species in a protein-rich environment (Table 3.2). Acrylyl-CoA (also known as propenoyl-CoA) is a highly reactive metabolite (Peters *et al.* 2014) and is generated from the catabolism of certain amino acids (L-valine, ornithine, methionine, isoleucine and threonine) (Kuwajima *et al.* 2021) or odd-chain fatty acids (Wongkittichote *et al.* 2017). It is highly toxic if its immediate conversion to succinyl-CoA is impeded (Peters *et al.* 2014; Wongkittichote *et al.* 2017; Kuwajima *et al.* 2021). Succinyl-CoA is then fed into the TCA cycle for energy. Therefore, the increase in acrylyl-CoA abundance in a protein-rich environment may simply reflect energy demand, maximising all potential energy sources, should the amino or fatty acids (from which it is derived) not be needed elsewhere. However, it is also thought that branched-chain amino acid catabolism is a conserved regulator of physiological ageing (Mansfeld *et al.* 2015; Canfield and Bradshaw 2019; Green and Lamming 2019; Richardson *et al.* 2021). Therefore, the elevated abundance of acrylyl-CoA may signify a comparatively increased rate of ageing caused by increased protein turnover (Basisty *et al.* 2018). Therefore, reducing the metabolism of amino acids under DR may be inherently geroprotective. Acrylyl-CoA could directly contribute to the negative consequences of such metabolism or represent a biomarker of more amino-acid weighted metabolism. A specific role for acryl-coA or its associated metabolism is supported by the effects on longevity when specific amino acids are manipulated, such as methionine (Pamplona and Barja 2006; Mirzaei *et al.* 2014; Canfield and Bradshaw 2019; Green *et al.* 2022).

The role of ecdysone metabolism in DR.

The concentration of 2,22-Dideoxy-3-dehydroecdysone was found to be increased across all three species in a protein-rich environment (Table 3.2). Interestingly, this was also true in our previous investigation using *D. melanogaster* (Chapter 2, Table 3.2). Ecdysone signalling is key during metamorphosis (Baehrecke 1996) and has been found to affect post-developmental processes such as stress resistance, behaviour, reproduction and lifespan (Schwedes and Carney 2012). Environmental stimuli, including starvation, are also shown to increase ecdysone abundance (Terashima *et al.* 2005). A low concentration of ecdysteroid is required for normal oogenesis. However, nutritional shortage causes the ovaries to retain produced ecdysteroids resulting in apoptosis if they exceed a tolerated threshold, consequently limiting oviposition (Terashima and Bownes 2004; Terashima *et al.* 2005). However, we suggest that the elevated level of 2,22-Dideoxy-3-dehydroecdysone observed did not exceed the tolerated threshold and is respondent to the increased demand for yolk-protein synthesis (Terashima *et al.* 2005), as the rate of oviposition is greatest following a protein-rich diet (McCracken, Adams, *et al.* 2020).

The level of protein mannosylation decreases in response to DR.

The concentration of GDP-4-Dehydro-6-deoxy-D-mannose was significantly decreased and directionally concordant in two of the three species investigated (*D. ananassae* and *D. virilis*) in response to DR (Table 3.2). GDP-4-Dehydro-6-deoxy-D-mannose is formed from the dehydration of GDP-D-Mannose, a nucleotide sugar that is the donor of activated mannose for all glycosylation reactions and is essential for all eukaryotes (Stewart *et al.* 2005). C-mannosylated peptides have important roles in modulating the functions of acceptor proteins in the cell (Ihara *et al.* 2021). Insufficient O-mannosylation results in muscle defects, degeneration, and neurological phenotypes (Nakamura *et al.* 2010). A reduction in GDP-4-Dehydro-6-deoxy-D-mannose could indicate an overall

reduction in mannosylation. Whether a reduction (but not elimination) in mannosylation under DR conveys an inherent geroprotective effect remains unclear.

Conclusion

Here we used conservation of metabolic responses across multiple species subjected to continuous DR to identify candidate mechanisms of DR's longevity benefits. We identified a small set of metabolites that show similar responses across the three species studied. We used the application of short-term dietary manipulations together with continuous DR in *D. melanogaster* previously (Chapter 2). Further support for the conservation of DR on the metabolite level is found by comparisons with this previous work. Although the overall annotation overlap between all four species was low (Figure S3.3), conclusions may be drawn from the shared pathways in which annotated metabolites are involved and possibly the directional concordances between species. Though some metabolites (such as acrylyl-CoA and possibly PA) appear to be an arguably passive metabolic consequence of the reduction of protein content in the diet, some candidate mechanisms identified (such as the folate, purine and possibly mannosylation pathways) we suggest are more probable to be causal to the longevity benefits observed under DR. PA is again shown to accumulate across a further three species of *Drosophila* under DR conditions. Excess mortality observed during refeeding following a period of DR could be considered a short-term effect due to excessive energy expenditure. Once supplemental energy reservoirs become depleted (which PA oxidation provides), then the mortality should become approximate to those of continually rich-fed flies, which they do (Chapter 1; McCracken, Adams, *et al.* 2020). Both AICAR and MTHF indicate a possible elevated role of regeneration and DNA repair under DR. These results partially confirm our previous findings (Chapter 2: PA, uridine) and have identified a possibly novel mechanism of DR physiology (mannosylation) that is conserved across multiple species. Across species comparisons of the DR response hold promise to further unpick the DR response and targeted metabolomics especially, allowing qualitative as well as quantitative comparisons across a wider range of Drosophila species could prove especially useful.

Supplementary

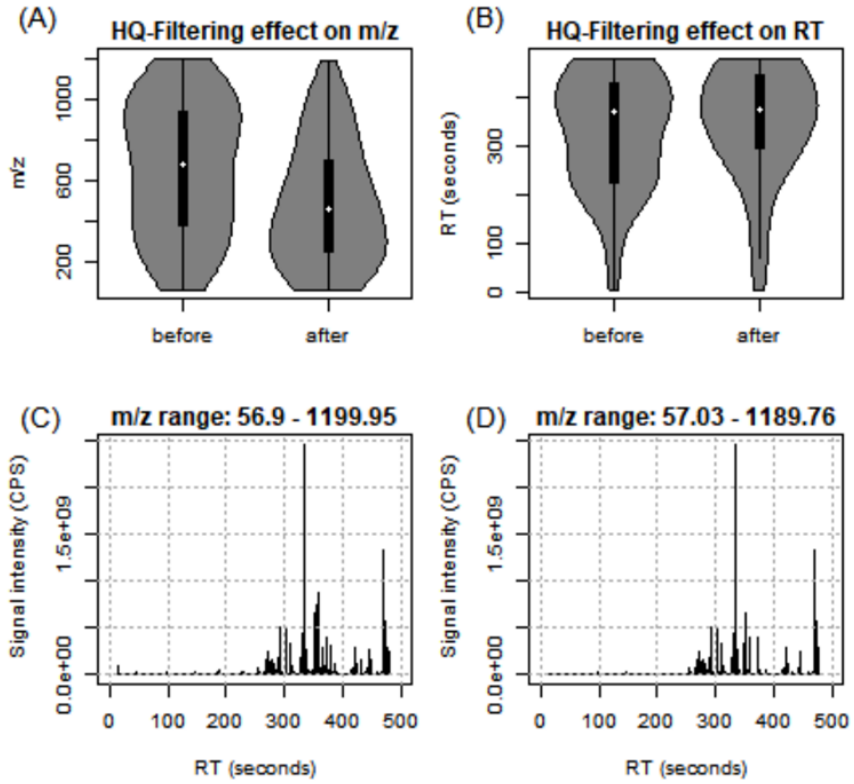

Figure S3.1 | The effect of high-quality filtering.

Violin plots represent the changes to both m/z (**A**) and RT (**B**) as high-quality filtering is applied. The median m/z value was significantly reduced (678.8098 to 465.6570) following high-quality filtering (Two-sample Mann-Whitney test; V = 86,038, P < 0.0001, 95% confidence intervals: 442.4755-487.0319). The median RT value was also significantly reduced (368.6736 seconds to 361.3697 seconds) following high-quality filtering (Two-sample Mann-Whitney test; V = 239,742, P = 0.0417, 95% confidence intervals: 355.4074-368.4199 seconds). Base peak chromatogram (BPC) plots represent the summed intensity of the most intense peak at every point in the analysis. BPCs before (**C**) and after (**D**) the application of high-quality filtering visualise the effect on overall intensity.

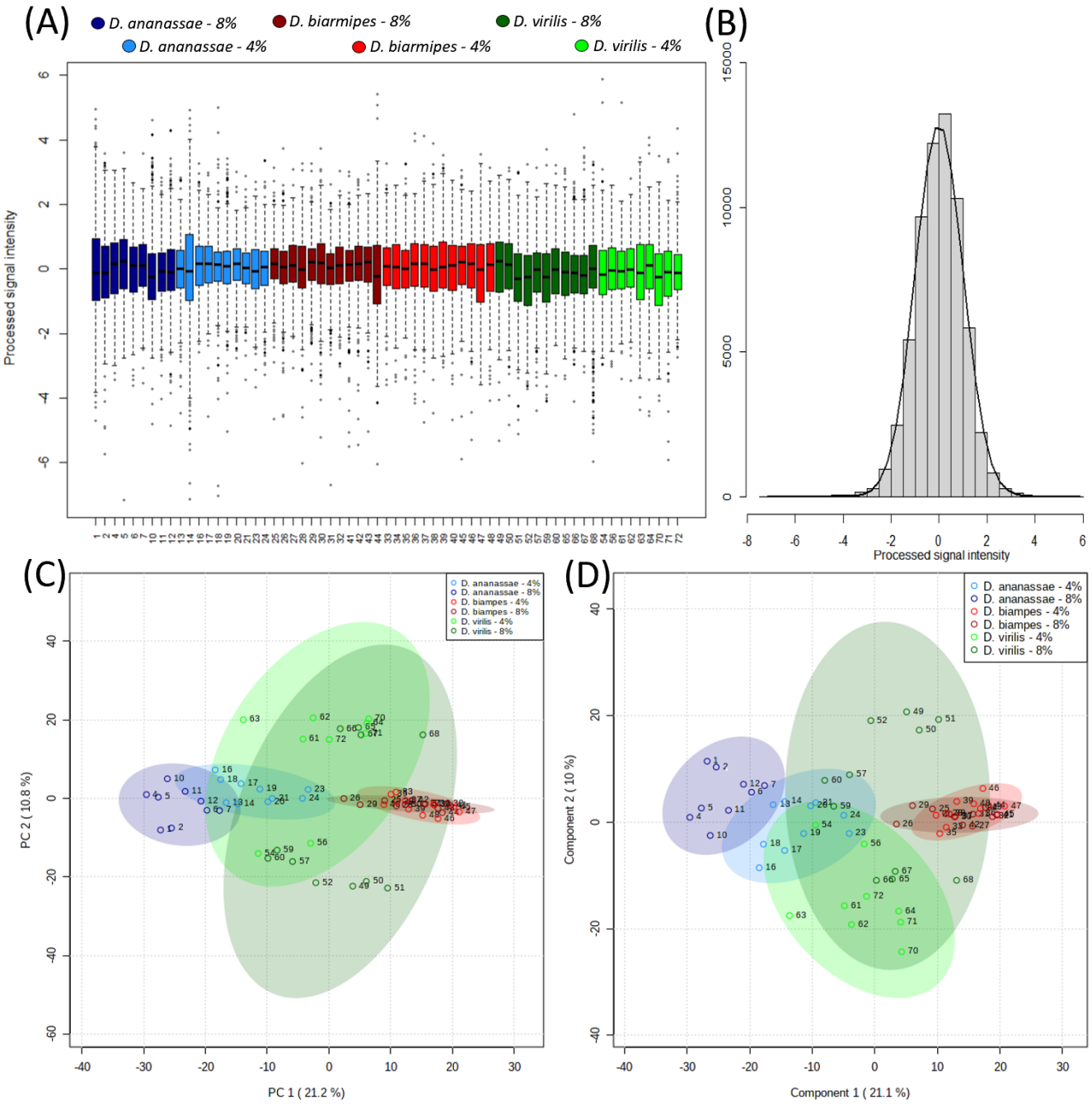

Figure S3.2 | Post-processing characterisation of the interspecies metabolome.

Total sample ion intensities (**A**) were plotted to visualise possible differences between both samples and groups following the data processing. Histogram of processed feature intensities (**B**) approximate a normal distribution (curved line). Initial principal component analysis (**C**) separated samples according to class, so partial least squares-discriminant analysis (**D**) was used to maximise this separation and identify differences in metabolites (chemical shifts) that are the basis of this class separation. Characterisation was performed at the feature level (non-annotated data) to capture the maximal metabolomic variance resulting from dietary perturbation (n = 1,017).

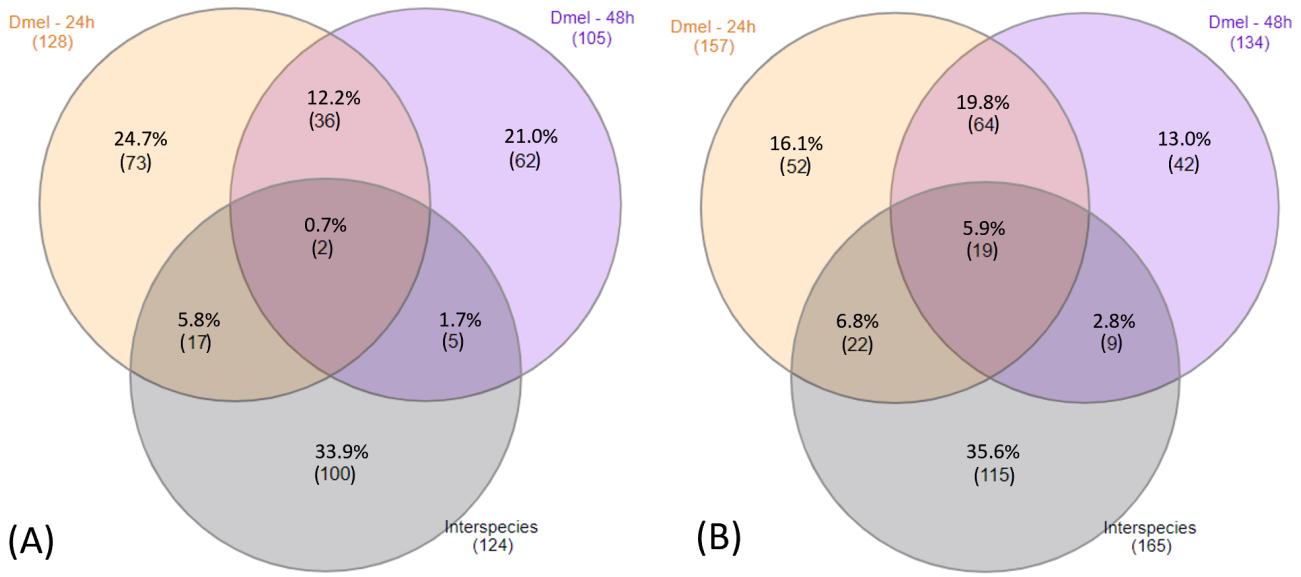

Figure S3.3 | Annotation overlap between interspecies and time-series datasets.
Venn diagrams representing the total metabolic overlap between both time point datasets (used in Chapter 2) and the interspecies dataset used here. (**A**) Comparison between unique KEGG terms (as used in Figure 3.2). (**B**) Comparison between the same terms, however, annotations containing multiple KEGG entries have been pooled and are treated individually. A single unique KEGG annotation (not multiple match) is shared between all three datasets (C05823). This increased to 19 possible KEGG entries when individual queries that match multiple entries were pooled together.

Chapter 4: Proteomics after diet switches in *Drosophila melanogaster* identifies candidate mechanisms of dietary restriction

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Abstract
Dietary restriction (DR) results in a significant reduction in age-specific mortality across a wide variety of species, yet mechanistically, this remains poorly understood. Here we investigated the relationship between the effects of DR and the proteome of *Drosophila melanogaster* to identify candidate mechanisms of DR's longevity benefits. We find that long-term dietary restriction results in a significant adjustment of proteostasis, with over half of all detected proteins showing a significant adjustment to long-term DR application. Refeeding after a period of DR, resulted in a dramatic shift in proteomic activity, with indications that this is fueled by the mobilisation of lipid reserves attained under DR, possibly to the detriment of health. Most surprisingly, there was no detected proteomic response to the onset of DR. Current hypotheses of the mechanisms of DR are rooted in nutrient signalling networks and changes in overall cellular biology, which are unlikely to be responsible for the lack of any change in the proteome. We suggest that the immediate reduction of mortality by DR in the fly is unlikely caused by adjustments of the proteomic cellular machinery but is perhaps a result of a metabolic shift towards a more overall 'healthy state'. Steps to characterise this molecular phenotype may be decisive in attaining the longevity benefit provided by DR.

Contributions:
AJC, TIG, and MJPS conceived and designed the experiment. AJC, MJPS, and LH conducted the experiment. AJC analysed the data and wrote the manuscript with input from all authors.

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Introduction

Dietary restriction (DR) is the limitation of a specific macronutrient, often protein content, without malnutrition and generates an inherent longevity benefit as demonstrated in a wide variety of species, including monkeys (Kemnitz 2011), rodents (Simons *et al.* 2013), nematodes (Pontoizeau *et al.* 2014), flies (McCracken, Adams, *et al.* 2020) and most probably also including humans (L. Fontana *et al.* 2010; Trepanowski *et al.* 2011). DR is shown to acutely counteract the causes of ageing within the fly (Good and Tatar 2001; Mair *et al.* 2003; Kenyon *et al.* 2010; Santos *et al.* 2016). Yet the exact mechanism(s) responsible for DR's health benefits are still largely unknown. No single DR mimetic or mimetic regime currently captures the longevity benefit provided by DR in its entirety (Roth and Ingram 2016; Liang *et al.* 2018).

We used metabolomics previously to study the molecular response to DR (Chapters 2 & 3). The benefit of untargeted metabolomics is that it is unbiased and more closely related to actual functioning when compared to genomic or transcriptomic investigations. Many mutations in the genome have no phenotype (Akashi and Schaeffer 1997), and many transcripts will be preferentially degraded (Towler

and Newbury 2018), thus possibly not resulting in functional change. Here we use another mass spectrometry-based technique, proteomics, to investigate the relationship between the effects of DR and the full proteome of *Drosophila melanogaster*. The proteome represents the complete set of proteins expressed by an organism and depicts the functional outcome of translation (Wang *et al.* 2006). As such, functional approaches which use proteomics have one major benefit over metabolomics; it can benefit from the vast repertoire of sequence-based resources (Adams *et al.* 2000; Stark *et al.* 2007). Modern proteomics allows 'sequencing' of small digested peptides from the total proteome that allows confident (although to a lesser degree than RNAseq) identification of the protein expressed and its abundance. Compared to transcriptomics (Whitaker *et al.* 2014), proteomics has the benefit that it is most closely related to function (Chapter 6), as not all transcripts turn into protein and levels of protein are not solely regulated by transcriptional regulation.

Existing proteomic investigations into the molecular response to DR in the fly reveal hundreds, if not thousands, of differentially expressed proteins in response to long-term DR (Gao *et al.* 2020; Campion *et al.* 2021). However, long-term physiological adjustment to an unvaried dietary composition is unlikely to explain the rapid and strong reduction of age-specific mortality observed in demographic studies of DR (Chapter 1; Good and Tatar 2001; Mair *et al.* 2003; McCracken, Adams, *et al.* 2020). Therefore, investigation of the short-term adjustments to DR may prove key in identifying mechanisms with a possible longevity benefit (switch to DR) and those which may explain the excessive mortality costs when returning from DR (refeeding). This switch-based paradigm (as outlined in Chapter 2, Figure 2.1) has proven effective in disentangling the phenotypic response to DR (Chapter 1; Chapter 2; Whitaker *et al.* 2014; McCracken, Adams, *et al.* 2020). Here we measure the proteomic and phosphoproteomic response in this dietary paradigm for the first time.

Methods

Fly stocks, experimental design and proteome extraction.

The flies used in these analyses were sampled from the dietary switching experiment, of which the demography is presented in Chapter 1. Details of the animal husbandry procedures and the experimental switching design can be found in Chapter 1. Flies were snap-frozen in liquid nitrogen 48 hours after the diet switches across all four groups. Five samples were taken from each group containing ten flies per eppendorf (200 total). These samples were bead milled and processed through RNAeasy Mini kits (Qiagen) to obtain RNA. The column's flow-through was used to obtain the protein fraction from these samples. This workflow was preferred as we aimed to directly relate the transcriptome to the proteome for future work, and this way, both were obtained from the same biological sample. We tested this protocol by comparing two samples for which we followed a standard protein extraction protocol with two samples obtained from the RNAeasy column flowthrough and found similar coverage of the full proteome (slightly better coverage of the proteome for the RNAeasy method) and no interference with the downstream proteomics laboratory protocol (unpublished). Samples were sent on dry ice to the proteomics facility at the University of Bristol for analysis.

High throughput shotgun proteomics.

Samples were resuspended at the University of Bristol via acetone precipitation. Concentrations were equalised using Bradford assays. Then, samples were digested with trypsin and split into two groups (Set 1: samples 1-10. Set 2: samples 11-20). A pooled sample containing an approximate equal amount of protein from all 20 samples was included as the 11th sample in each set. Each set was labelled with tandem mass tag (TMT) reagents in an 11-plex configuration according to the manufacturer's protocol (Thermo Fisher Scientific). Samples were then fractionated by strong cation

exchange using an Ettan LC system (GE Healthcare) before multiplex analysis (11 samples, 15 fractions per set) on an Orbitrap Fusion LCMS machine (Thermo Fisher Scientific). Two additional fractions were phosphopeptide-enriched (TiO_2 and FeNTA) to facilitate phospho-regulated proteomic 'phosphoproteome' study. The samples were demultiplexed *in silico* using the TMT tags.

Data processing and analysis.

R (R Core Team 2021) version 4.1.0 was used to conduct all analyses unless otherwise stated. Proteome Discoverer 2.1 (Thermo Fisher Scientific, University of Bristol) was used to process and normalise the initial raw data using the included total pool samples (Figure S4.1A), resulting in 5,567 unique protein identifications using the UniProt *Drosophila melanogaster* reference protein database. A total of 32 protein contaminants were identified and were removed *in silico* prior to subsequent processing steps. A total of 6.5% of the data contained missing values. Proteins with ≥ 50% missing values (1,066 proteins) were removed. The remaining missing values were imputed using the k-nearest neighbours algorithm (Batista and Monard 2003). Samples 5, 16 and 18 were identified as outliers and removed from the analysis (Figure S4.1B). This process was repeated for the phosphopeptide-enriched dataset (phosphoproteome), identifying 2,328 proteins with specific phosphopeptide modifications (Figure S4.2A-B). Initial proteomic characterisation revealed significant batch effects (Figure S4.1C-D & S4.2C-D) which were corrected using the ComBat algorithm (Johnson *et al.* 2007). Samples then underwent generalised logarithm transformation (Durbin *et al.* 2002) and autoscaling (van den Berg *et al.* 2006) following the MetaboAnalyst workflow (Pang *et al.* 2021) as done for the metabolomics chapters in this thesis. The intensity and distribution of proteomic features were characterised using multivariate analysis (Figure S4.3 & S4.4), showing complete separation of continuous dietary groups. Significance testing of features between dietary groups was conducted using unpaired unequal variance t-tests using Welch approximation. P values were adjusted for multiple comparisons using the Benjamini & Hochberg (1995) method. Only significant ($\alpha < 0.05$) features following this false discovery rate (FDR) correction were retained. To test for under and over-representation (depletion/enrichment) between Venn diagram overlaps of dietary comparisons (Figure 4.2), hypergeometric testing was conducted using the 'phyper' function from the stats package in R. This test is equivalent to a one-tailed Fisher's exact test. As no significant overlap was detected between the switch to DR and continuous DR comparison, enrichment testing of other overlapping regions (including the centre) was not needed. Significance testing of dietary correlations was conducted using Mann-Whitney tests using the correlation matrix used to generate the clustered heat maps (Figure 4.1), with self-correlated values (equal to 1) excluded. KEGG pathway enrichment was conducted using the 'limma' package (Ritchie *et al.* 2015), which tests for over-representation of KEGG pathways associated with the protein queries on the gene level. No FDR threshold was applied as false-positive rates are unclear for these analyses, and corrections will tend to be too conservative (Ritchie *et al.* 2015). The denominator of genes in a pathway, i.e. the 'universe', was based on the total genes encoding the proteome identified across samples.

Results

The dynamics of systemic proteomic change in response to protein concentration.

Investigating changes in the proteome in response to a switch in the diet allows a general test of how quickly the fly responds on a systemic level, as we did previously for the metabolome (Chapter 2). When looking at the proteome as a whole (Figure 4.1), flies that are switched to DR respond minimally when compared to a strong response when moving from DR to a rich diet. Flies switched to DR fail to separate from the original rich group after 48 hours (Figure 4.1). Refed flies now only separate into a unique dendrogram cluster on the same dietary branch (whereas they transitioned to the rich branch previously). Similar conclusions can be drawn from principal component analysis (Figure S4.3 & S4.4). The correlation of protein abundances across samples between continuous low-

and high protein diets were found to be significantly different (Proteome: W = 240, P < 0.0001. Phosphoproteome: W = 204, P = 0.0011) (Figure 4.1). Thus, there is a significant difference in the proteomic regulation between dietary states on the whole proteome level, with DR appearing to be more highly regulated (measured as inter-correlation) than that of a rich diet (Figure 4.1). We also show that there is not a significant difference in correlation of protein abundances between the continuous rich and switch to DR diets (Proteome: W = 88, P = 0.2195. Phosphoproteome: W = 76, P = 0.0899) as suggested by their lack of separation (Figure 4.1). However, there is a significant difference in the correlation of protein abundances between the continuous DR and switch to rich diet groups (Proteome: W = 136, P = 0.0002. Phosphoproteome: W = 120, P = 0.0060). Together, this indicates that the proteome is highly responsive to refeeding, but not necessarily to the start of DR.

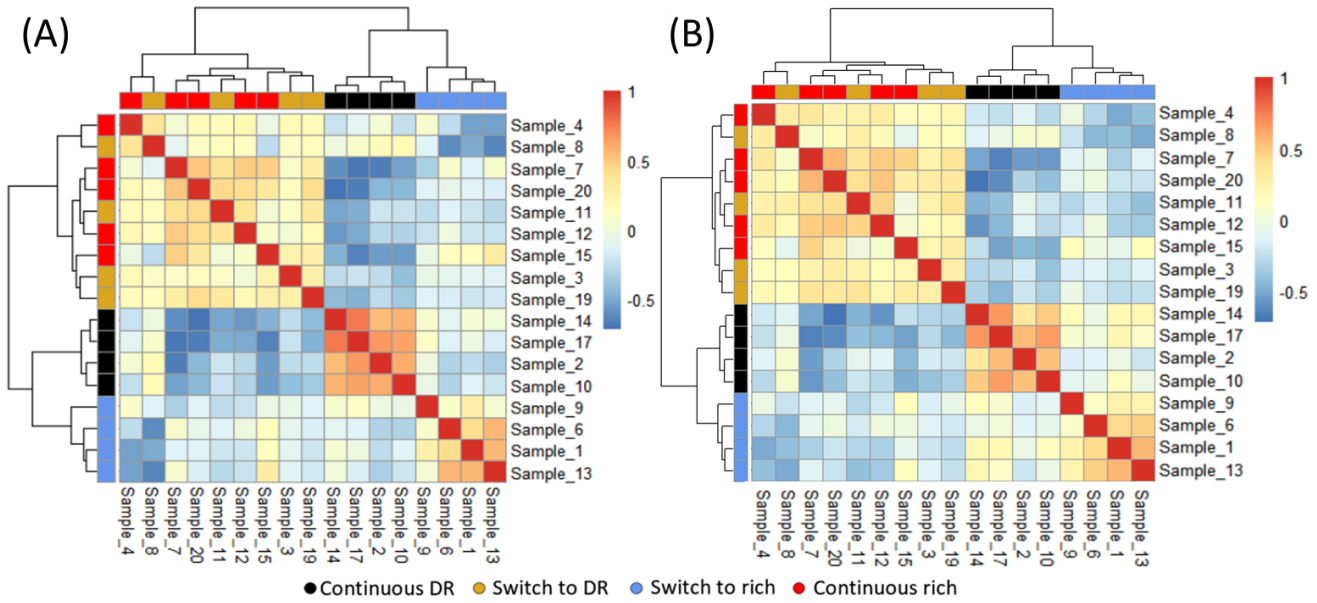

Figure 4.1 | Systemic proteomic change across dietary groups.
Clustered heat maps were generated using the correlation between samples and all detected protein intensities. **A** = Proteome (n = 4.465). **B** = Phosphoproteome (n = 2,328). Samples are reflected along the X and Y axis. Clustering method: Ward. Correlation coefficient: Pearson. Sample correlation was performed using intensity information from all detected proteins.

Dietary switching separates out proteins related to DR.

The application of DR in the long-term resulted in a highly significant adjustment of the proteome (2,241 / 4,465 proteins, FDR α < 0.05) and phosphoproteome (1,141 / 2,328 proteins, FDR α < 0.05). Refeeding also resulted in a strong proteomic adjustment, possibly reflecting the switch to a nutrient-enriched environment (Proteome: 1,691 / 4,465 proteins, FDR α < 0.05. Phosphoproteome: 444 / 2,328 proteins, FDR α < 0.05). However, the application of DR was not found to result in any significant proteomic adjustment after 48h in either proteomic dataset. As a result, enrichment analysis could only be performed on proteins from the refeeding groups which interacted with long-term DR physiology, where we found significant enrichment (Both data sets: P < 0.0001). A large portion of the proteomic refeeding response overlapped with long-term DR (Proteome: 1,324 / 4,465 proteins, Phosphoproteome: 376/2,328 proteins), which suggests that it relates closely to DR-based physiology.

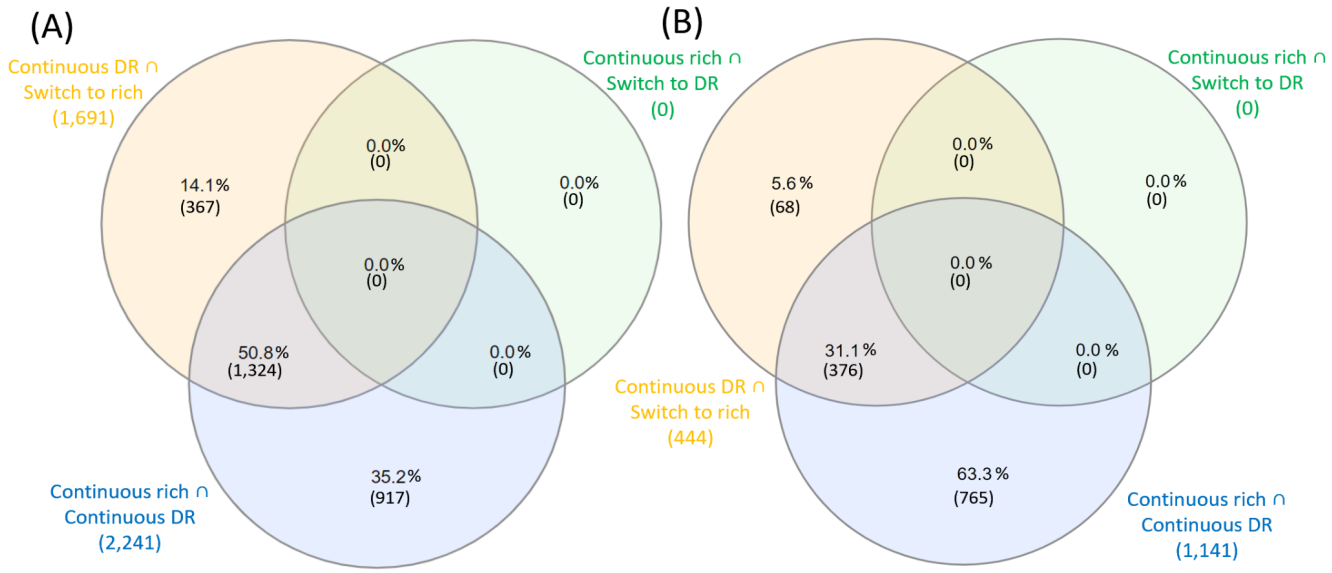

Figure 4.2 | Interaction of significant diet-related proteins.
Venn diagrams of the number of significantly changing proteins between diet conditions. **A** = Proteome (n = 4.465). **B** = Phosphoproteome (n = 2,328).The interaction between dietary conditions is denoted by the symbol '∩'. Each section is reported as a percentage to aid comparison between diet conditions. Counts are listed in brackets.

Pathway enrichment analysis of dietary conditions.

Pathway enrichment was conducted for each Venn diagram region (Figure 4.2) to further identify the possible mechanisms involved. Enrichment of proteins that changed in abundance in both refeeding and long-term DR (Figure 4.2, overlapping regions) revealed significant overrepresentation of several pathways, notably those associated with the ribosome, inositol phosphate metabolism and phosphatidylinositol signalling, and ether lipid metabolism (Table 4.1). Long-term DR (Figure 4.2, blue circles) resulted in significant changes in DNA replication and mismatch repair processes and also pathways of protein-based metabolism and energy generation (Table 4.2). Refeeding (Figure 4.2, orange circles) revealed significant modulation of retinol, xenobiotic, fatty and amino acid metabolism (Table 4.3). Interestingly, ribosome biogenesis and amino biosynthesis pathways were also enriched in this group, potentially representing the transition to the fully fed state, enriched for ribosome and proteasome (Table 4.1), probably resulting in higher transcriptional and protein metabolism.

Table 4.1 | Pathway enrichment of proteins with a shared response to refeeding and long-term DR.
All significant proteins in the dietary overlap between long-term DR (Continuous DR ∩ Continuous rich) and refeeding (Continuous DR ∩ Switch to rich) were used to perform enrichment analysis (n = 1,700). All significant terms (P < 0.05) were included below. Pathway ID = KEGG pathway identification number. n = total number of proteins found in this pathway in the proteome in this experiment. DE = number of differentially expressed proteins. P = probability of overrepresentation.

Pathway	Pathway ID	n	DE	P
Ribosome	dme03010	102	79	< 0.0001
Proteasome	dme03050	37	19	0.0041
ECM-receptor interaction	dme04512	6	5	0.0100
Phosphatidylinositol signalling system	dme04070	21	11	0.0229
Nitrogen metabolism	dme00910	9	6	0.0230
Inositol phosphate metabolism	dme00562	19	10	0.0286
Ether lipid metabolism	dme00565	5	4	0.0288

Table 4.2 | Pathway enrichment of proteomic terms unique to long-term DR.

All significant proteins unique to long-term DR (Continuous DR ∩ Continuous rich) were used to perform enrichment analysis (n = 1,682). All significant terms (P < 0.05) were included below. Pathway ID = KEGG pathway identification number. n = total number of proteins found in this pathway in the proteome in this experiment. DE = number of differentially expressed proteins. P = probability of overrepresentation.

Pathway	Pathway ID	n	DE	P
Mismatch repair	dme03430	13	9	0.0002
DNA replication	dme03030	22	12	0.0005
Nucleotide excision repair	dme03420	23	11	0.0033
Other types of O-glycan biosynthesis	dme00514	3	3	0.0089
Amino sugar and nucleotide sugar metabolism	dme00520	23	10	0.0114
2-Oxocarboxylic acid metabolism	dme01210	8	5	0.0122
Base excision repair	dme03410	8	5	0.0122
Spliceosome	dme03040	78	24	0.0230
Biosynthesis of nucleotide sugars	dme01250	19	8	0.0283
Citrate cycle (TCA cycle)	dme00020	23	9	0.0337
Arginine biosynthesis	dme00220	7	4	0.0377

Table 4.3 | Pathway enrichment of proteomic terms unique to refeeding.

All significant proteins unique to long-term DR (Continuous DR ∩ Continuous rich) were used to perform enrichment analysis (n = 435). All significant terms (P < 0.05) were included below. Pathway ID = KEGG pathway identification number. n = total number of proteins found in this pathway in the proteome in this experiment. DE = number of differentially expressed proteins. P = probability of overrepresentation.

Pathway	Pathway ID	n	DE	P
Retinol metabolism	dme00830	5	3	0.0053
Drug metabolism - cytochrome P450	dme00982	22	6	0.0082
Metabolism of xenobiotics by cytochrome P450	dme00980	24	6	0.0129
RNA polymerase	dme03020	13	4	0.0196
Peroxisome	dme04146	34	7	0.0215
Ribosome biogenesis in eukaryotes	dme03008	34	7	0.0215
Metabolic pathways	dme01100	482	53	0.0233
Ascorbate and aldarate metabolism	dme00053	8	3	0.0245
Biosynthesis of amino acids	dme01230	36	7	0.0288
Drug metabolism - other enzymes	dme00983	36	7	0.0288
Fatty acid biosynthesis	dme00061	9	3	0.0345
Butanoate metabolism	dme00650	10	3	0.0463
Tyrosine metabolism	dme00350	10	3	0.0463

Evidence of proteomic toxicity, or maladaptation to rapid dietary protein influx.

If the mortality observed during refeeding is due to toxicity effects, we could expect certain proteins or metabolites (Chapter 2) to be in excess compared to the constantly high fed group. We find a small number of proteins (11) that become significantly more abundant than under long-term rich fed conditions (Table 4.4). These proteins may provide evidence of proteomic maladaptation or toxicity (or compensation thereof) resulting from the sudden influx of dietary protein. Enrichment analysis of these 11 proteins against all detected proteins showed that inositol phosphate metabolism was significantly enriched (1 out of 11 proteins [gene name = Inos] are annotated in this pathway, P = 0.04).

Table 4.4 | Proteins with possible toxic effects under refeeding.

Dietary comparison: **(A)** Continuous DR ∩ Continuous rich, **(B)** Continuous DR ∩ switch to DR. The continuous DR dietary condition was used as the base for each dietary comparison. Positive log_2(FC) values represent a larger metabolite intensity in the continuous DR dietary group, while negative values represent a larger metabolite intensity in the continuous rich or switch to rich dietary group. All proteins included are significantly greater in the switching category (FDR α < 0.05). C% = Total coverage or the reference protein query by all associated peptide sequences reported as an overall percentage. GN = the first gene name of the UniProtKB entry. PE = Protein Existence is the numerical value describing the evidence for the existence of the protein (1 = Experimental evidence at the protein level. 2 = Experimental evidence at the transcript level. 3 = Protein inferred from homology. 4= Protein predicted). SV = Sequence Version is the version number of the sequence.

Accession	C%	Description	Gene Ontology	(A) log_2(FC)	(B) log_2(FC)
A0AP75	43.4	CG12909 protein GN=CG12909 PE=4 SV=1	nucleus; DNA binding; metal ion binding	-0.2393	-0.6297
A1Z8D2	25.5	GH14288p2 GN= CG18003-RC PE=2 SV=1	peroxisome; FMN binding; oxidoreductase activity	-1.5732	-1.8156
C0MLG6	25.3	CG8326-PA GN=CG8326 PE=4 SV=1	NA	-0.2727	-0.5653
O97477	35.4	Inositol-3-phosphate synthase GN=Inos PE=1 SV=1	cytoplasm; inositol-3-phosphate synthase activity; inositol biosynthetic process; phospholipid biosynthetic process	-0.2395	-0.5777
Q058Z5	20.4	IP08630p1 (Fragment) GN=CG32708-RA PE=2 SV=1	nucleolus; RNA binding	-0.2251	-0.5055
Q8MR24	44.9	Ribosome biogenesis regulatory protein (Fragment) GN=CG32409 PE=2 SV=1	nucleolus; preribosome, large subunit precursor; endonucleolytic cleavage in ITS1 to separate SSU-rRNA from 5.8S rRNA and LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA); ribosomal large subunit biogenesis; ribosomal large subunit export from nucleus	-0.2558	-0.4496
Q9V3W1	45.5	Tetratricopeptide repeat protein 2 GN=Tpr2 PE=1 SV=1	cytosol; nucleoplasm; heat shock protein binding; chaperone cofactor-dependent protein refolding	-0.3124	-0.5189
Q9V9Z9	43.3	Probable rRNA-processing protein EBP2 homolog GN=CG1542 PE=2 SV=1	nuclear periphery; nucleolus; preribosome, large subunit precursor; RNA binding; ribosomal large subunit biogenesis; rRNA processing	-0.3168	-0.6617
Q9VBR6	29.4	RE74917p GN=tobi PE=2 SV=1	hydrolase activity, hydrolyzing O-glycosyl compounds; carbohydrate metabolic process	-1.1878	-2.2190
Q9VM11	51.8	HL01515p GN= Dmel\CG5973 PE=2 SV=2	phosphatidylinositol bisphosphate binding	-0.3175	-0.7227
9W306	51.2	GEO08256p1 GN= Dmel\CG9691 PE=1 SV=1	NA	-0.1954	-0.5899

Discussion

Dietary restriction results in a large adjustment of the proteome and phosphoproteome when applied continuously. Approximately half of all detected proteins identified in the proteome and phosphoproteome were significantly different between the two continuous dietary groups (Figure 4.2). Most probably, a considerable amount of this shift is due to long-term physiological adjustments on the cellular, tissue, and perhaps tissue composition level (Price *et al.* 2022). As such, a large proportion of the proteins or genes identified in this manner will not underlie the response of interest: the DR-longevity response. However, by utilising the switching paradigm, it is possible to further pick apart DR's physiology. Enrichment analysis of proteins uniquely responsive to the process of long-term DR (Table 4.2) indicates a significant overrepresentation of somatic maintenance pathways. In our previous metabolomic investigations, we concluded a similar investment into the soma during DR (Chapters 2 & 3). However, DR's effect on mortality and the mortality cost of refeeding is rapid (Good and Tatar 2001; Mair *et al.* 2003). Therefore, comparing the overlapping response of dietary switches against steady-state conditions is key in identifying novel mechanisms core to DR-based physiology. From our analysis, key general mechanisms can even be put in doubt. DR is thought of as a highly regulated response interwoven with nutrient-sensing pathways (Kapahi *et al.* 2004; Ma *et al.* 2018). However, not a single protein was found to be differentially changed when DR was applied after 48 hours in both proteomic data sets investigated. Therefore, changes in the abundance of cellular machinery, or regulation of the phosphoproteome are unlikely responsible for the mortality reduction seen soon after DR is applied (Chapter 1).

Possible toxicity or maladaptation after refeeding.

We recently described a significant excessive overshoot in mortality in response to refeeding after a period of DR (Chapter 1; McCracken, Adams, *et al.* 2020). Though we provided an initial mechanistic insight into this phenomenon via the metabolome (Chapter 2), there was no evidence of metabolic toxicity or maladaptation to nutritional change. At least as defined by a significant difference of metabolite abundance in the switching category compared to the continuous control. As such, we suggested that the excess mortality associated with refeeding is the result of costs associated with the intake or metabolism of dietary components alone, as there was no indication of a regulated response. In the proteomic response to refeeding, we do however detect a small number of proteins that could exert a possible toxic or maladaptive effect (Table 4.4). Why these 11 proteins become significantly over-expressed (compared to the continuous long-term DR control) in response to a sudden influx of dietary protein is unclear. These proteins may be initially over-expressed due to proteomic maladaptation to a change in the dietary environment. However, what is not known is whether this poses an inherent mortality cost, or whether there are possible toxic effects when found in an elevated abundance. One possible mechanism is inositol phosphate metabolism.

Inositol is a naturally occurring sugar alcohol found in various plants and animals (including humans) that can be obtained from the diet or through the endogenous conversion of glucose. It is used as a mediator of several signalling pathways (Mishra and Bhalla 2002) and is associated with an increased growth rate (Lee and Bedford 2016). Inositol synthases hydrolyse phosphatidylinositol bisphosphate to produce inositol compounds (Wiradjaja *et al.* 2001), both of which were significantly overexpressed after refeeding (Table 4.4). Inositol's main function is to serve as a key phospholipid component of the plasma membrane (Lee and Bedford 2016). As such, it is possible that the return to a nutritionally-rich environment results in a short-term increase in cellular membrane activity or proliferation and turnover, driving inositol demand. Though at a significant excess, inositol is unlikely to pose a toxic risk as external supplementation is shown to improve cellular homeostasis in an adverse environment (Krause *et al.* 2007).

The shared response to refeeding and long-term DR results in cellular growth.

Anabolic pathways largely drive the shared refeeding response with DR (Table 4.2). Energy-dense fat reserves are metabolised to facilitate this increased energy demand, providing a short-term energy pool to bolster cellular proliferation. Lipids, including lipid ethers, are shown to become elevated under a high-sugar diet (Tuthill *et al.* 2020), and an elevated lipid content under DR has been previously observed in the fly, possibly owing to its relatively increased caloric component from sugar-based sources (Piper *et al.* 2005; Laye *et al.* 2015). Furthermore, the enrichment of lipid metabolism pathways has been previously implicated in studies of DR (Plank *et al.* 2012). We previously suggested reasoning from the metabolomics results that this rapid energy expenditure of lipid reserves accumulated under DR corresponds to the temporary increase in mortality compared to a continuous rich diet (Chapter 2).

The proteomic analysis appears to also support this hypothesis. Lipid catabolism and nitrogen metabolism pathways are significantly enriched (Table 4.2), with the latter previously shown to indicate a high-fat diet in the fly (Heinrichsen *et al.* 2014). Most specifically perhaps, the mobilisation of ether lipids (Table 4.2) could indicate that anabolic activity is prioritised over health. Ether lipids are lipids which carry an ether-linked fatty alcohol at the *sn*-1 position of the glycerol backbone (Schooneveldt *et al.* 2022). They have an important role in several homeostatic mechanisms, notably that of the membrane fusion process and the reduction of oxidative stress (Dean and Lodhi 2018). The extracellular matrix receptor interaction pathway was also significantly enriched during refeeding (Table 4.2), suggesting an elevated requirement of cellular structural support, nutrient transport or intercellular communication, which may ultimately determine cell fate (Kim *et al.* 2011). Other cellular functions, such as the proteasome (also significantly enriched; Table 4.2) may partly compensate for the catabolism of ether lipids, but possibly not as a whole (Lecker *et al.* 2006; Lefaki *et al.* 2017). However, elevated protein turnover facilitated by proteasome activity may in itself be harmful (Basisty *et al.* 2018).

The metabolism of lipid reserves culminates in a significant increase of available cellular energy that is then directed toward cellular growth, as suggested by the enrichment of the ribosome (Grewal *et al.* 2007). This supports our previous hypothesis that the mortality cost observed during refeeding results from excessive energy expenditure and increased resource investment into anabolism which may be hazardous to health (López-Otín *et al.* 2016). Even though refed flies have poorer reproductive output compared to continuously rich fed flies (McCracken, Adams, *et al.* 2020), energetic requirements and a shift towards more reproductive activities could underlie these effects and possibly the observed mortality costs.

Proteins unique to the refeeding response are diet responsive.

Proteins unique to the refeeding response are unlikely to be causative of the physiology associated with DR. The pathways enriched within this section are likely to simply be responsive to a nutritionally-rich environment. Alternatively, there is a possibility that the proteomic response observed may explain the exacerbated mortality observed during refeeding. Retinol metabolism is the most enriched pathway considered in this category. Plank *et al.* (2012) were the first to associate retinol metabolism with dietary restriction; our previous findings (11-cis-Retinyl palmitate, Chapter 3, Table 3.2) supports this observation and offers a possible explanation of this effect. 11-cis-Retinyl palmitate is a retinyl ester composed of retinol (vitamin A) and palmitic acid (PA) and is the most abundant form of vitamin A storage in animals (O'Byrne and Blaner 2013). If the PA is catabolised for energy (as suggested in Chapters 2 & 3), retinol is liberated, resulting in the observed pathway enrichment (Table 4.3). This is supported by a concordant enrichment of fatty acid biosynthesis, which converts existing fatty acid reserves into palmitic acid, which can then undergo beta-oxidation for energy release (Angelini *et al.* 2019). This is again supported by the significant enrichment of the peroxisome (Table 4.4), which facilitates the oxidation of palmitate (Kondrup and Lazarow 1985) and is found to be significantly overexpressed compared to a continuous rich diet (Table 4.4).

Finally, ribosome biogenesis (and RNA polymerase) was also enriched in this group. The ribosome is an important mediator of DR-physiology (Table 4.2). However, ribosome synthesis is extremely costly and is estimated to account for ~15% of the total protein synthesis budget (Schleif 1967). Therefore, enrichment of its creation represents a significant investment into the gain of translational function provided by additional ribosomal activity under the refeeding paradigm. However, studies have shown that the increased demands of protein synthesis reduce both ribosomal accuracy (Conn and Qian 2013) and efficiency (Borkowski *et al.* 2016). Therefore, the investment into ribosome biosynthesis might alleviate the rapidly increased demand for protein synthesis following a period of DR, not present in a continuously-rich diet (Table 4.4). This issue is likely compounded by the fact that DR leads to a mild but widespread inhibition of transcriptional and translational activity with a concordant depression of protein synthesis (Ding *et al.* 2014), which may result in a significant reduction of associated cellular machinery (such as the ribosome) if applied for an extended period.

Changes to proteomic regulation do not mediate the DR-longevity benefit.

We previously observed that the mortality risk reduction when switching to DR was relatively more rapid than the induction of exacerbated mortality on the switch to a rich diet (McCracken, Adams, *et al.* 2020). However, this was not reflected in adjustments to the metabolome (Chapter 2), corroborating the proteomic results presented here (Figure 4.2). Critically, our results indicate that there is no rapid regulation of gross protein abundance, or change to the phosphoproteome in response to the onset of DR (Figure 4.2). Such a response would be expected if DR is a regulated cellular response in terms of nutrient signalling for example. Moreover, proteins may be catabolised to yield energy. However, they are not a readily available form of energy (Chatterjee and Perrimon 2021) and are thus unlikely to become significantly depleted in response to short-term DR. The immediate benefit to mortality is therefore unlikely obtained through significant adjustments in the abundance of cellular machinery. Such benefits are also unlikely to be obtained via phosphoproteomic adjustments. However, phosphopeptide-enrichment was the only post-translational modification mechanism investigated in this study; it is possible that alternative mechanisms of post-translational modification such as glycosylation, ubiquitination, nitrosylation, methylation, acetylation mediate the DR effect, which are possible to detect using mass-spectrometry techniques with sufficient adjustment to the experimental design (Dunphy *et al.* 2021). Many databases and tools exist for post translational investigations (Ramazi *et al.* 2021). It is possible therefore that other PTM mechanisms may show a response to the onset of DR which was not revealed in this study. However, additional PTM investigation would have complicated the already challenging analysis conducted here; therefore, only the phosphoproteome was considered as it could be investigated under the same experimental design.

Some studies find a rapid transcriptomic response to the onset of DR; Whitaker *et al.* (2014) discover some 144 genes which significantly change in expression upon a switch to DR. However, they found that folate biosynthesis was the only pathway to become upregulated in response to DR (which we also observed in the metabolome; Table S2.4) with the remainder associated with a significant down-regulation of genes involved in carbohydrate and fatty acid metabolism. We do not currently see a reflection of this within the proteome. It is likely that the 48 hour sampling time used here is insufficient to reflect the changes in gene expression and to allow for proteasomic degradation (Chondrogianni *et al.* 2010). Our investigation of the phosphoproteome also failed to suggest any PTM-linked regulation (though other mechanisms may be present). However, if we were to consider the rapid increase of carbohydrate and lipid utilisation in response to refeeding (observed across both metabolomic and proteomic investigations), given time, such decreases in carbohydrate and lipid metabolism pathways should become evident in response to DR, in line with the observations made by Whitaker.

The longevity benefit observed when switched to a DR diet is both significant and rapid (Chapter 1), and though we observe a strong and significant modulation of the proteome in response to refeeding, we do not see any response from the application of DR. It is possible that tissue-specific differences in regulation may mask any globally observed effects (Price *et al.* 2022), and are worthy of further investigation. Perhaps the benefits of DR are simply a result of a metabolic shift towards a more healthy state. Such a state could be reflective of an escape of costs paradigm (McCracken, Adams, *et al.* 2020), where such instant 'unregulated' benefits of DR might be expected. If DR's benefits could be obtained in this manner, it may explain why its key mechanisms remain hard to pin down, as the cellular state and molecular machinery are essentially the same; the animal simply operates in a manner that is more supportive of life (as a result of the absence of additional mortality costs). However, it is also possible that this 'healthy state' is due to a partial reversal of age-related change (Wuttke *et al.* 2012). If this is the case, then the small number of effectors (two as identified in Wuttke *et al.*'s study: Ime1 and Ndt80) may be masked by pathway analysis (used by studies of large datasets as done so here; Tables 4.1 - 4.4) and returning to a normal diet would soon negate any such benefit. Whatever may be the case, we suggest that defining this healthy state and how to induce it pharmacologically (without having to avoid mortality costs) might hold the key to unlocking the health benefits of DR for our own species.

Supplementary

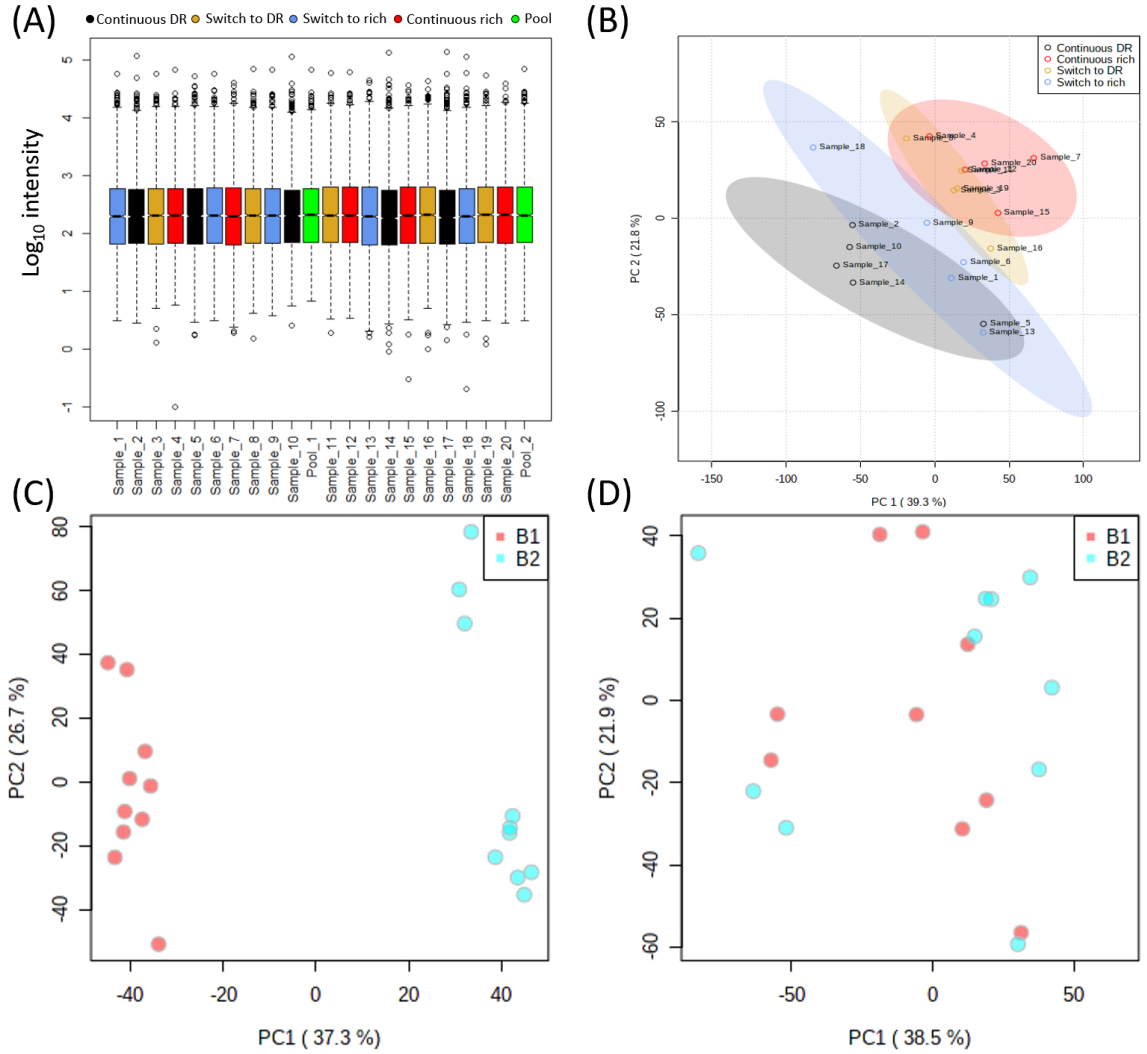

Figure S4.1 | Initial proteome characterisation.
Total sample ion intensities (5,567 proteins) were plotted to visualise possible differences between both samples and groups following data acquisition with Proteome Discoverer 2.1 (**A**). Initial principal component analysis (**B**) of dietary groups revealed possible outlier samples (5,16&18). Principal component analysis of proteins before (**C**) and after (**D**) batch correction with Combat. Batch effects are clearly apparent (B1 v B2) prior to adjustment.

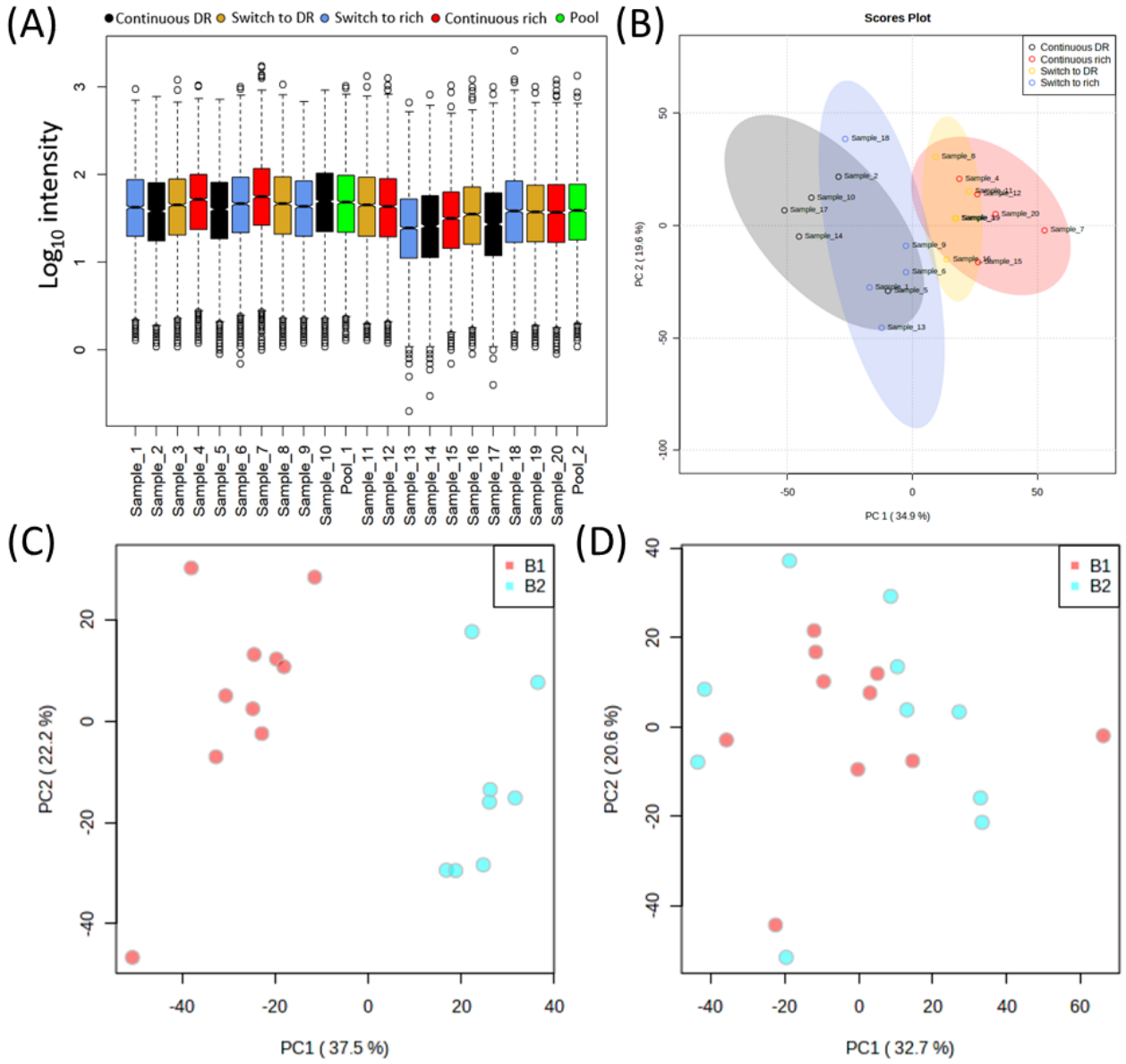

Figure S4.2 | Initial phosphoproteome characterisation.

Total sample ion intensities (2,328 proteins) were plotted to visualise possible differences between both samples and groups following data acquisition with Proteome Discoverer 2.1 (**A**). Initial principal component analysis (**B**) of dietary groups revealed possible outlier samples (5,16&18) as identified previously. Principal component analysis of proteins before (**C**) and after (**D**) batch correction with Combat. Batch effects are clearly apparent (B1 v B2) prior to adjustment.

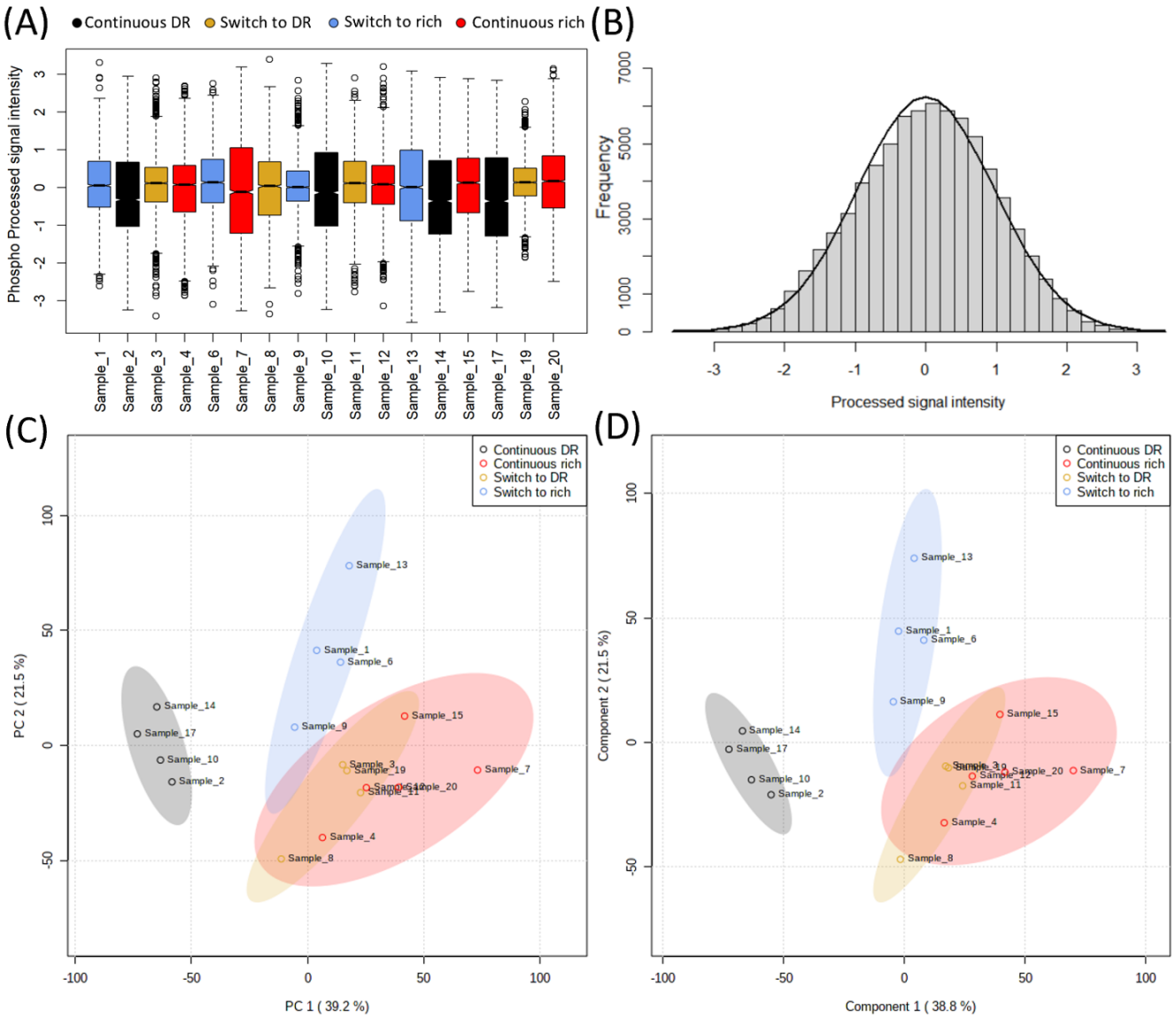

Figure S4.3 | Post-processing characterisation of the proteome.

Total sample ion intensity (**A**) was plotted to visualise possible differences between both samples and groups following the data processing. Histogram of processed protein intensities (**B**) approximates a normal distribution (curved line). (**B**). Initial principal component analysis (**C**) separated samples according to class (clear separation between continuous diets). Partial least squares-discriminant analysis (**D**) was used to maximise this separation using grouping information.

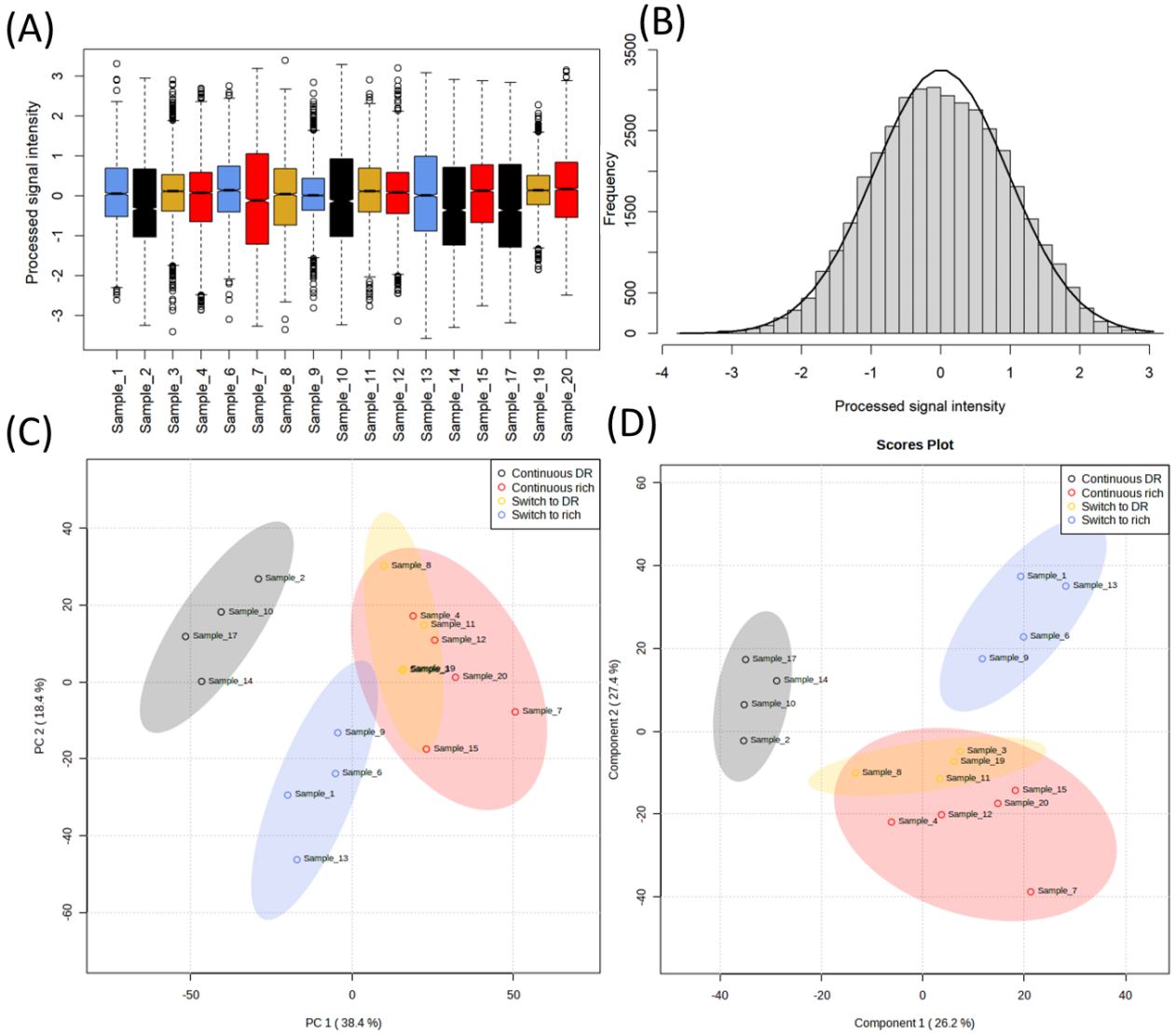

Figure S4.4 | Post-processing characterisation of the phosphoproteome.

Total sample ion intensity (**A**) was plotted to visualise possible differences between both samples and groups following the data processing. Histogram of processed protein intensities (**B**) approximates a normal distribution (curved line). (**B**). Initial principal component analysis (**C**) separated samples according to class (clear separation between continuous diets). Partial least squares-discriminant analysis (**D**) was used to maximise this separation using grouping information.

Discussion

My thesis used omics combined with unique dietary and interspecies approaches with the aim to identify the elusive physiology of DR. I was able to identify a set of candidate mechanisms, but whether or how much of the mechanisms of DR are explained by these remains to be determined. Manipulations of these mechanisms and measuring the DR longevity response would be a first step. Furthermore, there is progress to be made in combining different omics (namely transcriptomics) into the approach employed here. Measuring the physiological response to DR, from transcriptomic, to proteomic to metabolic and distilling shared mechanisms across these, has a lot of promise to be able to distil the identified mechanisms further and/or identify mechanisms missed in my thesis.

As employed in chapters 2 & 4 of my thesis, metabolomics will be a challenge to combine with other omics, for example, the transcriptome. This incorporation with sequence-based methods will help delineate the implicated pathways more easily. As sequence-based methods (relatively) unambiguously identify which genes are involved, they suffer less from ambiguity in annotation and identification inherent to mass-spec (Li *et al.* 2011). For metabolomics, even with perfect identification of a metabolite, there are many metabolites of unknown *in vivo* production, or metabolites that can be derived from multiple physiological processes. As such, and also a key limitation of my work, it is hard to pinpoint specific mechanisms without further experimentation or additional evidence. However, the benefit of metabolomics, and proteomics in part, is that it is more closely related to actual function when compared to genomic or transcriptomic information. Many mutations in the genome have no phenotype (Akashi and Schaeffer 1997), and many transcripts will be preferentially degraded (Towler and Newbury 2018), thus possibly not resulting in functional change. Integrating the end products of physiology, i.e. metabolites, into sequence-based approaches would thus strengthen inference across omics. Such integration I predict would be especially powerful in complex phenotypes such as ageing and DR.

Technical considerations

On a technical level, sequence-based approaches are heavily dependent on bioinformatics tools and pipelines to process and analyse experimental data (Crowgey *et al.* 2015; Doig *et al.* 2017; Lassmann *et al.* 2020; SoRelle *et al.* 2020). This allows the end-user to distance themselves from many technological aspects and focus on the biological matter at hand. However, such a level of support is not available for mass-spectrometry based analytical methodologies, especially in the more recently established field of metabolomics (Kuehnbaum and Britz-McKibbin 2013; Pinu *et al.* 2019). The simple biochemical procedure required for sample preparation maintains a low barrier to entry like that of transcriptomic or even proteomic based approaches. However, those who choose to perform metabolomic analysis are then either forced to purchase expensive, proprietary and often outdated software packages matched to the analytical platform used, or craft their own data handling solutions from scratch (Worley and Powers 2014). I used the latter option in my thesis, creating a novel workflow from mostly R based metabolomics packages to process the data output from the Waters analytical platform. The lack of adequate resource materials and standardisation makes metabolomics a less-attractive option than established next-generation sequencing (NGS) technologies (Beger *et al.* 2016; Markley *et al.* 2017; Pinu *et al.* 2019). The complete genomic sequence of *Drosophila melanogaster* was first published in 2000, containing approximately 14,000 protein-coding genes (Adams *et al.* 2000). The most recent UniProt reference contains 13,821 genes and 22,084 proteins. There are just 868 reference metabolites in the KEGG *Drosophila melanogaster* database, a fraction of what is available for the sequence-based resources. We identified 363 metabolites in total across all metabolomic investigations (Chapter 2 & Chapter 3), covering 42% of the metabolome. We identify 5,567 unique protein sequences in the proteome of *Drosophila melanogaster* (Chapter 4), yet this represents just 25% of the proteome. The discrepancy here is

probably even worse, as arguably, we will have identified nearly all functional genes in the fly (~14,000), but metabolites in their many subforms are more likely in the hundreds of thousands (Wishart *et al.* 2022).

Improvements to the detection capability of metabolomic techniques remain a priority within the field (Beger *et al.* 2016). However, the benefit of an unbiased, quantitative and non-targeted metabolomic approach (such as that utilised here) is also one of its greatest weaknesses (Roberts *et al.* 2012; Zampieri *et al.* 2017). Though there are a greater number of metabolites identified when compared to targeted approaches (thus maximising discovery potential) (LaBarre *et al.* 2021), the annotation confidence is lower than targeted approaches (resulting from the lack of standards for each metabolite), and the identification of specific metabolites are not always guaranteed (Matsuda 2016; Markley *et al.* 2017). Metabolites represent the terminal point of molecular regulation and cell activity and, as such, offers a holistic approach to understanding the phenotype of DR (Kuehnbaum and Britz-McKibbin 2013). Ultimately a targeted approach of all 868 metabolites could reveal the full extent of the changes to metabolomic regulation in response to DR, but more probably is limited in scope as it still would not capture potentially unknown physiology that could be key. A lack of annotation coverage and confidence is thus a key limitation in metabolomics, especially when the aim is to uncover presently unknown physiology. Though even with these limitations, insight into complex biology such as DR can be made.

Furthering our mechanistic understanding of DR

This thesis does not provide a complete understanding of the molecular processes which underpin the DR-longevity effect. It does, however, expand on our current knowledge in several general and specific ways which would not have been possible had each organisational layer been tested in isolation. DR results in a rapid reduction of age-specific mortality within the fly (Good and Tatar 2001; Mair *et al.* 2003). Demographically, I replicate this effect again within 2-4 days following the application of DR (Chapter 1). The expectation is therefore that the regulation of mechanisms responsible for this reduction in mortality should in part approximate that of the flies continuously fed a DR diet. The change towards this state should be very rapid to be able to explain the almost instantaneuous lowered mortality shortly after DR is imposed. However, we found only a slight separation of switching diets from their original diets after 24 hours in the metabolome (Chapter 2, Figure 2.2A). This separation increased at 48h, where there were strong indications of metabolic state switching (Chapter 2, Figure 2.2B). However, it was clear that a complete metabolomic switch was yet to complete. Combined with the observed demographic effects, this suggests that a small proportion of metabolites (and pathways) are responsible for the observed changes in age-specific mortality, and not a full mirroring of the metabolomic state during continuous DR. Within the proteome the changes observed over the same 48h period do not even reach significance, suggesting after short term DR the proteome is still locked into the continuous fed rich state. Furthermore, analysis of the phosphoproteome also failed to reveal any significant switch in the regulation of protein function (Chapter 4); however, it is possible that other forms of post-translational control are in effect which warrants further investigation.

Full- or rich- feeding methodologies have been criticised for generating an unnatural burden of metabolic cost (Hayflick 2010; McCracken, Adams, *et al.* 2020), the DR-longevity benefit is not simply an escape from these costs alone (Baur *et al.* 2010; Luigi Fontana *et al.* 2010). For this to be true, pro-longevity pathways would not be actively upregulated under DR. We find evidence for and against this. Our metabolomic investigations reveal a number of pro-longevity candidates which respond to a sudden decrease in dietary protein (Chapter 2). Yet this is contrasted by our findings which show that there is no significant adjustment in proteostasis (Chapter 4). Although it is possible that the immediate mortality benefit observed under DR is the result of metabolic regulation, or some other mechanism such as post-translational modification (that is not mediated by the phosphoproteome)

(Chapter 4; Hu *et al.* 2019). The complete lack of proteomic response to the onset of DR suggests that changes in the abundance of cellular machinery or phosphorylation state are unlikely to be responsible for the mortality reduction seen soon after DR is applied (Chapter 1) which is surprising, considering that DR is thought of as a highly regulated response interwoven with nutrient-sensing pathways (Kapahi *et al.* 2004; Ma *et al.* 2018). We have confidence in the technological approaches employed, as known long-term mediaries of DR, such as changes to lipid (Anthony *et al.* 2013) and amino acid metabolism (Mirzaei *et al.* 2014) are repeatedly uncovered in each analysis (Chapters 2, 3 & 4). These are again found in the short-switches, but not to the same extent, which suggests that elements of these pathways are more responsive to the sudden onset of DR than others, and may play a more significant role in the observed longevity benefit. However, though this adds to the current knowledge base, it is not complete. It is possible that the elusive mechanisms of DR are largely external to the technologies used in this investigation, they are possibly tissue-specific, or that they are masked by the reliance on pathways analysis.

However, we do propose that the excess mortality observed during refeeding is the result of additional costs and possible maladaptation to a sudden protein influx, which concomitantly accelerates the ageing process. This hypothesis is supported by findings across multiple organisational layers and species of *Drosophila*. This is shown by the elevated accrual of a known ageing biomarker, dolichol, during refeeding, which indicates that the rate of biological ageing becomes accelerated, compounding the costs resulting from an increased protein intake and turnover (Basisty *et al.* 2018). Flies switched to a rich diet did not contain any metabolites at a significantly greater concentration than those found in flies continually fed a rich diet which would indicate possible toxicity. As only a fraction of the theoretical metabolome is covered in our study, it is possible therefore that the lack of complete metabolomic coverage masks such an effect. Still, widespread metabolic toxicity is unlikely as we did not find a single suggestion of toxicity or imbalance. However, a total of 11 proteins were significantly overexpressed under refeeding (Chapter 4, Table 4.4), though it is probable that these are responsive to diet, and not inherently hazardous at an increased concentration. However, we also find possible evidence for maladaptation of protein synthesis in response to refeeding. We find significant investment into ribosome biogenesis possibly reacting to the overburdening demand of protein synthesis, an issue compounded by the decrease in protein synthesis (and associated machinery) under DR (Ding *et al.* 2014). Furthermore, the widespread mobilisation of lipids, such as ether lipids with known homeostatic benefits, signifies a prioritisation of anabolism over other cellular activities. It is possible that the highly anabolic environment of the fly upon a return to nutrient-rich conditions is exhaustive, placing an overwhelming demand upon cellular regulation at a detriment to longevity. To confirm this hypothesis, we propose that future works which would allow for a controlled and gradual return to a rich diet following a period of DR be undertaken to avoid such mortality overshoot.

Several interesting candidate mechanisms were revealed in this thesis with both known and unknown pro-longevity functions. However, there appears to be some disparity in our findings between the observations we make between the metabolome and the proteome. Metabolically, pathways of oxidative stress resistance (Haigis and Yankner 2010; Yang *et al.* 2010) and repair mechanisms (Moroz *et al.* 2014; Schultz and Sinclair 2016) were upregulated as a result of DR (Chapters 2 & 3), which is partly observed in the proteome (Chapter 4). External supplementation of these compounds (AICAR, ceramide, ether lipids, ecdysone, MTHF, NMN and PP-IX), or modulation of their endogenous quantities by genetic manipulation may reveal whether they have any inherent pro-longevity benefit when tested in isolation. However, it is probable that the excessive mortality costs seen when flies return from DR to a rich environment soon outweigh any fitness benefit provided by this investment into somatic maintenance during periods of DR. The novel metabolic candidates of DR we identified, notably xylitol and palmitate, warrant further experimental investigation to test for possible pro-longevity benefits.

However, we do not find any evidence of longevity-associated change within the proteome. One reason for this is that pathway association over the interpretation of single genes (or metabolites) might be less sensitive to pick up these effects. The integration of genomic, transcriptional, proteome and metabolomic information could help identify candidate mechanisms more reliably. Another reason could be that the longevity benefit observed under DR indeed reflects the escape of costs paradigm (McCracken, Adams, *et al.* 2020), where the subject simply operates in a 'healthy state' and such instant 'unregulated' benefits of DR might be expected. If DR's benefits could be obtained in this manner, it may explain why its key mechanisms remain hard to pin down, as the cellular state and molecular machinery are essentially the same; the animal simply operates in a manner (i.e escapes costs) that is more supportive of life. The transcriptome also shows little functional change in response to the onset of DR; Whitaker *et al.*, found that folate biosynthesis was the only upregulated pathway in response to DR and all other 'strong candidates' which they identified as proximal mediators of reduced mortality under DR were downregulated. The proteomic analysis conducted here (Chapter 4) found no evidence of proteomic change in response to DR. Therefore, these transcriptomic adjustments possibly attenuate the DR effect in the long-term, but are not responsible for the immediate benefit observed when DR is applied (Figure 1.2). Therefore, a shift in the metabolomic state is most likely to explain the mortality benefit observed under DR in line with the escape of costs paradigm. However, until there is a time in which metabolomic resources match that of the proteome and transcriptome, we have a limited scope of the metabolic environment which is further hampered by the lack of standardisation and overall underutilisation. Future work should revisit this experiment at a time when the technology has matured (containing hundreds of thousands of compounds in the *Dmel* metabolomics database, and not the 868 it does so presently) as it may reveal the complete picture of metabolomic change, and not the fragment which is currently available.

Though we have shown it is possible to assess the metabolic and proteomic state in response to DR, it is perhaps simply not as regulated as we might expect. It is likely that the longevity benefit generated by DR is a combination of factors that the experimental design employed by this thesis was unable to fully decipher. Steps to characterise this molecular phenotype may be decisive in understanding the longevity benefit provided by DR, at least the portion that is regulated. Metabolomics or *in vivo* measures of cellular or systemic regulation (such as mitochondrial function or autophagy) would, I argue, be the most successful tools to identify such mechanisms over the investigation of gross-quantities between conditions, which may be enhanced through tissue specific investigations. Though such an experimental design would be both complex and expensive, they may hold the key to understanding complex biological interactions (Dunphy *et al.* 2021; Rollins *et al.* 2019) and allow us to pin down the mechanisms of DR in their entirety (Macklin 2019). For now, we show that the traditional 'omics approaches in combination with the switching paradigm fails to provide the complete picture of the DR effect; however, it has shown where we should possibly look next.

Conclusion

Only when the ease, cost and resource availability, and standardisation of mass-spectrometry based analytical approaches match that of NGS technologies may we see an increased rate of adoption within multi-omic studies (Beger *et al.* 2016; Hasin *et al.* 2017). Metabolomics in particular is the most technologically challenging (as it cannot leverage sequence-based resources), but holds the greatest potential to answer questions of DR as the technology matures. Each method has the potential to complement the shortfalls of the other, building a more complete picture of DR. Despite the technological difficulty, my use of metabolomics and proteomics has been fruitful in confirming known and identifying novel mechanisms of molecular regulation generated from the application of DR (Chapters 2, 3 & 4). Though my work does not provide a complete understanding, it improves the current comprehension of DR-based physiology and identifies novel candidates for experimentation. Perhaps most importantly, it questions whether regulated cellular machinery that changes in response to DR is fully responsible for its longevity benefits.

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