Exome-matching of dietary amino acids in flies and mice

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PhD Thesis

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See dying vegetables life sustain,
See life dissolving vegetate again:
All forms that perish other forms supply,
(By turns we catch the vital breath, and die),
Like bubbles on the sea of matter born,
They rise, they break, and to that sea return.

Alexander Pope, An Essay on Man (1733-34), Epistle III, lines 15-25

Molecular illusion, going on to confuse our minds…
…There reality is grey, with no hope or faith
I start a journey to inside, waking my first term
I'm just returning to my dream, where I'm coming from

Eloy – Inside (1974)

Declaration

I declare that the work presented in this thesis is my own except where duly noted.

George A. Soultoukis

22.02.16

Abstract

Dietary restriction (DR) is a moderate reduction in nutrient intake that improves health and prolongs longevity across most species tested. The restriction of specific nutrients, rather than overall food intake, is particularly important in mediating the beneficial effects of DR. In flies dietary protein mediates DR-induced lifespan-extension, and imbalance of single amino acids (AAs) dictates longevity and fecundity. In a conserved fashion in mice and rats, restriction of protein or of single essential AAs (EAAs) also extends lifespan. However, the definition of what constitutes a limitation or excess in the intake of an AA can be dubious as many past attempts to determine a reliable measure for dietary AA requirements have had limited success. A possible general predictor of the AA requirement of an organism could be its genome, potentially allowing a rational design for a balanced dietary AA ratio. Here it is reported that the exome composition of an organism may provide a suitable template for its AA requirements. Matching the dietary AA supply of flies or mice to their exome promotes anabolic traits including growth and fecundity compared to amino acid profiles found in commonly used protein sources including yeast and casein. In flies, the presence of exome-defined limiting amino acids lowers the amount of bioavailable protein in the diet. However this response also depended on the identity of the limiting amino acid. Therefore the amino acid profile of a food source is an important factor when considering the usage of dietary nitrogen, anabolic traits, and lifespan.

Zusammenfassung

Eine reduzierte Nahrungsaufnahme (dietary restriction, DR) verbessert die Gesundheit und erhöht die Lebensdauer der meisten untersuchten Spezies. Dabei ist die Verminderung spezifischer Nährstoffe, und nicht die reduzierte Nahrungsaufnahme wesentlich für die positiven Effekte von DR. In Fliegen ist der Proteinanteil der Nahrung für die DR-induzierte Verlängerung der Lebensspanne verantwortlich; ein Ungleichgewicht von einzelnen Aminosäuren bestimmt die Lebensdauer sowie Fertilität. In den Säugetiermodellen Maus und Ratte verlängert eine verminderte Aufnahme von Proteinen oder einzelnen essentiellen Aminosäuren ebenfalls die Lebensspanne. Jedoch ist immer noch unklar, was eine Limitierung oder ein Übermaß bei der Aufnahme von Aminosäuren darstellt, da frühere Versuche, ein verlässliches Maß für die erforderliche Menge an Aminosäuren zu finden, nicht besonders erfolgreich waren. Eine mögliche generelle Vorhersage des Aminosäurebedarfs eines Organismus könnte sich aus seinem Genom ergeben, das potentiell ein sinnvolles Design einer Diät mit einem ausgewogenen Aminosäurverhältnis erlauben könnte. In der vorliegenden Arbeit wird gezeigt, dass die Zusammensetzung des Exoms eines Organismus eine brauchbare Vorlage für die Berechnung des Aminosäurebedarfs des Organismus darstellt. Die Anpassung der Aminosäurezusammensetzung der Nahrung an das Exom von Fliegen und Mäusen begünstigt anabole Prozesse wie Wachstum und Fertilität im Vergleich zur Aminosäurezusammensetzung von herkömmlichen Proteinquellen für Nahrung wie Hefe oder Kasein. In Fliegen reduziert eine durch das Exom definierte limitierende Aminosäure die Menge des bioverfügbaren Proteins im Futter. Jedoch ist diese Reaktion auch von der Art der limitierenden Aminosäure abhängig. Deshalb ist die Aminosäurezusammensetzung der Nahrungsquelle ein wichtiger Faktor bei der Untersuchung der Lebensspanne, anaboler Prozesse oder der Nutzung des Stickstoffes der Nahrung.

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Abbreviations-Acronyms

DR – dietary restriction

AA – amino acid

EAA – essential AA

NEAA – non EAA

BCAA – branched-chain AA

IIS – insulin and insulin-like growth factor 1 (IGF1) signalling

mTOR – mammalian target of rapamycin

SB – splanchnic bed

TCA cycle - tricarboxylic acid cycle

TAGs – triacylglycerides

ATF – activating transcription factor

GCN2 - general control nonderepressible 2

AMPK - denosine monophosphate-activated protein kinase

AAR – amino acid response

ASNS – asparagine synthetase

CNS - central nervous system

GAP activity - GTPase-activating protein (GAP) activity

GEF activity - guanine nucleotide exchange factor (GEF) activity

GPCR - G-protein-coupled receptor

GLP-1 - Glucagon-like peptide-1

PYY - peptide YY

CCK – Cholecystokinin

FOXO - transcription factor Forkhead box O

BMI – body mass index

Publications arising from this thesis

1. Matthew D W Piper, Eric Blanc, Ricardo Leitão-Gonçalves, Mingyao Yang, Xiaoli He, Nancy J Linford, Matthew P Hoddinott, Corinna Hopfen, **George A Soultoukis**, Christine Niemeyer, Fiona Kerr, Scott D Pletcher, Carlos Ribeiro, and Linda Partridge. A holidic medium for Drosophila melanogaster. ***Nature Methods*** **11**, 100–105 (2014).

2. **George A. Soultoukis** and Linda Partridge. Protein, Metabolism and Ageing. ***Annual Reviews of Biochemistry*** 85, xxx-xxx, (June 2016).

Chapter 1. Introduction: Protein, metabolism, and ageing

1.1 Abstract

Dietary restriction (DR), a moderate reduction in food intake, improves health during ageing and extends lifespan across multiple species. Specific nutrients, rather than overall calories, mediate the effects of DR, with protein and specific amino acids playing a key role. Modulation of single dietary amino acids affects traits including growth, reproduction, physiology, health, and longevity in animals. Epidemiological data in humans also link the quality and quantity of dietary proteins to long-term health. Intricate nutrient-sensing pathways fine-tune the metabolic responses to dietary amino acids in a highly conserved manner. In turn, these metabolic responses can affect the onset of insulin resistance, obesity, neurodegenerative disease, and other age-related diseases. Here it is discussed how amino acid requirements are shaped, how ingested amino acids regulate a spectrum of homeostatic processes, and finally how the use of related nutritional strategies provides a unique opportunity of using to improve human health during ageing.

1.2 Introduction

1.2.1 Diet and health

Obesity and its associated metabolic diseases are a global health problem, linked to reduced life expectancy. Both quantity and quality of food intake are clearly important in the development of obesity, with excess fat (1) and carbohydrate intake detrimental to health and lifespan in flies (2), mice (3), and humans (4). Clearly diets that promote obesity should be avoided, as should those that induce nutritional deficiencies. But what dietary compositions best promote health, and why? Does the optimal balance of macronutrients vary with age, gender, genotype or disease state? Defining the macronutrient composition of a healthy diet, and identifying the molecular and physiological mechanisms by which it promotes health, are important challenges. The introduction of this thesis focuses particularly on the roles of dietary protein.

1.2.2 Dietary restriction

A nutritional intervention that has clear health benefits is dietary restriction (DR), a moderate reduction in food intake that protects against multiple ageing-related diseases and impairments, and extends lifespan in most animals tested. The severity of DR can range from ~10 to ~50% of ad libitum intake levels, and the lifespan increase can be as modest as a few percent or as high as three-fold (5) . In rodents and primates, DR protects against ageing-related loss of function and disease, including cardiovascular disease, obesity, multiple cancers, neurodegeneration, nephropathy, loss of sensory, motor and immune function, and diabetes (5, 6) . Short-term DR in humans also benefits glucose and energy homeostasis, increasing insulin sensitivity and reducing body fat (5) . However, DR is not a practical intervention for most humans because it is difficult to implement and sustain. Moreover, DR can decrease wound healing capacity and increase susceptibility to viral infections (5). Thus an important aim is to identify the nutrients that mediate the health benefits of DR. Understanding the physiological and molecular mechanisms by which these key nutrients exert their effects may pave the way to DR-mimicking diets, as well as pharmacological interventions to improve health during ageing with minimal side-effects.

1.2.3 Dietary protein and amino acids

Recent findings have increasingly pointed to a causal role of the protein component of the diet in promoting the health and lifespan benefits of DR. In the fruit fly *Drosophila*, restriction of dietary yeast, the fly's usual protein source, but much less so of carbohydrate or total calories, extends lifespan (7), an effect attributable to the amino acids (AAs) (8) and the protein-to-carbohydrate ratio of the diet (2). In mice (3) and rats (9), reducing dietary protein, thereby decreasing the diet's protein-to-carbohydrate ratio, also increases healthspan and lifespan. The beneficial effects of protein restriction outweigh those of carbohydrate or fat restriction (3, 10, 11). Indeed, dietary carbohydrates and fats are largely interchangeable without detrimental effects in many species (1, 12). Moreover, specific AAs or their ratio can determine

health and ageing, because reduced intake of methionine in *Drosophila* (8), and of methionine or tryptophan in rodents (13, 14) results in improved health during ageing and increased lifespan. Additionally, increases in circulating branched-chain AAs (BCAAs) stimulate the target of rapamycin (TOR) and IGF/insulin signaling (IIS) pathways in rodents (3, 15), which may be detrimental for health, since suppression of TOR and IIS signaling is often beneficial for a healthy lifespan (5). The mechanisms by which individual dietary amino acids affect metabolism and health are starting to be understood, and are revealing potential targets for improvement of organismal health during ageing.

1.3 Dietary restriction, health, and ageing

1.3.1 Protective effects of dietary restriction

The physiological, metabolic, and molecular changes through which DR extends healthspan and lifespan are becoming clearer, although a complete account is lacking for any organism. Reduction in nutrient intake triggers modulations in the activity of nutrient-sensing pathways, which stimulate protective mechanisms over most aspects of health during ageing. From flies to humans, the DR response is highly conserved and involves an extensive array of protective metabolic changes that include an increase in stress resistance, detoxification capacity, and genome stability, and promotion of proteostasis and energy homeostasis (5, 16). The IIS and TOR pathways are also conserved in humans, and so are their responses to DR (5). Long-term and short-term DR trials in humans result in marked reductions in IIS and TOR signaling, decreasing multiple risk factors such as obesity, insulin resistance, and cardiovascular disease (17). These evolutionarily conserved responses to DR present an opportunity for significant health-promoting applications in human nutrition (11). Apart from its effects on energy homeostasis, DR also reduces cancer propensity. A reduction in tumour incidence (lymphomas, pituitary, and thyroid neoplasms) accompanies DR treatment in mice (6). Furthermore, primate studies also implicate dietary protein in health during ageing. Two recent Rhesus monkey lifespan trials found conflicting results, with one (WNPRC), but not the other (NIA), reporting a lifespan extension as a response to DR (11), although both found multiple improvements in health in the DR animals. Among several experimental differences between the two trials, differences in the amount and type of dietary protein were prominent (5, 11). In humans too, DR reduces IGF-I and decreases the risk of cancer (17). However, DR in humans only lowers circulating IGF-I if protein intake is also restricted (5). Human trials also highlight a role for protein quality, because diets containing low, plant-based AAs promote multiple aspects of healthspan (18). Such findings emphasize a difference between protein sources, for example plant versus animal protein, discussed further below. Moreover, recent work suggests that increased activity of the transsulfuration pathway is required for the extension of lifespan by DR in *Drosophila* (19). Indeed, hydrogen sulfide production by the transsulfuration pathway is associated with extension of lifespan by DR in yeast, flies, and mice (20).

There is thus an emerging role of dietary protein, and of specific AAs, in modulating the health benefits of DR in humans, suggesting the potential of interventions in protein intake to improve human health.

1.3.2 Protein content and AA imbalance

In agreement to what is observed in DR animals, restriction of dietary protein or AAs reduces wound healing capacity and increases susceptibility to viral infections (21). Also in accord with what is seen in DR animals, protein-restricted mice have protected cognitive function and live longer (3, 22). However, in contrast to DR animals, protein-restricted mice can show an increase in body fat and insulin resistance (3). Decreasing dietary protein also increases body fat in humans (23), but a high intake of dietary protein and AAs promotes insulin resistance and adversely perturbs glucose homeostasis (24). In consequence, only 10-15% of energy intake as protein is recommended for humans (25), although for weight loss management the absolute amount of protein consumption is of greater importance than the percentage of energy (23).

The effective protein uptake depends on the efficiency of a protein's usage. This is the result of the combined effects of (i) how much protein is ingested and metabolized, (ii) its essential to non-essential AA ratio (EAA:NEAA), and (iii) its precise AA composition (26). Adequate protein intakes can be achieved with lesser amounts of high quality protein than of low quality protein, where quality is determined by its efficiency for anabolic traits. The effects of a dietary AA imbalance are more severe when overall AA intake is low (27), as AA imbalances further decrease AA usage. A low EAA:NEAA can also be inefficient for anabolic traits at low AA intakes. Therefore, the protein content and effective macronutrient ratio of protein to carbohydrates and fats greatly depends on the AA proportions of the ingested protein. Consequently, the AA proportions of a diet are critical for health, both through effects on total protein usage, and through mechanisms mediated by specific AAs. We highlight the metabolic fate of AAs, from ingestion to effects upon metabolism and health, in the following sections.

1.3.3 Multivariate complexity of defining a dietary AA-imbalance

Identifying a diet with a healthy mixture of nutrients is complicated by the multivariate nature of diets, which poses a challenge for experimentation, and by the synergistic effects of multiple essential macro- and micro-nutrients (2). Accordingly, complex interactions between individual AAs render phenotypic responses to different AA ratios hard to interpret. Moreover, an interaction of AAs with other nutrients such as vitamins can also modulate metabolism (28), which further increases the complexity of nutritional space and confounds biological interpretations. Consequently, defining a balanced AA intake is a challenge.

To simplify the nutritional landscape, recent methods dissect nutritional interactions and their physiological effects in multidimensional space (2). Such

a representation of nutritional space, called the geometric framework, can better describe the responses of metabolic, lifespan, and other traits, and can reconcile apparently contradicting results (2). Multi-dimensional approaches are desirable but sometimes impractical, and progress can also be impeded due to the lack of standardized methods. In flies, the recent development of holidic diets enables the accurate analysis of the effects of single dietary AAs (29), and such tools would benefit work in other model organisms such as mice and rats. This kind of experimental standardization will aid the systematic analysis of multivariate AA interactions and their effects on health and ageing.

It is difficult to discern the physiometabolic effects of subtle AA imbalances that occur under conditions of normal nutrition, which is usually characterized by the intake of varied dietary protein sources. Even in laboratory model organisms, with carefully controlled conditions using chemically defined diets, problems can persist. Autoclaving and irradiation, both common sterilization steps in laboratory rodent food preparation, can degrade certain AAs including lysine, methionine, and cysteine, as well as vitamins like A and B_1 (28). In everyday human nutrition, food processing such as cooking may alter the AA contents of a protein source. Food texture can also have dramatic effects upon metabolism, as soft foods increase nutrient efficiency and adiposity. In humans, ageing also leads to anorexia and weight loss, largely due to the progressive functional decline of the digestive system (30), which also likely results in deceased AA absorption. All these factors render practical diet design challenging.

1.3.4 Anabolic traits and their experimental assessment

Although health and lifespan responses to AA sources are sometimes evaluated, experimentally the balance of an AA source is typically defined by its ability to maximize production traits (28).

With regards to the growth effect of single EAA limitations in vertebrates, the principle of the minimum is typically applied. The principle states that when all essential nutrients required for growth are abundant in a diet except for one, the limiting essential nutrient, incremental additions of this limiting essential nutrient only will increase growth (31). For EAAs, this has been repeatedly demonstrated experimentally in rodents (26). In addition, the principle is coupled to the law of diminishing returns, according to which each succeeding increment of the limiting essential nutrient will produce a smaller increment of growth than the preceding increment (31, 32). However, the nature of the link between anabolic traits and long-term health is complex and is discussed below with respect to protein and AA intakes.

1.3.5 Link between anabolic traits and long-term health?

Growth: Both DR and protein restriction can suppress growth and extend lifespan in rodents (3, 5, 26). The developmental theory of ageing holds that a prolonged lifespan is caused by retarded development, and this notion was quickly adopted as an explanation of the lifespan response to DR (33). In flies and mice, the reduced growth observed upon DR or protein restriction

respectively seems to be effected by a reduced cell number, implicating the suppression of IIS (34, 35). Life-extending tryptophan or methionine restrictions also reduce growth in mice and rats via reduction in circulating IGF-I (13, 14). Reduced IGF-I signaling modulates the negative correlation between body size and lifespan in mice (36) and dogs (37). Several rodent studies show a negative correlation between body size and longevity in both genders (36, 38), as do several genetic models of extended longevity under reduced IIS (39). All these observations together strongly suggest an inverse correlation between growth in body size and lifespan.

However, growth depression is not a prerequisite for lifespan extension. Body growth has been uncoupled from longevity both in mice and in flies (39). Thus, manipulations of growth signaling can extend longevity with no effect on body growth. Moreover, although anabolic traits are often used in the nutritional evaluation of a dietary protein, such traits are not reliable predictors of health during ageing. In rodents, some AA imbalances that do not cause growth depression can be detrimental for health and cause fatty infiltration of the liver (26). Therefore, reduction in growth signals can cause growth suppression and lifespan extension, but the former is not a prerequisite for the latter. From this perspective it remains possible that specific dietary AA intakes could optimize anabolic traits and lifespan, avoiding trade-offs between them.

Reproduction: Fecundity depends on nutrient utilization, and animal models are used to evaluate the link between fecundity and lifespan. In flies, DR reduces fecundity and increases lifespan (40). Fecund females allocate much of their ingested nutrients to reproductive processes, a proportion diminished in DR flies (41). In rodents, some long-lived models show a marked reproductive capacity reduction (40). Apart from extending rodent lifespan, protein restriction also suppresses fecundity (28). Reproductive output is also negatively associated with lifespan in dogs (42) and humans (43). However, in several fly models lifespan extension is not characterized by a lower reproductive output (40). Supplementing methionine in a methionine-restricted fly diet can rescue fecundity with no lifespan shortening (8), indicating that dietary modulation of AAs can promote longevity without impairing fecundity. Therefore, suppression of reproduction may not be indispensible for lifespan extension, and the design of diets that optimizes both traits is possible.

1.4 Amino acids: ingestion, absorption, and systemic availability

1.4.1 Dietary protein and amino acid absorption

The intake of AAs is achieved through the consumption of either whole protein or free AAs in the diet. Following their ingestion, the identity and amount of the AAs that become available to cells and tissues depends on AA absorption by epithelial enterocytes. The digestibility of whole dietary proteins is confounded by numerous factors (44), which makes it difficult to establish the identity and amount of bioavailable AAs after the ingestion of whole protein foods (45). Yet, whether derived from digested peptides or free AA diets, AAs

show substrate antagonism and other physicochemical properties that can complicate estimations of their availability (44-46). In contrast to whole protein diets, free AA diets avoid many of the confounding factors influencing whole protein digestion, and are more readily absorbable (29, 47). Moreover, in contrast to oligopeptide transporters, characterization of the free AA transporters in the human intestinal epithelium is comprehensive (46). Therefore, free AA diets are more suitable for the assessment of post-absorptive effects of dietary AAs upon metabolism, health, and ageing.

1.4.2 Dietary AAs and the microbiota

The uptake of AAs is influenced by gut bacteria, and this can greatly affect the response to a dietary protein or AA source. Instead of epithelial enterocytes, free AAs in the gut's lumen may first encounter gut microbes. Gut bacteria are both consumers and producers of AAs, but the net exchanges between host and microbiota of specific AAs, and the factors that influence this exchange, are not yet fully understood (48, 49). Nevertheless, many AA exchanges between the host and the microbiota have been characterized. Gut bacteria synthesize all essential AAs (EAAs), and contribute up to 10% of mammalian plasma metabolites, including the EAAs tryptophan, phenylalanine, and lysine (49, 50). Although such contributions for other EAAs are not yet described, they are likely to occur. In addition, during the host-colon nitrogen cycle, microbes further contribute towards AA re-absorption by the host (49). However, when rodents with a gut microbiota are fed single EAA-deficient diets, their health quickly deteriorates, which suggests that bacterial EAA contributions are modest (26). The effects of the deficiency also depend on the identity of the AA deprived (26), and on how much of each EAA gut bacteria can provide to the host. However, ruminants with a rumen microbial load able to synthesize all EAAs, still require an ample dietary AA supply to achieve high levels of growth or milk production (51). Therefore, the contribution of EAAs from gut bacteria to the host appears limited. In contrast, the microbiota consumes substantial amounts of AAs, with up to 50% of fecal nitrogen being of bacterial origin (1, 4). However, more work is needed to establish how much of each ingested AA can be used by gut bacteria (48). Therefore, the gut microbiota can shape AA availability to the host. This can be especially important in low protein diets, because small changes in AA availability can have a proportionally greater effect on the available AA profile. In turn, this can also influence the useable dietary protein and the macronutrient ratio, which shapes health and ageing.

Changes in the microbiota are also associated with the risk for obesity, diabetes, and heart disease, and in mice DR enriches microbiota phylotypes associated with increased longevity (52). Gut bacteria adapt to ingested nutrients and can shift focus from dietary carbohydrate to dietary AA metabolism (53), while they also benefit from ample dietary fibre, which increases fecal nitrogen and decreases net AA uptake by the host (48). By adapting to macronutrients and metabolizing fibre, gut bacteria can stimulate the secretion of intestinal growth factors or satiety hormones (54, 55). Apart from the health risks of a chronically over-stimulated growth axis, such interactions also complicate estimations of efficiency of dietary AA sources

that rely on the evaluation of anabolic traits (growth) or behavioural traits (food intake). Also, the metabolism of AAs by gut bacteria may be influenced by circadian rhythms, or the host's age and immune status. Due to such complications, quantifying the net AA exchanges between host and microbiota is easier in animals with a small number of gut bacteria species and chemically defined diets, such as fruit flies (29, 54). Understanding of the factors affecting AA usage by gut bacteria, the mechanisms by which gut microbes influence AA availability to the host and the health consequences, will be aided by approaches in which the amount or composition of the microbiome is experimentally manipulated.

1.4.3 The splanchnic bed and systemic AA availability

The amount of each AA that becomes systemically available is critical for metabolism, affecting health through AA-sensing mechanisms discussed in the following sections. However, this amount greatly depends on how many AAs are metabolized immediately after absorption in the gut. Once absorbed by enterocytes, free AAs enter into the splanchnic bed (SB), which comprises the gut, liver, spleen, and pancreas, where free AAs can be metabolized. In general, up to a third of all dietary AAs are metabolized by the SB (48), greatly shaping the AA profile that reaches circulation to become systemically available. Metabolism of AAs in the SB also depends on AA identity. Despite arterial supply of AAs, enterocytes greatly rely on dietary AAs (56), and a low AA intake may contribute to enteral atrophy. Enteral usage of threonine is particularly high, presumably for the synthesis of threonine-rich mucins (48, 56). In the liver, methionine enters many transulfation, transmethylation, and folate metabolism reactions (56). Glutamate, valine, isoleucine, leucine, and phenylalanine are also largely used by the SB. For all these AAs, an estimated 35%-100% of dietary intake is used by the SB, never reaching systemic circulation (48, 56). In contrast, arginine, alanine, tyrosine, and proline undergo minimal usage by the SB (56). An AA's conformation can also determine its SB usage. In flies, mice, and rats, D- and L-methionine are highly bioavailable, while in humans D-methionine is only ~30% bioactive (26, 57, 58). In contrast, all other AAs are fully usable only in the L- form across the four species (26, 57). Thus, SB metabolism of free AAs substantially affects their systemic availability depending on the individual AA's identity.

Following their passage through the SB, free AAs pass into circulation, to become part of the free AA pool. Free AAs represent a very small fraction of a body's total AA contents, but are metabolically significant as they form the systemically available AA profile. Indeed, free AAs are associated with lifespan across species. In flies low levels of glutamine, lysine, and alanine are linked to extended longevity (59). In mice, circulating metabolites including glutamine, methionine and proline decrease with age (60). This decrease is countered by acute DR, which increases circulating methionine, glutamine, alanine, and valine indicating a shift towards gluconeogenesis and energy conservation (61). However, an opposite metabolic shift has been suggested in dogs, where lower levels of isoleucine, leucine, phenylalanine, and valine are associated with the health benefits induced by DR (62). The same association has been made in humans too, where a plasma decrease in isoleucine, leucine, valine, lysine, phenylalanine, and histidine was linked to a

reduced carbohydrate metabolism and an increased AA catabolism (63). Moreover, depleted levels of circulating methionine and BCAAs have been observed in long-lived IIS mutant mice (64). Finally, in mice elevated circulating BCAAs stimulate their catabolism in the liver (15). Thus, it is currently difficult to interpret the mechanisms involved in plasma AA changes with age or with DR, or the consequences of these changes for health. Some possible mechanisms linking such free AA modulations to lifespan are discussed in more detail in following sections.

From circulation, free AAs can enter interstitial fluids and cells, to become part of tissue intracellular pools. Although the circulated free AA pool is available to all tissues reached by circulation, cell-specific AA availability depends on AA transporters whose abundance can vary between cell types (65, 66). Abundance of transporters is well characterized for enterocytes, hepatocytes, pancreocytes, nephrocytes, and in the brain, as is substrate antagonism between AAs for transporters (46, 66, 67). Thus, despite equilibrium between circulatory and intracellular pools for most free AAs, substantial differences in concentrations between the two pools are seen in some cases. In humans, glycine, glutamate, and glutamine are 10-50 times more concentrated in intracellular pools (4). And it is also noteworthy that the free AAs in a tissue do not match the AA composition of the tissue's proteome. In rat muscle, compared to protein-bound AAs, there are depleted levels of phenylalanine, methionine, and BCAAs (4). Therefore, circulating, intracellular, and protein-bound AA profiles differ significantly, but circulating and intracellular AAs fluctuate more dynamically and play a prominent metabolic role.

The dynamics between transporter abundance and tissue-specific AA availability need more clarification, as do the effects of bidirectional transport between specific AAs, such as glutamine and leucine (68) upon AA tissue specific AA availability. Nonetheless, some physiological effects of AA antagonisms are clear. Antagonisms between the BCAAs can result in growth depression upon supplementation of one of these three AAs in the diet (26). Similarly, antagonisms between lysine and arginine can suppress growth upon addition of lysine or arginine only in the diet (26). Excess leucine or methionine deppress rat growth independently of food intake, with excess leucine increasing the growth requirement for tryptophan (26). However, there is a lack of long-term studies, and the effects of an AA imbalance-induced decrease in growth signaling upon health and lifespan await further study.

1.4.4 Metabolic fate of ingested amino acids

The metabolic fate of intracellular AAs is important in determining the effects of AA intake upon health and ageing. An outline of AA metabolism is given in Figure 1.1. Once in the splanchnic bed, free AAs can be used for protein biosynthesis (e.g. in liver or intestinal muscle cells), or can be broken down to their carbon skeleton and amine groups. Amine groups are typically excreted, but carbon skeletons can have a diverse fate. They can be used in the biosynthesis of acetyl-coenzyme A (acetyl-CoA) or acetoacetyl-CoA, the main precursors of fatty acids, which are in turn stored as triacylglycerides (TAGs)

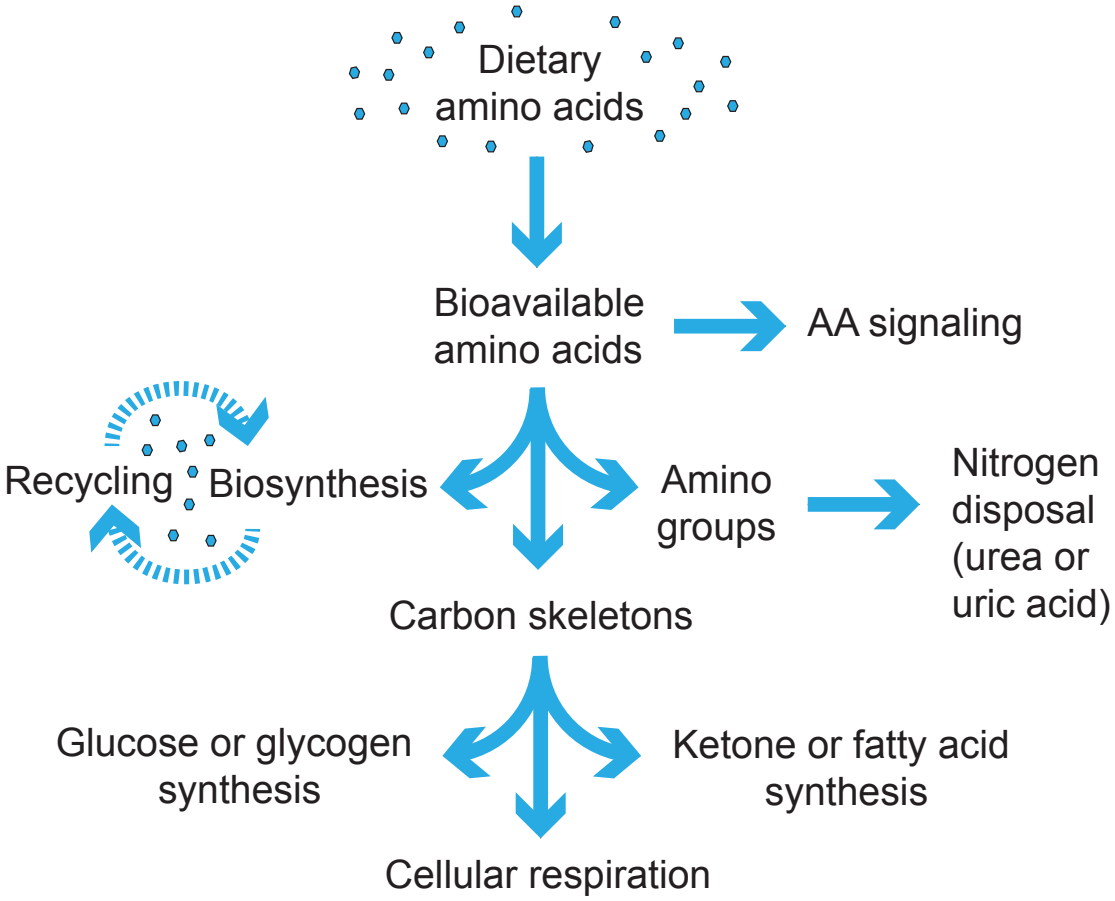


Figure 1.1

The metabolic fate of ingested amino acids. Modified from Berg et al. 2006 (69)

in adipose tissue. Alternatively carbon skeletons can be used to synthesize pyruvate and oxaloacetate, the precursors of glucose (stored as glycogen) fueling the tricarboxylic acid (TCA) cycle. Finally catabolism of the carbon skeleton can also be used for cellular respiration and energy production in the form of ATP. Those AAs not metabolized in the splanchnic bed can enter circulation, from where they can be absorbed by cells and tissues. Consequently, the proportion of AAs in the diet can affect many of these metabolic pathways. This aspect is discussed separately for AA limitations and excesses in the following sections.

1.5 Detection of AA limitations and regulation of metabolism and health

The sensing of both circulating and intracellular free AAs occurs through various mechanisms, at both the cellular and systemic levels. These two modes of AA-sensing determine many of the metabolic and physiological responses to fluctuations in AA availability.

1.5.1 GCN2-dependent detection of AA limitation

A metabolic response to limited AAs can only occur after their limitation is detected. In flies, nutrient perception involves chemosensory sensila, enteroendocrine, and gustatory signals (70). The consequences of the fly's nutrient sensing can be uncoupled from its actual food intake, because stimulating odorant receptors can reverse the benefits of DR upon lifespan independently of food or protein intake (71). The fly's selection of an AA source is partly mediated by the serine/threonine-protein kinase general control nonderepressible 2 (GCN2) acting in dopaminergic neurons in the brain (72). The mammalian central chemosensor detecting decreased circulating essential AAs is found in the brain's anterior prepiryform cortex (APC) (73). Here, low levels of EAAs stimulate GCN2, which suppresses anabolism and promotes catabolism through the AA response (AAR) pathway (73-75) (Figure 1.2). This GCN2 activation is independent of the AA's identity, because GCN2 senses the AA deficiency by binding non-specifically to any uncharged transfer RNA (73, 76). However, branched chain AAs, and in particular leucine, the most abundantly used AA in mammalian proteomes, appear to play a predominant role (77). Upon binding an uncharged tRNA, GCN2 changes its conformation to promote inhibitory phosphorylation of its primary downstream translation activator, the eukaryotic initiator factor 2 alpha (eIF2α) (73). This leads to global down-regulation of transcription and translation through changes in mRNA levels or mRNA stabilization, growth arrest and reductions in lipid and carbohydrate anabolism, activation of AA transporters (e.g. asparaginase synthetase ASNS), and changes in neuronal glutamatergic activity, intracellular calcium and GABAergic signaling (65, 73, 74, 78). A common downstream effector of GCN2 activation upon methionine or leucine restriction is fibroblast growth factor 21 (FGF21), which represses liver fatty acid synthesis and increases fatty acid mobilization (13, 79). Although GCN2 is a key modulator of the systemic response to decreased levels of circulating AAs, it is also expressed across tissues and may also act in a cell-autonomous manner (73). In rodents, three isoforms of GCN2 are found: α, β, and γ. The α and γ isoforms have no functional GI (GCN2/Impact)

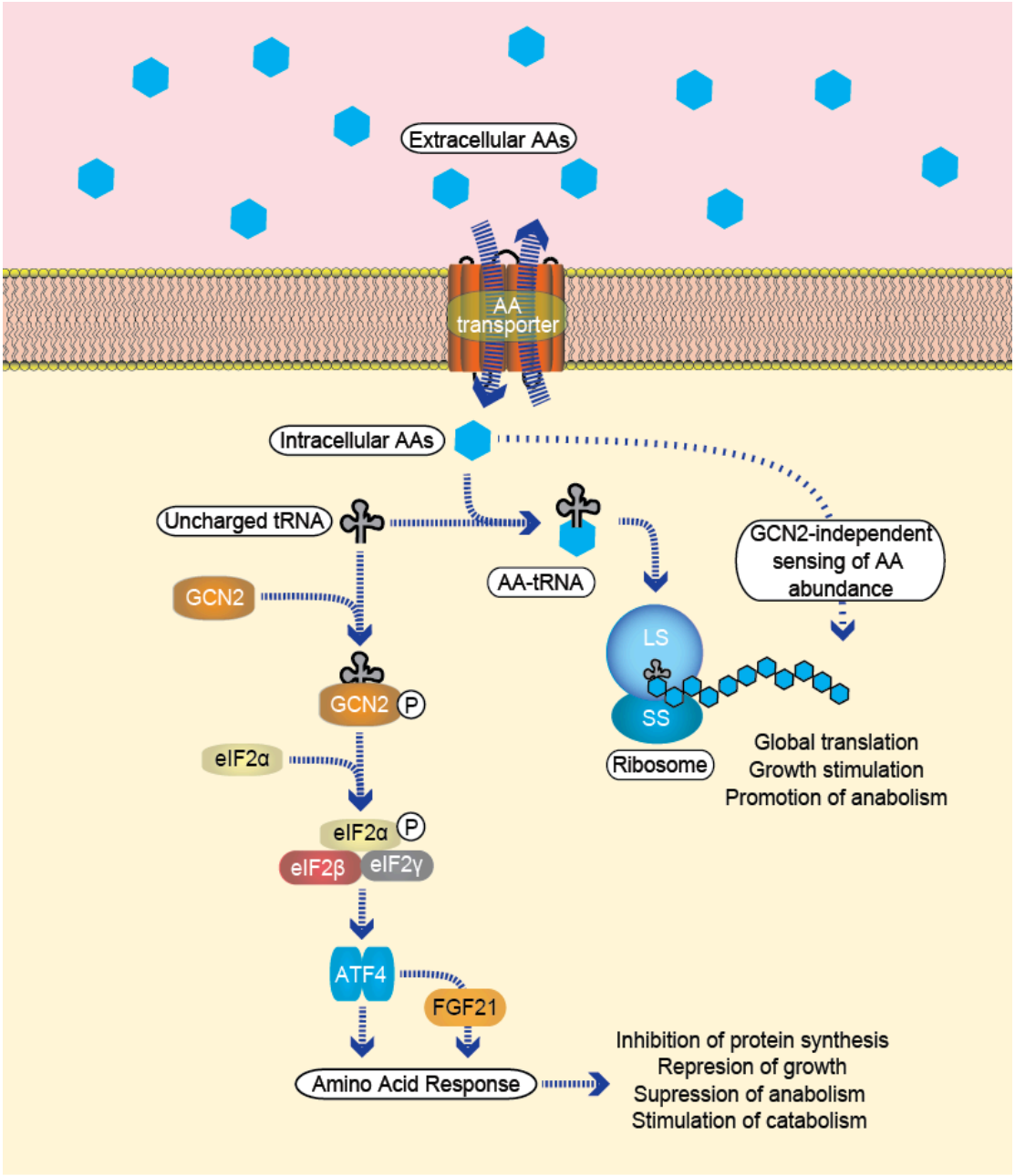


Figure 1.2

GCN2-dependent sensing of AA limitations. Extracellular amino acids (AAs) can activate their cognate tRNA, which is now available for ribosomal protein synthesis (LS=large subunit, SS=small subunit). In contrast, uncharged tRNAs bind to and activate by phosphorylation GCN2, which in turn phosphorylates eIF2α. This activates the eIF2 complex to stimulate ATF4, which induces FGF21 to trigger the amino acid response (AAR), which inhibits anabolic processes, and promotes catabolism (see main text).

domain to bind GCN2 to its activator GCN1, and are expressed tissue-specifically, while β has a functional GI domain and is expressed similarly across tissues (76). However, mice lacking GCN2 specifically in the brain fail to show the normal aversive behaviour towards AA-imbalanced diets. AA sensing in the APC thus overrides peripheral GCN2 activity with regards to feeding behaviour (74). Therefore, low circulating AAs result in stimulation of the AAR pathway through the activation of GCN2, which orchestrates cell-autonomous and non-cell autonomous effects to promote catabolism, suppress anabolism, and thereby induce a metabolic maintenance mode.

1.5.2 GCN2-independent detection of AA limitation

Although much evidence supports a role for GCN2 in the sensing of ingested AAs, some studies cast doubt over its significance in the physiological and behavioural responses to AA-deficient foods. Recently, GCN2 has been shown to have no effect on the detection of AA-deficient diets in mice (80). In addition, sensing of AAs such as alanine or glycine in hypothalamic neurons also occurs via excitatory signals that are modulated within seconds from the moment an AA is supplied (81). Such response mechanisms are GCN2-independent, as transcriptional changes modulated by GCN2 would likely require a longer time period. Indeed, some preliminary findings in GCN2 knockout mice indicate that the response to methionine restriction is not dependent on GCN2 (13). Therefore, given the central role of the hypothalamus in modulating feeding behavior, obesity, and metabolism (82), it will be interesting to see how different ingested AAs give rise to an organism's hypothalamic and consequent metabolic response to ingested AAs.
Dietary AA restriction also regulates gene expression via multiple GCN2-independent pathways, including transcriptional (e.g. Cxcl10) or post-transcriptional (e.g. Dusp16) responses (74, 83). Although the molecular mechanisms behind the activation of such responses by low AAs are not known, such changes are able to increase catabolic processes and metabolic efficiency independently of the GCN2-mediated AAR response. For example, efficiency of AA uptake is increased by up-regulation of asparagine synthetase ASNS, or of plasma membrane AA transporters such as the neutral AA transporters SNAT2 and LAT-1 and the cationic AA transporter CAT-1 (65, 74, 84). Other GCN2-independent responses can include transcription factor adjustments (ATF2-5, C/EBP and other ATF/CREB TFs), and changes in ribosomal proteins that affect translation (65, 74, 84).
Although GCN2 is the only kinase exclusively responsive to AA deprivation, phosphorylation of eIF2α can also be effected by other kinases including heme-regulated inhibitor kinase (HRI), double-stranded RNA-activated protein kinase (PKR), and PKR-like endoplasmic reticulum-resident kinase (PERK), and considerable overlap has emerged in the activation of downstream effectors between PERK and GCN2 upon methionine restriction in mouse liver (78). Moreover, internal ribosomal entry sites (IRESs), such as that in the CAT-1 mRNA, allow preferential translation by phosphorylated eIF2α, and the role of such IRESs in PERK or GCN2 activation also requires further characterization (78). Another GCN2-independent mechanism may involve the AMP-activated protein kinase AMPK, which senses low-energy states by detecting high AMP levels (85). AMPK functions both cell-autonomously and

non-cell-autonomously and is also activated upon low AA status (85, 86). Importantly, increased AMPK activation extends worm and fly lifespan (85).

The dynamics of GCN2-independent responses can vary. As mentioned, sensing of supplied AAs by hypothalamic neurons can occur within seconds, thereby comprising a rapid response (81). In contrast, other modulators can vary across a wide range of time. For ASNS, a translational surge upon AA limitation is followed by a more sustained transcriptional activation (65). Moreover, plasma AA responses to dietary AA limitations indicate that the limited AA drops in the plasma initially, but after several days its levels are restored (87). As a response to an AA-imbalanced diet, growth and food intake depression also subside after several days (88), perhaps through reconstitution of hormonal homeostasis (discussed below). In contrast, short-term responses relying on GCN2 do not require hormonal adjustments (73). However, some AA-sensing mechanisms require further elucidation. For example, eukaryotes detect misloaded tRNAs with different efficiencies depending on the identity of the AA (89). A domain in glutaminyl-tRNA synthetases allows the accurate sensing of loaded $tRNAs^{Gln}$ to glutamine (89), but the metabolic or proteostatic causes and effects of such differential accuracies in correct tRNA loading, as well as related non-GCN2 signaling effects, are unclear. Thus, although several GCN2-independent responses to limited AAs have emerged, further clarification of the molecular mechanisms and characterization of the short- versus long-term responses is needed.

1.5.3 Effects of AA limitations on ageing

All limitations or excesses of dietary AAs are sensed in a way that modulates anabolic and catabolic processes and, ultimately, homeostasis. Suppression of anabolism and growth signaling can extend lifespan and is induced by AA limitations. The identity of essential AAs is conserved between rodents and humans, and human cell culture work on AA limitations shows consistent results to murine cell systems implying conserved molecular mechanisms (90). In rodents, methionine and tryptophan limitations suppress anabolism and translation, and promote catabolic processes (13). Glucose, insulin, thyroid hormones, and IGF-I levels are also reduced in methionine-restricted mice. Yet, generally, low levels of circulating AAs reduce IGF-I function largely independently of their identity (91). Lack of the AA building blocks for anabolic traits also results in induction of apoptosis by IGF-I, which activates the apoptosis inducer CHOP (65). Stimulation of apoptosis aids the recycling of molecular building blocks, including AAs. Therefore, AA limitations deplete growth signaling and can thus induce a maintenance mode that benefits long-term health.

By increasing catabolism, methionine or tryptophan restrictions also reduce fat storage in rodents (13, 14). However, although across multiple organisms and humans DR results in leanness or rescue from obesity to confer multiple metabolic advantages that favour longevity, the role of fat loss per se in promoting health is not clear. For instance, diets low in protein increase adiposity in mice because of increased food intake, but these mice are as

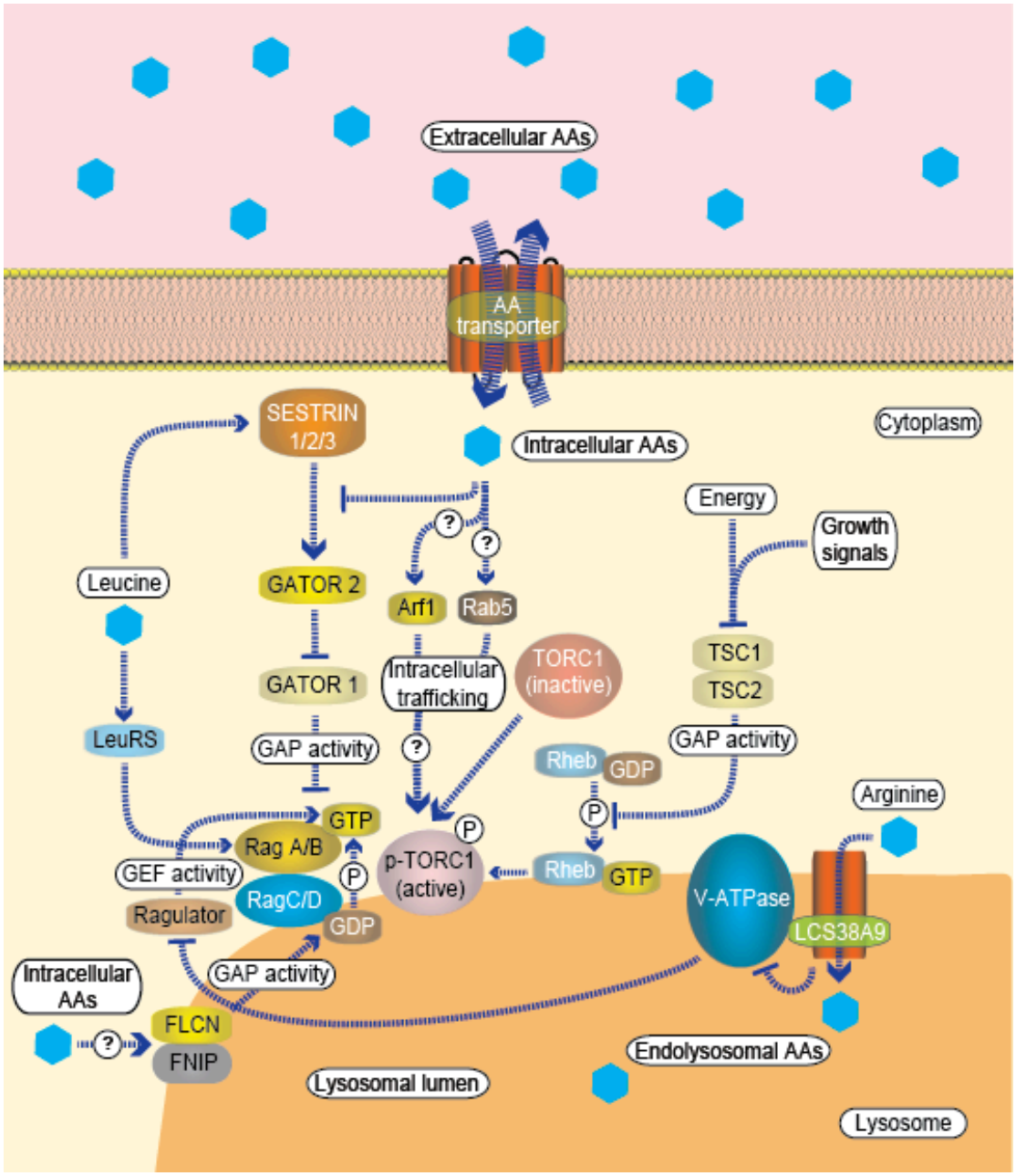


Figure 1.3

TOR-dependent sensing of AAs. Intracellular AAs activate TORC1 through multiple TOR-associated factors (see main text for details). GAP - GTPase-activating protein; GEF - guanine nucleotide exchange factor (GEF)

healthy as DR mice (2, 3). Additionally, the ability of animals to maintain their adiposity despite DR appears to mediate the beneficial effects of DR (92). The decline of mTOR expression with age in rat white adipose tissue is also prevented by DR (93). Therefore, as the role of fat deposition in DR is unclear, and given that different types of fat affect health differently, the role of fat deposition in mediating the health benefits of protein or single AA restriction requires further investigation.

1.5.4 TOR-dependent detection of AA abundance

In mammals, the cell-autonomous AA response upon excess of AAs relies primarily on the mammalian target of rapamycin (mTOR), which occurs in two complexes, mTORC1 (rapamycin/nutrient sensitive) and mTORC2 (rapamycin/nutrient insensitive). Absence of AAs results in the TORC1-inhibitory recruitment of the tuberous sclerosis protein TSC2 onto the lysosomal membrane (94). Mechanisms of activation of mTORC1 in the presence of AAs are shown in Figure 1.3. Sensing involves a complex interplay between numerous molecules recruited in or around the lysosome, which is a key site of AA recycling, and intracellular/intravacuolar AA sensing (77, 95). Intracellular AAs prevent the inhibitory association of Sestrins with the GATOR2 complex (96, 97). This lowers GATOR1's GAP activity upon Rag A/B, which is bound to Rag C/D and is important for the activation and translocation of TORC1 onto the lysosomal membrane (96). Intracellular AAs are taken into the lysosome by transporters such as SLC38A9, which has a particularly high affinity for arginine(98). This transport induces conformational changes in the endolysosomal V-ATPase, which dissociates from the Ragulator/Rag complex (98). The Ragulator then enables the activation of Rag A/B through its guanine nucleotide exchange factor activity (98)(88). Intracellular AAs can also promote the GTPase activity of the folliculin complex FLCN/FNIP (99), which results in the RagC/D complex being loaded with GDP, and stimulates Arf1 and Rab5, which are involved in intracellular trafficking inducing TORC1 activation (100).

The identity of the AA also determines how it is sensed by TORC1. Leucine activates Rag A/B through Sestrin 2 (97), glutamine sensing involves Arf1 and the V-ATPase, but not Rag A/B, and arginine sensing involves SLC38A9 (101). Other factors involved in TORC1 activation by AAs may involve the kinase Vps34 and SH3BP4 (95). Importantly, inhibition of mammalian TORC1 activation extends lifespan and, although activated TOR is critical in regulating adiposity by inducing lipid synthesis (95), insulin resistance is effected primarily by mTORC2 (102). The interplay between TOR and AMPK in sensing AAs also requires further characterization. Inhibition of rat muscle mTORC1 with rapamycin has no effect on AMPK, but activation of AMPK suppresses mTOR signaling and insulin resistance (103). Accordingly, reduced AMPK activity precedes mTOR activation by glucose or leucine, leading to insulin resistance (103).

As discussed for GCN2, not all AAs stimulate mTORC1 equally. In rodents leucine is a particularly strong activator (77). It has been reported that intracellular leucine is uniquely sensed by leucyl-tRNA synthetase (LeuRS),

which activates the mTORC1 complex (Figure 1.3) (104). Nonetheless, it is now known that Sestrin2 possesses a leucine pocket to bind to, and sense, cytoplasmic leucine levels (105). Some obese animal models deplete their circulating glucogenic AAs, leaving higher circulating sulphur (106) and BCAAs including leucine (15)), which can result in chronic TOR activation. In contrast, a low protein diet decreases circulating BCAAs and mTOR activation (3) and sensitizes animals to AA imbalances (26). Importantly, TOR activation can stimulate the secretion of hunger or satiety hormones in the GI tract and brain, such as ghrelin and leptin, respectively (24), and recent evidence suggests TOR is a mediator of the enteroendocrine hormonal responses to dietary proteins and AAs (107). Therefore a regularly high dietary intake of AAs induces chronic mTOR activation, which is detrimental to health and lifespan, whereas AA imbalances or limitations can inhibit TOR. However, TOR activation with respect to single AA modulations requires further elucidation both in invertebrates and vertebrates.

1.5.5 TOR-independent detection of AA abundance

Multicellular organisms have both intracellular and extracellular AA sensors, as well as neural sensors that respond to the intake of nutrients. Although TORC1 senses endocellular AAs and can stimulate satiety signals, in the GI tract extracellular AA sensors also play a prominent role. At least some mammalian G-protein-coupled receptors (GPCRs) are transceptors. Transceptors are transmembane transporters of nutrients, including AAs, that also act as receptors involved in inducing endocellular signaling. In mammals, GPCR receptors of the T1R family are activated mostly by L-AAs in the digestive tract (108), and even modulate TOR signaling independently of intracellular AA levels (66). Transmembrane GPCRs in enteroendocrine cells can stimulate the release of appetite-regulating incretins or decretins (104, 109). Specifically, high luminal AA concentrations increase secretion of satiety incretin hormone GLP-1 by enterocytes (110). Additionally, in response to food or AA intake, density-, stretch-, and other chemo-receptors in the GI tract release neural signals of satiety to the CNS (55, 111). Cholecystokinin (CCK) is secreted in response to luminal AAs, and CCK receptors stimulate vagal afferent signals to the nucleus of the tractus solitaries (NTS) of the brainstem, which relays signals to the hypothalamus (111). Other secreted peptides by the GI tract or along the splanchnic bed include leptin, insulin, and peptide YY (PYY), which suppress appetite in response to bulk food intake or protein intake, while ghrelin increases it (111) (Figure 1.4). Furthermore, recent evidence strongly supports unique ingested amino acid-specific signaling to the CNS, involving vagal afferents and the area postrema (112). All the above responses have some degree of conservation between invertebrates and vertebrates as similar mechanisms are involved in the fly's intestinal nutrient sensing (70). Thus, neural and hormonal modulations in the gut can function independently of intacellular AA sensing by TOR. In this way, ingested AAs trigger the release of hormones to regulate homeostatic processes in the whole organism (Figure 1.4).

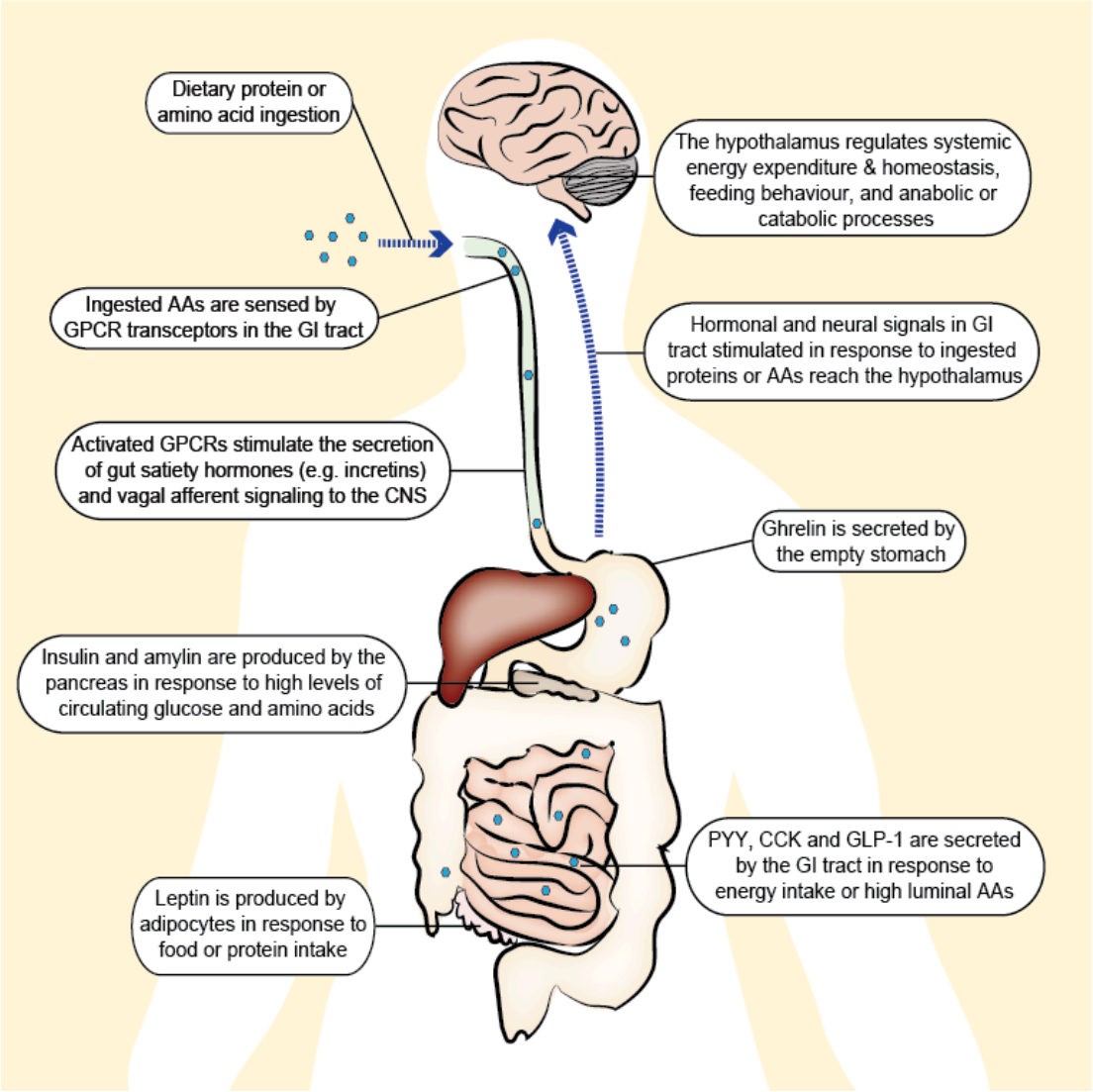


Figure 1.4

Sensing of dietary proteins and AAs along the gastrointestinal (GI) tract. Ingested AAs activate transceptors (e.g. GPCRs) that relay hormonal and neural signals of satiety to the brain. Protein or amino acid intake can also stimulate the secretion of a range of appetite suppressors including PYY, CCK, and GLP-1 (see main text). Such hormonal signals are targeted to the hypothalamus, which regulates a range of systemic and metabolic processes that are associated with homeostasis and health during ageing.

1.5.6 Responses to AA surpluses that affect physiology and ageing

High protein diets increase satiety and decrease food intake in many organisms, including flies (29), mice (2), and humans (23), an effect referred to as 'protein leverage' (2), with the main driver of appetite a target protein intake. Therefore the reduced obesity and insulin resistance of animals and humans fed a high protein diet ad libitum can be explained by their decreased food intake (24). However, dietary AA-induced chronic stimulation of the IIS/TOR pathways is detrimental for health (3, 24, 86, 95, 113). In yeast and worms, AA restrictions can inhibit TOR and extend lifespan (5, 114, 115). Inhibition of TOR by rapamycin or of S6 kinase (S6K), a downstream effector of TOR, also extends fly and rodent lifespan (113). Another main effector of mTOR is the translation repressor 4E-BP, which is activated upon TOR inhibition by DR in flies (113) or methionine-restriction in rodents (116). In humans, high levels of protein or AA intake also result in TOR activation (24) and insulin secretion (23), while excess acidifying AAs or sulphur AAs also raise blood pressure (23). With ageing, mTOR activity in mouse hypothalamic neurons increases, silencing anorexic neurons and contributing to age-related obesity (16). Moreover, TOR function can affect diverse systemic processes, including cell and tissue growth signaling, immune function, proteostasis, neurodegeneration and cognitive function, tissue and stem cell physiology, and others (95, 113). Thus, activation of TOR by high AA intakes can in the long-term be detrimental for health, promoting age-related disease such as neurodegeneration (117).

Although AAs promote growth signaling and TOR, excess AAs can also suppress growth, and therefore growth signaling, through antagonistic interactions. Mechanistically, this may occur if an AA is ingested in amounts that saturate specific AA transporters due to the AA's higher abundance, substrate affinity, or kinetics. In this case, it is possible that in some tissues intracellular levels of out-competed AAs become limited, thereby triggering the AAR to inhibit anabolic processes. Specific examples of moderate additions of AAs inhibiting growth in rodents were discussed earlier. Unpublished data in our laboratory also indicate that moderate additions of AAs can inhibit growth or growth signaling in both flies and mice. Importantly, such inhibitions of growth signaling may also affect health and longevity. In worms, addition of some AAs extends lifespan significantly through TOR inhibition (114). However, the identity of the AA is important, as addition of some AAs in the worm's diet had no effect, or even decreased lifespan drastically (114). In rats, an excess of threonine can be well tolerated but a similar excess of tyrosine can cause pathological lesions (26). Therefore the identity of the AA ingested in excess determines its effects upon health. More life-long studies will further clarify these interactions upon long-term health and ageing.

1.5.7 Convergence of AA sensing pathways

There are many interactions between the multiple nutrient and AA sensing pathways discussed above (83). Phosphorylation of some translation initiation factors by TOR changes their conformation to allow accessibility by other kinases or phosphatases (including GCN2 downstream effectors) (118). Protein synthesis inhibition by GCN2/eIF2α stimulation occurs in conjunction

with mTOR inhibition, and some cancer drugs deplete circulating AAs and trigger GCN2 to decrease mTORC1 signaling (119). In yeast, the AAR pathway is most responsive when mTORC1 is inhibited by rapamycin (76). In worms, there is also a convergence of GCN2 and TOR upon AA limitation towards inhibition of global translation and down-regulation of FOXO transcription factors (120). Stimulation of FOXO transcription factors modulates the life-extending effect of IIS downregulation across species (5, 16). Therefore, the orchestration of these two nutrient sensing pathways (TOR and GCN2) modulates AA sensing, although a detailed characterization of this interaction, especially with respect to individual AAs, remains to be established.

1.5.8 Food aversion, protein leverage, and growth signaling

Because imbalanced protein sources prevent the usage of excess and therefore total AAs, adequate protein intakes can be achieved with lesser amounts of high quality protein than of low quality protein. In order to achieve the target AA intake as driven by protein leverage, a less usable protein will therefore be consumed in greater amounts than a highly usable protein. For instance, whey promotes growth more than does casein, and also induces a higher satiating effect in humans (121, 122). However, imbalanced AA sources can result in deficiencies for specific essential AAs, and so animals must also have protective aversive responses to direct them to alternative, balanced AA sources (27). Thus the motive for increasing the intake of an imbalanced AA diet to achieve a target protein consumption may conflict with the motive to avoid a detrimentally imbalanced AA intake. The thresholds distinguishing between such conflicting motives are unclear, as is the impact of the imbalanced AA's identity on such effects.

Rodents are more sensitive to limited than they are to excess AAs. Very small AA limitations are detectable by rats, representing a 0.009% w/w change in the diet (73). Such limitations are not reflected in the plasma, but are seen in the APC region within 15 minutes of feeding on the imbalanced diet, resulting in loss of appetite (73). In contrast, growth suppression is detectable upon changes that represent >0.1% w/w of the limiting AA in the diet (26). In addition, ad libitum fed rodents on severely AA-limited diets decrease their food intake and growth but, if the animals are made to eat equal amounts, growth returns to normal (26). Therefore, appetite is more malleable in response to ingested AAs than is growth signaling. Moreover, responses to ingested AAs also depend on the identity of the imbalanced AA. Restriction of specific AAs (lysine, threonine, or isoleucine) alters food preference but not food intake in rats (73) . In mice, excess consumption of some AAs (e.g. methionine, tryptophan) suppresses food intake and growth more than does excess intake of others (e.g. threonine) (26). Therefore, the response to an imbalanced AA ingestion depends on the AA identity and on the physiological (e.g. growth) or behavioural (e.g. appetite, food choice) trait assessed. Further understanding of these aspects and interactions will be important in elucidating how AA modulations regulate metabolism and ageing, and in designing nutritional applications for humans.

1.5.9 Distinct bioenergetic and metabolic roles of amino acids

Because of their different molecular structures, free AAs are broken down through distinct biochemical reactions. According to their catabolism, AAs can be glucogenic (all AAs except lysine and leucine), leading to the generation of glucose, or ketogenic (lysine, leucine), resulting in ketone bodies, although some AAs can be both (isoleucine, threonine, phenylalanine, tyrosine, and tryptophan). Glucose and ketones are the body's main energy sources, and cellular energy production from AA catabolism can represent 10-15% of total energy production (23). Importantly, the energy density of glucose is typically lower than that of ketones. Moreover, the energy expenditure for the metabolism of different AAs varies. Glutamate is the most energetically efficient AA (123), which may explain the central role of glutamate in providing TCA cycle precursors (67). Each AA also has a different metabolic efficiency for anaplerotic reactions, i.e. reactions that produce TCA cycle intermediates from precursors including AAs. Therefore, the metabolism of specific AA can uniquely affect energy homeostasis, which may impact on ageing. In worms, dietary supplementation with the ketogenic beta□hydroxybutyrate (114), the ketone derivative α-ketoglutate (124), or with several TCA cycle metabolites extends lifespan (114). This longevity gain in worms is thought to be mediated by anaplerotic reactions (114, 124). The energy sensor AMPK senses and modulates the metabolic and energy homeostasis changes of these long-lived worms, and the DAF-16/FOXO pathway is also activated by higher levels of TCA cycle intermediates (114).

The ketogenic or glucogenic potential of ingested AAs may also affect long-term health and ageing in rodents and humans. In mice, highly ketogenic diets reduce the catabolism of ketogenic AAs to prevent further ketogenesis, but do not alter lifespan (12). However, a modest increase in the intake of ketogenic compounds may be beneficial for mouse lifespan. Increasing the intake of the ketogenic AA leucine contributes to the mouse lifespan extension by BCAA supplementation (125), while in a mouse cancer model two different ketogenic compounds, butanediol and ketone ester, significantly increased survival independently of DR (126). In humans, ketogenic or leucine-supplemented diets may decrease food intake, adiposity, insulin resistance, sarcopenia, and cognitive deterioration with age (127-130). However, the TCA cycle is amphibolic, i.e. it is both anaplerotic and cataplerotic. This makes it difficult to quantify its bioenergetic modulations upon intake of different AAs. Therefore ketogenesis, TCA metabolite levels, and energy flux are coordinated by different AA ingestions to induce health and longevity gains across species. However, more investigations are needed to further elucidate how AA catabolism impacts on health and ageing through such modulations.

1.5.10 Health biomarkers of specific AA-imbalances

The AA profile of a dietary protein is generally the primary determinant of the protein's nutritional value. Several studies have identified effects of different dietary proteins with distinct AA profiles upon health and ageing. Soy and whey proteins improve a range of health markers and longevity, including increased insulin sensitivity and reduced adiposity (Table 1.1). In contrast, milk or casein proteins increase circulating IGF-I, insulin, and satiety

hormones compared to other protein sources, and such chronic IIS over-stimulation can be detrimental for ageing (Table 1.1). The molecular mechanisms mediating the effects of such different protein sources implicate their AA contents. Soy (Figure 1.5A) and whey (Figure 1.5B) proteins are low in methionine and tryptophan content (1, 131), while casein has a higher methionine content than soy protein (Figure 1.5C). Tryptophan (132) and methionine (13) promote growth hormone (GH) secretion, so diets with lower levels of these AAs decrease IGF-I, thereby promoting long-term health (131). Whey protein is also high in the BCAAs leucine and isoleucine (Figure 1.5B), which may explain its growth-promoting and appetite-suppressing effects in animals (122), and its prevention of muscle loss in older humans (129). Egg protein is a high-quality protein for growth (122), but is not necessarily optimal for long-term health as it causes high postprandial levels of circulating glucose accompanied by a low appetite suppression effect (121). Therefore specific protein sources with distinct AA profiles can down-regulate IIS and increase healthspan and longevity.

Other endocrine modulations involve thyroid hormones, with soy protein lowering parathyroid (PTH) hormone secretion (Table 1.1), which in humans is linked to BMI and mortality, at least under some pathological conditions. Along with growth hormones, secretion of thyroid hormones is also reduced by DR (6) and tryptophan restriction (14). In mice, increased plasma levels of BCAAs are associated with decreased lifespan (3), but high dietary BCAAs have also extended lifespan presumably through different protective mechanisms (125) that require more detailed investigation. In humans, increased plasma BCAAs are linked to insulin resistance and type 2 diabetes (133).

Fish protein is also linked to human health benefits, including increased insulin sensitivity and reduced circulating low-density lipoproteins (Table 1.1) (23, 24). A comparison of the AA content in >10 fish species shows that the two most limiting AAs in fish are tryptophan and methionine (Figure 1.5d), with cysteine as the most limiting non-EAA. Some long-lived human populations, like Okinawans, Sardinians, or Ikarians (134) are located in areas were fish is a predominant protein source (135, 136). Thus it is tempting to draw a link between the reduced sulphur AAs and tryptophan and the insulin sensitivity and lifespan-extension observed in these populations.

In humans, several cohort studies show that high intakes of animal protein, which is typically methionine-rich, are positively associated with chronic and age-related disease, and this association is abolished when the dietary protein source is plant-based (11). However, the age of an individual also determines the health response to the ingested protein (11), as discussed below.

The bioenergetics of different AA sources may also contribute to health effects. The metabolic efficiency of different dietary proteins integrates their AA composition and the energy used in the catabolism of each AA to produce one ATP molecule (123). A comparison of the metabolic efficiency of different

Species examined	Physiological effects observed	Measured effect on health and/or lifespan	Reference(s)*
Homo sapiens	Increase in circulating IGF levels, mTORC1 activation, increase in circulating AAs and in growth rate, reduced insulin sensitivity, changes in calcium signaling	Negative (milk and dairy protein)	Crowe et al. 2009, Melnik et al. 2013, Tucker et al. 2015
Homo sapiens	Reduced plasma lipids and adipocity, reduced prostate and breast cancer, and improved insulin and glucose homeostasis	Positive (soy)	Tremblay et al. 2007
Homo sapiens	Compared to animal meat, soy protein decreased protein synthesis, protein oxidation, energy expenditure, and thermogenesis.	n.d.	Westerterp-Plantenga et al. 2009
Homo sapiens	Whey increased circulating GLP-1 levels, thereby increasing satiety	n.d.	Westerterp-Plantenga et al. 2009
Ratus norvegicus	Soy reduced serum parathyroid hormone and nephropathy	Positive (soy)	Kalu et al. 1988
Homo sapiens	Fish protein increases insulin sensitivity, reduces plasma LD and increases HDLs	Positive (fish)	Tremblay et al. 2007
Ratus norvegicus	Rats fed whey protein had reduced plasma and liver cholesterols	Positive (whey)	Zhang et al. 1993
Mus musculus	Mean lifespan increase, increased liver and heart glutathione levels, increased insulin sensitivity	Positive (whey)	Bounous et al. 1989, Shertzer 2011
Homo sapiens	Reduction in adipocity, increase in muscle protein, protection from high blood pressure	Positive (whey)	Coker et al. 2012, Jakubowicz 2013
Ratus norvegicus	Casein increased circulating IGF levels	Negative (casein)	Noguchi 2000
Homo sapiens	Plant proteins decrease circulating IGF-I levels compared to animal proteins, and increase the IGF-I inhibitor IGFBP-3	Negative (plant and soy protein)	McCarty et al. 2009, O'Neill 2010
Homo sapiens	Whey increased satiety, decreased post-prandial levels of circulating glucose, and decreased subsequent food intake compared to egg and turkey proteins	Positive (whey)	Pal 2010

Table 1.1

List of findings relating dietary protein sources to healthspan and lifespan, including effects upon the IIS and TOR pathways, and on circulating metabolites linked to health-related parameters(23, 24, 30, 121, 130-132, 137-144).

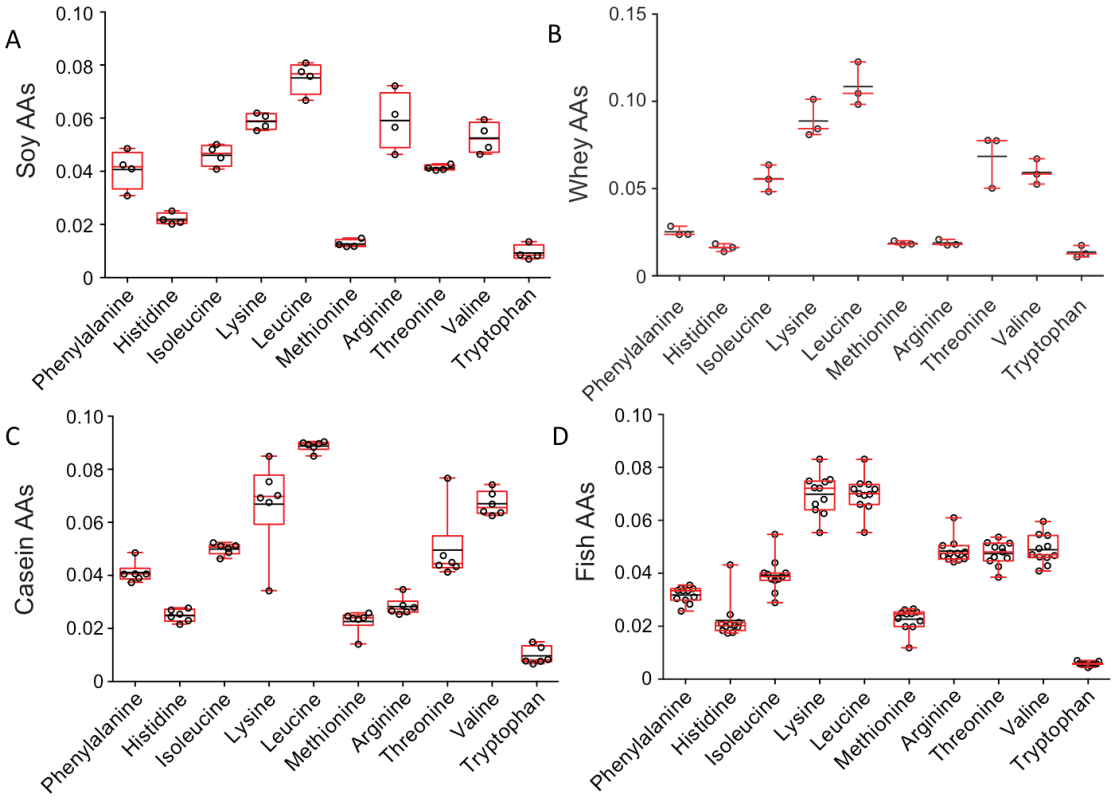


Figure 1.5

A) Four published soy EAA profiles show considerable batch-to-batch variability and low levels of tryptophan and methionine. Molar proportions are shown.

B) The essential AA proportional representation in three published whey profiles. Five of the essential AAs, including methionine and tryptophan, are particularly low, but leucine is higher than in soy, fish, or casein proteins.

C) Six bovine casein EAA profiles indicate differences between casein sources within the same species (*Bos taurus*), low contents of tryptophan, and higher levels of methionine compared to whey or soy proteins.

D) An analysis of the AA profile of ten teleost fish species indicates limitations in tryptophan, histidine, and methionine. However, due to the severe limitation in cysteine, the limitation of methionine likely surpasses that of histidine, making methionine the second most limiting AA. Also, a lower leucine level is seen compared to soy, whey, or casein proteins.

dietary proteins shows that proteins linked to beneficial effects for healthspan and lifespan in animals and humans (Table 1.1) tend to have an essential AA profile that has a higher metabolic efficiency (Figure 1.6a). The calculated % energy efficiency is lower for lactalbumin, egg, and casein, and higher for soy and fish proteins. Therefore it is possible, although not yet established, that a link between metabolic efficiency of AA catabolic reactions and health exists.

Interestingly, in the two recent DR primate studies, the WNPRC diet had higher contents of tryptophan and BCAAs (lactalbumin) than the NIA diet (fish-soybean-wheat-corn-alfalfa) (Figure 1.6b). Milk and dairy proteins such as lactalbumin can induce TOR activation and insulin resistance in humans (24, 132). Thus, AA intake differences between the two studies could contribute to differences in mortality and cancer incidence, as a higher intake of BCAAs and tryptophan could have lead to a chronically higher TOR/IIS stimulation (5).

In conclusion, a number of observations suggests an important role of dietary AA intake upon health and ageing in humans.

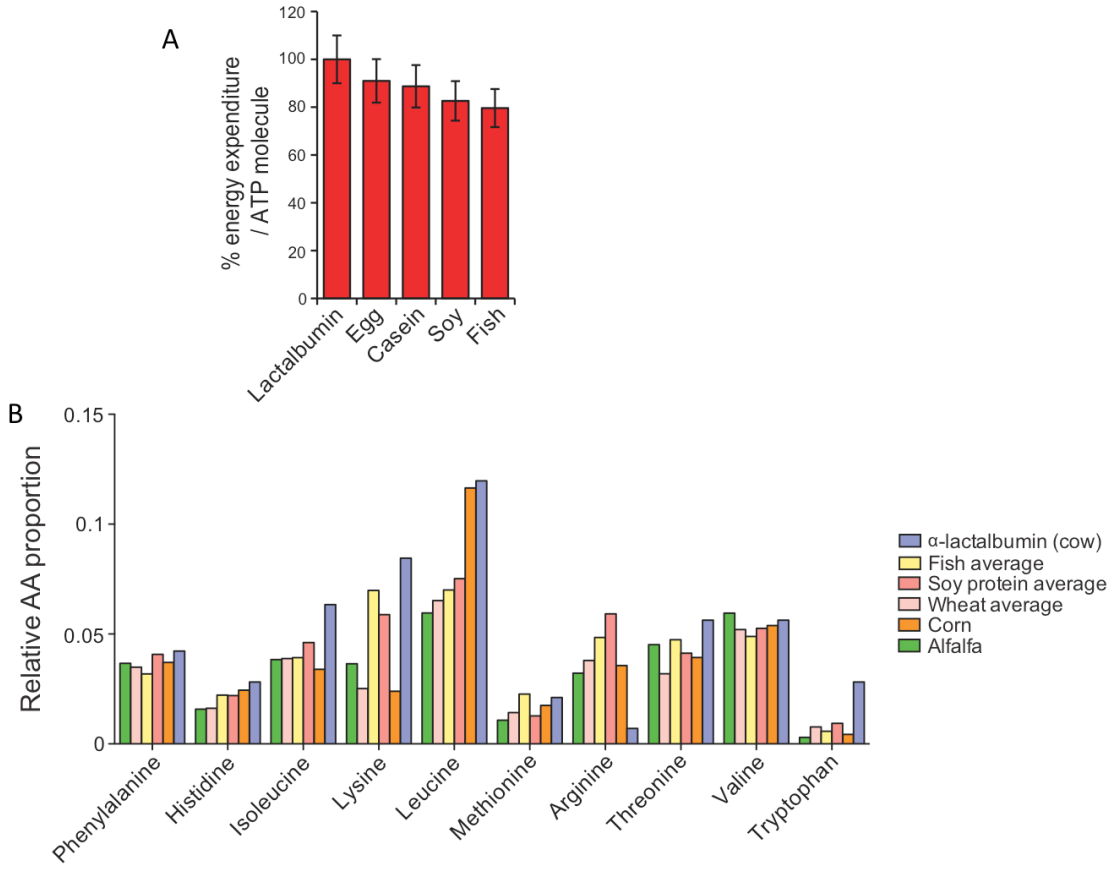


Figure 1.6

A) The calculated % energy expenditure for the production of one ATP molecule based on non-integral P/O ratios (Milgen 2002), shown for the essential AA composition of five common types of dietary protein: lactalbumin, egg, casein, soy, and fish. Differences are due to the range of carbon chain and cofactors that result from essential AA catabolism. Some protein sources associated with health benefits (Table 1.1) appear to have a proportionally higher metabolic efficiency than proteins associated with detrimental effects.

B) Comparison of the mean EAA content (from published data) of the six protein sources used in the two primate studies (see main text for discussion). Lactalbumin has a particularly high content in tryptophan and isoleucine, as well as leucine.

1.6 Optimal amino acid intake

1.6.1 Variation in requirement for amino acids.

The AA needs of individuals, populations, and species, are influenced dynamically by internal state and environmental factors. The main sources leading to variation in AA requirements are discussed here.

Although not much studied beyond inborn errors of metabolism, genetic background across and within species impacts greatly on the response to AA consumption. Inter-strain variability for the requirement of some AAs (e.g. glycine) has been shown to be significant in Drosophila (145). In rodents and humans, genetic background profoundly affects body size, AA requirements, and food intake (4, 28). Wild strains of worms and flies live longer upon DR (146), and mice of different strains respond diversely to a single DR regime (147). The genetic background may also contribute to differences in the DR response observed in the recent primate studies (5). Single DR regimes do not indicate the response of a mouse strain across different restriction levels (2), but do suggest that genetic constitution profoundly impacts on the response to reduced intake of nutrients including AAs. Some recent approaches assess single nucleotide polymorphisms (SNPs) to identify specific genes and to explain how genetic variation in inbred mouse populations determines traits of interest (148). Similar approaches could be employed to evaluate the role of genetic determinants in the DR response, both in rodents and in humans. Such information will inform our understanding of how natural genetic variation predisposes the DR response both in model organisms and in humans, and even aid the design of individualized nutritional interventions.

All growing or reproducing mammals, including humans, have higher AA requirements than adults (25, 28). Consequently young children are more susceptible to protein malnutrition and related diseases such as Kwashiorkor. For laboratory animals there is a clear distinction between diets optimized for breeding or growth stages versus diets optimized for long-term maintenance (28). Adjusting the dietary protein supply to match AA requirements with age promotes health and longevity. Providing high dietary protein to young animals and lower to mature ones extends lifespan in rats (9, 149) and mice (6). In rodents, protein absorption reduces with age as older rats show a decreased ability to digest proteins and AAs (150). Moreover, mature rodents fail to show some of the adverse effects of ingesting AA-imbalanced diets (26, 27). Accordingly, BCAA stimulation of the IIS/TOR pathways is greater in younger, not older, animals (84). Therefore it is not surprising that early onset DR extends rodent lifespan significantly (151), but late onset DR is less effective (6). The protein source during early life also impacts on health during ageing. Although milk protein can chronically over-stimulate IIS and contribute to insulin resistance (Table 1.1), restriction of milk-protein during weaning only can significantly increase mouse lifespan (152). Requirements for AAs may also change qualitatively as an animal physically matures. Some evidence in mice suggest subtle changes in the body's AA composition with development (153). In humans, a low protein intake appears to benefit groups of 50-65

years of age, but may be detrimental when applied to older ages (11). Therefore, it is clear that age and life stage can affect both the requirement for AAs, and the response to AA intakes.

The EAA requirements of individual cells or tissues can vary depending on tissue-specific AA metabolism. For example, enterocytes secrete threonine-rich proteins, so require a higher threonine intake than other cells (48, 56). Hepatocytes require high levels of methionine to serve many transulfation, transmethylation, and folate metabolism reactions (56). The type and abundance of AA transporters also determines which AAs enter readily into which cells. These aspects require further study in conjunction with more systematic analysis of tissue-specific usage of individual AAs.

In humans, gender defines AA requirements as males require more AAs than non-pregnant females, which reflects body size differences to an extent (4, 25). Beyond inborn errors of metabolism, the maintenance of health requires adequate AA supply, as multiple immunological processes depend on AAs, and an imbalanced AA intake can suppress the immune system (21). Indeed, the efficiency of the immune response declines and the susceptibility to infections increases upon low AA intake (4). Thus, it is possible that infectious or disease conditions that increase the function of immunological processes may raise dietary AA requirements (21). In the future, more work is required to understand how specific disease states increase the requirement for specific AAs.

In addition, healthy physical activity increases the metabolic rate and promotes protein degradation, AA oxidation, and depression of protein synthesis in humans, thereby increasing AA requirements (4). The metabolic rate of individuals can also be modulated by environmental conditions, as lower temperatures can increase the metabolic rate in endothermic animals (4). Similarly, seasonal increases in day cycle duration can promote physical activity, thereby increasing the metabolic rate particularly at younger ages (4). Such increases in metabolic rate also translate in increases in AA requirements.

In summary, numerous findings from different branches of nutritional research clearly indicate that both environmental and internal state factors must be considered when estimating of AA requirements.

1.7 Conclusions

Identifying beneficial AA intakes can lead to improvements in human nutrition. In human populations, health benefits for older age groups mirror most mortality gains, and late life dietary interventions based on AA intake are beneficial (11). As AA intakes are critical to the DR response, dietary AAs provide a powerful intervention strategy for human health. Indeed, recent evidence shows that a fasting mimicking diet based on a limited plant-based AA intake benefits human health (18). Such dietary manipulations comprise a drug-free way of intervening towards healthy ageing. Moreover, nutritional efficiency can have diverse applications within our societies, as it can help to end starvation, to devise tools against obesity and disease, to enhance produce yield in the food industry, and to assist patients in numerous clinical applications including cancer.

1.8 Outstanding questions addressed in the current thesis

The work described in this thesis addresses some of the unresolved issues in the field of dietary protein and amino acids, metabolism, and ageing. More specifically, the following questions are addressed:

1. What defines dietary AA requirements - can they be approximated by the organism's exome AA usage? To answer this question several anabolic traits were assessed in flies and mice including developmental rate and viability, growth rate, fecundity, and lean mass accretion (Chapters 3 & 4).

2. Is the exome a better measure of AA requirements than other, commonly used measures of estimating AA needs, than commonly used AA sources, or than the weighted whole body proteome? A comparison of several historically accepted measures of defining AA requirements, as well as a direct comparison of the exome versus the weighted proteome AA profiles, was carried out focusing on anabolic traits both in flies and mice (Chapters 3 & 4).

3. Can the exome AA supply be used to quantitatively predict physiometabolic phenotypes? To address this point, models were designed for the prediction of the limiting dietary AA in exome-mismatched diets, and their accuracy in predicting an anabolic trait response (notably a fecundity response in flies) was examined (Chapters 3 & 4).

4. What are the behavioural and physiometabolic consequences of matching the dietary AA supply to the exome, against commonly used (exome-mismatched) dietary AA supplies? To address this point, several bahavioural, physiological and biochemical aspects were assessed in both flies and mice. These included food preference, food intake, fat metabolism, nutrient sensing, proteostasis, respiration rate,

glucose and glycogen homeostasis, nitrogen balance, bone mineral density, and others (Chapters 3 & 4).

5. Do any physiometabolic effects of an exome-matched AA supply affect ageing? To address this point, survival analyses were carried out in flies comparing the exome-matched diet to a range of exome-mismatched diets (Chapter 3).

1.9 Summary points list

1. Even with adequate intake of macronutrients, the protein and amino acid content of the diet are critical for health during ageing

2. An imbalanced supply of amino acids occurs when the requirement for dietary amino acids, usually determined by their effects on anabolic traits, is not matched by their intake

3. This requirement is affected by multiple factors including genetic diversity, gender, age, and health status

4. Amino acid absorption and availability is determined by the gut microbiota, the amino acid's identity, and first pass metabolism

5. Ingested and systemically available AAs are sensed by various mechanisms, involving TOR, GCN2, GPCRs, and other sensors

6. Excess intakes of amino acids can over-stimulate growth signaling, which can be chronically detrimental and decrease longevity

7. Limiting or imbalanced intakes of amino acids can down-regulate growth signaling, inducing a maintenance mode

8. Experimental animal models can inform human nutrition, increasing our understanding of how AA intakes affect human health and ageing

9. This thesis addresses several unresolved questions around dietary AA requirements, and the effect of a diet upon behavior, physiology, metabolism, and ageing

Chapter 2: Materials and Methods

2.1 Materials and methods for fly experiments

2.1.1 Media and flies

In all experiments except otherwise indicated, we used our laboratory stock of outbred wild-type D. melanogaster, Dahomey. Flies were maintained in large population cages or in multiple glass bottles with overlapping generations at 25 °C with a 12 h:12 h light:dark cycle. Flies wDah; Df(3L)ilp2-31, ilp5 deletion mutants (lacking (ilp2, ilp3 and ilp5) and Wolbachia-positive white Dahomey (wDah) controls are those previously reported (154). Dilp flies were backcrossed into the genetic background of their control for at least six generations prior to experiments.

For all experiments using adult flies, other than specified development assays, flies were reared on sugar, yeast food (155). Holidic media were prepared as previously described (156).

To generate age-synchronized adult flies, larvae were allowed to develop on SY food at standard density, transferred to fresh SY food upon emerging as adults and allowed 48 h to mate. Under light CO^2 anesthesia, females were separated from males and allocated to treatment vials at a density of ten flies per vial. Flies were transferred to fresh vials three times per week at which point deaths and censors were scored. Egg-laying was scored after flies occupied vials for ~18 h, and the value was expressed as the number of eggs per vial per female.

2.1.2 AA ratio calculations and diet design

We account for deficiencies in the conditionally EAAs, tyrosine and cysteine, by reducing the molar availability of their precursor essential amino acids, phenylalanine and methionine respectively, by one mole per mole of amino acid required. Methionine (M) is typically limiting in casein, however the batch used in our analysis was particularly limiting in tryptophan (W), another common limiting EAA in casein (157), thus making W as limiting as M in this ratio, when accounting for casein's cysteine deficiency using our above conversion. The soy protein composition was calculated from several reported soy protein compositions (158-161).

In flies, the whole larval body ratio was adopted from previously published data (162). The second instar larval ratio (L2AA) was selected for this comparison rather than the third instar larval ratio as the later was more limiting for tryptophan (rW(min)=0.026) than the former (rW(min)=0.031).

To enable comparisons, all diets were isocaloric, with constant micro- and macro- nutrient compositions (except for AAs), isonitrogenous, and with constant total mass and total moles of amino acids. Moreover, as under normal conditions in eukaryotes AA catabolism accounts for 10-15% of total energy production in the cell, we also considered that each AA's utilization towards ATP formation requires greatly different energy expenditures due to the variety of intermediate metabolic steps for each AA's catabolism (23). Thus we also ensured equal metabolic efficiency values across all tested ratios on the basis of recent biochemical data on energy expenditure of nonintegral P/O ratios (123). Therefore MouseAA is 1% less energetically

efficient than mSILACAA, and 2% less efficient than the mMMAA and the NRCAA, while it is 1% more efficient than CaseiAA.
To predict the most limiting essential amino acid in a diet, the proportion of amino acids in the food was divided by the proportional representation of amino acids in the translated exome of the consumer. The essential amino acid with the lowest value after this transformation was considered limiting. If the requirement for either of the conditionally essential amino acids tyrosine or cysteine exceeded the available supply, their requirement was met by subtracting a mole of phenylalanine or methionine, respectively, for each mole of amino acid to be synthesised. To design MMAA, the Euclidian distance from HuntAA to FlyAA in 20 dimensional space (1 dimension per amino acid) was determined. Another point, MMAA, that the found that was equidistant from FlyAA as HuntAA, but as far away as possible from HuntAA.

2.1.3 SILAC proteome amino acid usage

Using the weighted amino acid composition of set of proteins expressed in a tissue or organism (the proteome) would provide a better approximation of its amino acid content and provide more data for diet optimization. In this line, we used published mass spectrometry based proteomic data (163) in order to calculate protein copy numbers and the corresponding weighted amino acid composition. In order to calculate the protein copy numbers we used the Total Protein Approach, a label free quantification method which relies on the proportionality between protein abundance and its mass spectrometric signal (164). This method was initially developed for the quantitative analysis of cancer tissues and was shown to perform well in the calculation of protein copy numbers of label free or stable isotope labeled standard proteins (165). The raw data from published proteomic studies on fly (163) and mouse (166) was reanalyzed. Protein identification was carried out using MaxQuant (167) version 1.4.1.2 using the integrated Andromeda search engine (168). For fly, the raw data were searched against the gene translations fasta database (release FB2008_05), downloaded from Flybase, ftp://ftp.flybase.net/releases/FB2008_05/dmel_r5.8/fasta/dmel-all-translation-r5.8.fasta.gz, 21,070 entries. For mouse, the gene translations fasta database (release 54) was downloaded from Ensembl, ftp://ftp.ensembl.org/pub/release-54/fasta/mus_musculus/pep/Mus_musculus.NCBIM37.54.pep.all.fa.gz, 40732 entries. For the protein identification from the raw proteomics data, the fasta databases were automatically complemented with sequences of contaminating proteins by MaxQuant. For peptide identification, cysteine carbamidomethylation was set to “fixed” and methionine oxidation and protein N-terminal acetylation as “variable” modifications. For *in silico* digestion the enzyme was set to “LysC/P” allowing for cleavage after lysine, also when followed by proline with a maximum of two missed cleavages. The minimum number of peptides and razor peptides for protein identification was 1; the minimum number of unique peptides was 0. Protein and peptide identification was performed false discovery rate (FDR) of 0.01. The “second peptide” option was on allowing for the identification of co-fragmented peptides. In order to transfer identifications to non-sequenced or non-identified peptides in the separate raw files, the option “Match between runs” was turned on using a

“Match time window” of 1 min and “Alignment time window” of 20 min. Protein copy numbers per 1g of total protein were derived using the label free quantification based total protein approach (TPA) (164, 165). Protein copy numbers were calculated from the protein intensity data from MaxQuant’s ProteinGroups.txt output file. As MaxQuant reports identified proteins in groups when the presence of individual protein sequences cannot be unambiguously inferred, only the first protein ID in a protein group was used. For each mouse tissue or whole fly experiment only proteins that had recorded mass spectrometric intensity more than zero were used for analysis. For the whole fly data, the combined (heavy and the light SILAC) signal from the proteins was used. For the mouse data, only the light SILAC signal was used. Protein copy numbers (per 1g of total protein) were calculated by dividing the protein intensity values by the total intensity of all identified proteins. This value, which corresponds to protein concentration measured in mol per gram total protein, was divided by the protein MW arriving at protein copy numbers per 1g of total protein. Next, the protein copy number was multiplied by the number of each of the 20 amino acids arriving at the total amino acid numbers for each protein (data not shown). These latter values were used to calculate the total amino acid quantities for a mouse tissue or a whole fly and the respective weighted amino acid proportions (data not shown).

2.1.4 Measuring development, body mass, egg laying and lifespan.

For development assays, young age-matched flies were allowed to lay eggs on grape juice plates overnight. 24 h later, first instar larvae were picked onto holidic media, pre-warmed at 25 °C. The eclosion time of adults was scored daily at 24-hour intervals, and viability was calculated at the end of the experiment as the % of larvae eclosing. Approximately 50% of all eclosions were male and 50% female. For the body mass measurement in flies, approximately 20 pairs of 10-day old flies per treatment were allocated into 0,5ml Eppendorf tubes. Body mass was measured with the use of a microscale (Sartorius ME235S genius balance). Developmental viability was defined as the proportion of picked eggs eclosing successfully.
For lifespan assays, we generated age-synchronized adult flies: larvae were allowed to develop on SY food at standard density, transferred to fresh SY food upon emerging as adults and allowed 48 h to mate. Under light CO2 anesthesia, females were separated from males and allocated to treatment vials at a density of ten flies per vial. Flies were transferred to fresh vials three times per week at which point deaths and censors were scored. Egg-laying was scored after flies occupied vials for ~18 h, and the value was expressed as the number of eggs per vial per female. For mortality analyses, 1000 flies were allocated into 10 bottles of 100 flies each, and flies were transferred into fresh bottles three times per week as for vials.

2.1.5 Fly tissue dissection

Flies treated in the respective diets were immobilized on ice after 8 days of treatment (10 days of adult age) unless otherwise stated. Head and thorax, abdomen, fat body and digestive tracts of *Drosophila* were manually dissected in PBS with the use of fine forceps.

2.1.6 Fly protein extraction

10-day old flies treated in the respective diets were homogenized in 300 µl of RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF) and centrifuged for 5 min at 13,000 rpm (4°C). The supernatant was collected as the protein fraction.

2.1.7 BCA Protein Assay

The bicinchoninic acid (BCA) assay (Thermo Scientific, PierceTM BCA Protein Assay Kit 23225) was used for protein measurements. 25µl of fly homogenate (the number of flies/tissues used for each experiment indicated in captions) as well as 8 dillutions (0, 125, 1250, 500, 750, 1000, 1500 and 2000µg/ml) of protein standard (BSA) to plot the standard curve were transferred to a 96-well plate. Absorbance was measured at 562nm.

2.1.8 Western blot

Extracted protein was subjected to Tris/Glycine/SDS polyacrylamide electrophoresis and transferred to nitrocellulose membranes using a criterionTM blotter (Bio-Rad) for 1 h at 100 V. After blocking with TBST milk (50 mM Tris, 150 mM NaCl, 0.05% Tween20, 5% milk powder) the membrane was incubated with primary and subsequently secondary antibodies. The list of utilized primary and secondary antibodies indicated in the table below. An ECL (Enhanced Chemi-Luminescence) reaction was performed using Amersham ECL Select Western Blotting Detection Reagent (RPN2235, GE Healthcare Life Sciences). Imaging was performed using the ChemiDoc MP System, Biorad.

Antibody name	Company, product nr.	Origin	Concentration
Atg8	Custom-made	Rabbit	1:1 000
eEF2	CST 2332S	Rabbit	1:1 000
P-eEF2	CST 2331S	Rabbit	1:1 000
nonP-4E-BP1	CST 4923	Rabbit	1:1 000
P-4E-BP1	CST 2855S	Rabbit	1:1 000
LC3	MBL PM036	Rabbit	1:1 000
20S alpha proteasome subunit	Santa Cruz Biotech sc-65755	Mouse	1:1 000
Ref2P	Abcam ab178440	Rabbit	1:1 000
K48-linkage Specific Polyubiquitin	CST 4289	Rabbit	1:1 000
Alpha Tubulin	Sigma T9026	Mouse	1:1 000
VDAC	Merck Millipore MABN504	Mouse	1:1 000
anti-Mouse IgG (H+L) Secondary Antibody, HRP conjugate	G21040, Life Technologies	Goat	1:10 000
anti-Rabbit IgG (H+L) Secondary Antibody, HRP conjugate	G21234, Life Technologies	Goat	1: 10 000

Table 2.1: list of primary and secondary antibodies used in this study

2.1.9 Proboscis Extension Behaviour (PEB)

PEB assays were set up with 5-10 females per vial, for 10 vials (50-100 flies) per treatment, as previously reported (11). The flies were observed on several days for a period of 90 minutes, with feeding events recorded every 10 mins. Each assay was repeated >3 times on mated females every 2-3 days from day 3 to day 10 of age. The assay was carried out in a standard conditions behavior assay chamber (25°C / 65% humidity). Data points shown represent distinct biological replicates, each representing the mean of more than three 90-minute average values per treament at ages 3-10 days.

2.1.10 Blue dye assay

Food intake across the dietary AA ratios was quantified using blue-stained holidic media as previously described (169). For each assay, 50 mated female flies were placed in vials of 5-10 flies for each treatment. PEB assays were carried out, and at 10 days of age, following 2 days mating in 1SY media and 8 days on the respective holidic diet, the PEB-flies were used in the Blue Dye Assay. Flies were transferred into fresh vials containing Erioglaucine disodium salt (EDS) blue dye for exactly 30 minutes. Flies were then frozen and homogenized, and absorbance of the homogenate was measured at 629 nm along standards as previously described (169). Data points shown represent distinct biological replicates, each representing the mean of a trial of 50-100 flies per treament, at age 10 days.

2.1.11 Dietary preference assay

The apparatus used for the dietary preference assays is a modified version of that described in (170), which enables the experimental flies to choose between 4 vials containing a medium. We used a prototype scaled-down version of the chamber for 40 flies (we thank Wilfried Haider from the Max Planck Institute for Metabolic Research, who designed and constructed the chambers), and tested diet preference, population effects and time-monitoring to determine the experimentally optimal conditions. To avoid side-bias, the chamber was enclosed in a polysterene container, which only allowed diffuse light to enter the chamber, precluding light-dependent behaviour and eliminating spatial or other environmental cues (including temperature variations), while experiments were also reproduced with the chamber rotated hourly by 90°. Each assay differentiated between two diets only, each set placed diagonally. All the media used for the food choice assay were prepared according to recently described holidic medium recipies (156). All media contained the same quantities of all other nutrients (sugar, cholesterol, vitamins, ions, nucleic acids, etc. (156).

The dietary preference assay was carried out in female flies only. *Drosophila* undergoes a dietary switch after mating leading to increased preference for protein-enriched diets (171). Therefore, after emergence, females were mated for two days and were then treated with a diet lacking all AAs (0N) for 3 days. Following this, 5 day-old females were used for assessing dietary preference. Flies were sorted under light CO_2 anesthesia, and were placed in the apparatus. Each assay used 40 females, and flies where acclimatized in the chamber and allowed time to settle on the diet of their choice overnight for

approximately 14 hours before the start of preference measurements. This amount of time (14 hours) was previously found to be adequate for the detection of dietary preferences in females in this set up (unpublished data). To avoid any disturbances in the feeding behavior, measurements started in the morning and readings were taken hourly for ~8 hours. A strong positive correlation has been established in our set-up between the number of flies in a food surface and the number of flies feeding (as determined by proboscis extension behavior, data not shown). The feeding rate was quantified as the number of flies having a direct contact with the food surface as follows: DPI = (nr. of flies on surface of food A – nr. of flies on surface of food B) / (nr. of flies on surface of food A + nr. of flies on surface of food B). The DPI was calculated at each of the ~8 time points, and the mean DPI was calculated as the mean of these 8 values (the proportion of flies feeding at each time point remained constant; data not shown). We took the mean DPI to represent one biological replicate, and this value was combined to the mean DPIs of 5-10 independent assays, representing 5-10 biological replicates, for each experiment.

2.1.12 Triacylglyceride

Flies (25 flies per diet) were anesthetized using CO_2, homogenized (5 flies per MP Biomedical Lysing Matrix D tube) in 1 ml 0.05% Tween 20 lysis solution, heated at 70°C for 5 min, and centrifuged at 14,000 rpm for 5 min. Supernatant (50 µl) from each sample was then transferred to a 96-well plate, and 200 µl of Thermo Infinity Triglyceride solution (pre-warmed at 37°C) was added to each well. Samples were incubated away from light for 5 mins, and absorbance was measured at 540 nm. Whole-body TAG levels were measured in adult flies (10 days age / 8 days treatment) and normalized to total protein levels (25 flies per group).

2.1.13 S35-labelled methionine incorporation assay

Heads and thoraxes (5 tissues/sample) were dissected in DMEM medium (Ginco 41965-039). The medium was removed and tissues were washed with methionine- and cysteine- free DMEM, and subsequently incubated in methionine- and cysteine- free medium for 30 min to deplete intracellular methionine. 40µl/ml ^{35}S-methionine/cysteine was added to 200µl of methionine/cysteine-free DMEM. After 30 min incubation on a slow shaker, tissues were washed with PBS, centrifuged for 2 min at 13,000rpm and lysed in 24µl RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% Nadeoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF) with addition of protease inhibitor for 15 min on ice. Samples were centrifuged at 13,000rpm for 10 min. Protein concentration was determined with the use of BCA assay. Subsequently, samples were subjected to Tris/Glycine/SDS electrophoresis as described for Western blots. The gel was stained in Coomassie Blue for 30 min, incubated in 40% MeOH, 10% glacial acetic acid for 40 min, dried and exposed to film.

2.1.14 Proteotoxic stress assay

At least 80 female flies (at lest 8 replicates, 10 flies each allocated into glass vials) were sorted to the respective diets after mating for 2 days in 1SY bottles following emergence. Flies were pre-treated for 8 days with holidic diets of the

respective AA ratios, and then transferred to the same diet supplemented with Bortezomib 15 μM (PS-341; S1013 Selleckchem), proteasome inhibitor. Stress resistance was quantified by regularly scoring dead flies and plotted as survival curve for each treatment.

2.1.15 Mitochondrial Respiration

Female flies (10 days old) were dissected in PBS and tissues were collected (n=2, 8, 15 and 20 per replicate for thoraces, fat bodies, heads and guts, respectively). Tissues were resuspended in the respiration buffer (20 mM sucrose, 50 mM KCl, 20 mM Tris-HCl, 4 mM KH2PO4, 2 mM MgCl2, 1 mM EGTA, 0.05% BSA, 0.01% digitonin, pH 7.2). Oxygen flux was measured at 25°C using a high-resolution respirometry device called OROBOROS Oxygraph 2K. Complex-I driven respiration (PGMP3) was measured by adding substrates Pyruvate (10mM), Glutamate (5mM), Malate (5mM) and Proline (10mM), along with ADP (1.25mM) (state 3). Un-phosphorylated state (PGMP4) of respiration was measured by inhibiting complex-V activity using Oligomycin. The respiration (state PGMPc) was uncoupled using CCCP (0.3uM). Maximum flux (Max. flux) was measured by adding complex-II substrates Glycerol-3 phosphate (5mM) and Succinate (10mM). Rotenone sensitive flux (RS) was measured in presence of 3uM rotenone. Finally, dry weights of tissues were determined and normalized to the oxygen consumption flux.

2.1.16 Polyribosome profiling

Gradients of 17–50% sucrose (11 ml) in gradient buffer (110 mM KAc, 20 mM MgAc2 and 10 mM HEPES pH 7.6) were prepared in thin-walled, 13.2 ml, polyallomer 14 × 89 mm centrifuge tubes (Beckman-Coulter, USA). Heads and thoraxes were lysed in 500 μl polysome lysis buffer (gradient buffer containing 100 mM KCl, 10 mM MgCl2, 0.1% NP-40, 2 mM DTT and 40 U/ml RNasin; Promega, Leiden, Netherlands) using a dounce homogenizer. Samples were centrifuged at 1200 g for 10 min and protein concentration was measured with a BCA assay. Samples corresponding to 3μg of total protein were loaded on the top of the sucrose gradients. Samples were ultracentrifuged for 2 h at 40,000rpm in a SW41Ti rotor (Beckman-Coulter, USA) in the Optima XPN-100 ultracentrifuge (Beckman-Coulter, USA). Gradients were passed through an UA6 absorbance reader (Teledyne ISCO, USA) using a syringe pump (Brandel, USA) containing 60% sucrose. Absorbance was recorded at an OD of 254 nm.

2.1.17 Proteasome activity assay

Flies were homogenized in homogenization buffer (25 mM Tris-CL, pH 7.5) and centrifuged for 5 min at 13,000rpm at 4°C. Protein concentrations were determined by BCA assay. 20ug of fly protein was incubated at 37 °C with fluorigenic substrates Succinyl-Leu-Leu-Val-Tyr-7-Aminocoumarin (Suc-LLVY-AMC; Enzo BML-P802-005) and Leu-Leu-Glu-7-Aminocoumarin (LLEAMC; Enzo BML-ZW9345-0005) for 30 min and the release of free fluorescent AMC was measured every 2 min using a microplate fluorescence spectrophotometer (Infinite M200, Tecan), at excitation/emission wavelengths

of 360/460 nm. Reactions were carried out in black 96-well plates. 20S activity was calculated by the slope of free AMC release over time and expressed as pmoles of substrate degraded per minute and mg protein (pmol/min/mg protein).
Mouse proteasome activity assays were performed similar to the fly assay using a different homogenization buffer (172) (50mM Tris, 150mM NaCl, 5mM MgCl2, 1mM EDTA, 1mM DTT, pH 7.5). Proteasome activity was assessed on flash-frozen livers, muscles, guts and brains.

2.1.18 Uric acid quantification

Uric acid excretion was quantified separately in *ad-libitum* and in fasting regimes. For the *ad libitum* regime, after 8 days of treatment, mated 10-day old females were placed in fresh vials containing the respective holidic diet. Flies were maintained for 16 hours at 25 °C and 65% humidity standard conditions. Following this period, flies and media were removed from the vial, and the uric acid content of the vials was dissolved in 2 ml of 0.1 M sodium glycinate buffer (pH 9.2) and quantified spectrophotometrically (585 nm) using the conversion of uric acid to resorufin (Amplex Life Technologies kit A22181). For the fasting regime, after 8 days of treatment, mated 10-day old females were placed in empty vials and were maintained for 16 hours at 25 °C and 65% humidity standard conditions. After this time, flies were removed and uric acid was quantified as above.

2.1.19 Statistical analyses

All statistical analyses were performed using Prism software (V9). The nonparametric Wilcoxon rank-sum test was chosen to avoid assumptions of data normality. The log-rank test, a nonparametric test for survival analysis, was used to assess survival distributions of treatments, and Cox proportional hazard was used for survival experiments with more than one explanatory variable. Student's t-test and analysis of variance (ANOVA) were used where previous experimental data has conformed to the assumptions about normality and variance equality. Student's t-test was used for Western blot analysis, one-way analysis of variance (ANOVA) with Dunnet post hoc was used for proteasome activity assay analysis.

2.1.20 Reproducibility and experimental design

Sample sizes are at least, and in many cases beyond, what is required to give confidence in the results. These are consistent with established norms for research on *Drosophila* and aging. For lifespan and egg-laying assays, individuals that developed in the same vessels were assigned evenly and systematically between experimental treatments. Except for the dietary preference assay, none of the assays required scoring blindly. Moreover, the current mouse study was designed to meet rigorous criteria for ageing research as described recently (173).

2.2 Materials and methods for mouse experiments

2.2.1 Mouse strain selection

For each mouse experiment shown, twenty C3B6F1/J females per treatment were used. Our parental mice are two inbred strains. C57BL/6J and C3H. C57BL/6J is a sub-strain of C57BL/6, bred for F=227 generations at the Jackson laboratory; it is one of the most commonly used inbred strains and the first strain to have its genome sequenced. The N strain was separated at F=50 from the original inbreedings that generated the J strain. A recent report is in favour of 4-way cross hybrid animals (produced from the cross of two F1 hybrids derived from four genetically distinct parents, so that the F2 generation have four different grandparents), which it claims are preferable to 2-way cross hybrids for reasons of greater genetic diversity, heterozygosity, and elimination of genetic background effects (174). However recent findings suggest that the body size variability in such F2 hybrids is high (with adult BW often ranging from 25 to 60 grams), which renders such strains less appropriate for the accurate assessment of growth rates. Moreover, even though heterozygosity issues may be bypassed, the genetic variability in such hybrids is not as diverse as would be desired to eradicate genetic background effects.

2.2.2 Mouse housing

Mice were kept at a temperature range of 20-24°C (mostly 21-22°C). Individually housed mice weigh more, show more cancers, and a greater variance in body mass compared to group housed mice (175, 176). We therefore housed mice under SPF conditions in groups of 5 per cage to achieve a lower variance in growth rates.

2.2.3 Mouse food and water intake

Except for *ad libitum* fed animals, a pair-feeding regime was applied to ensure identical food intakes. Pair feeding is a method by which food intake in a control group is measured, and then applied on the next day to other animals to ensure equal cumulative feeding (177). To determine the food intake of the pair-fed groups, the mass of pellets consumed daily by the food-restricted group consuming the least amount of food was supplied to the rest of the groups at the following time point(s), until cumulative food consumption was equalized between all pair-fed groups.
For the *ad-libitum*-fed groups, food intake was measured for each cage separately (dry matter mass consumed during the feeding period) twice per week, and the mean of a treatment was calculated as the average of all 4 cages (N = 4 per treatment per timepoint).
Water intake for all treatments was also measured for each cage separately twice per week, and the mean of a treatment was calculated as the average of all 4 cages (N = 4 per treatment per timepoint).

2.2.4 Mouse diet design

Dietary nitrogen, energy, protein retention efficiency (PRE) and protein efficiency ratio (PER) are more efficient in the lower-protein diets, with a balanced NEAA:EAA (178). Moreover, low protein diets enable the detection of physiometabolic effects of dietary AA imbalance (26), therefore all our mouse diets were designed to have a low crude protein (CP) content of 6-8%.

2.2.5 Body mass

Body mass may be a good predictor for mouse lifespan at early ages (peaking at 5 months), but maximum weight is not a good predictor (36). We therefore measured mouse body mass twice per week, as reported previously for studies focused on the effect of AA limitation on growth (179), post-weaningly for a period of 20 weeks (until age 23 weeks of age). The linear growth curve portion (from 3.0 to 6.4 weeks) was used to estimate growth rates and dietary efficiency (180).

2.2.6 Mouse SILAC amino acid usage

For the calculation of AA usage in the whole mouse and in specific mouse organs, we employed the same method as described for flies, and used published mass spectrometry based proteomic data (166) in order to calculate protein copy numbers and the corresponding weighted amino acid composition in C57BL/6 mice. We expressed AA abundance in the proteomic profiles of both whole mice (mouse SILAC AA, referred to as mSILACAA) and of tissue-specific proteins (tissue-specific mouse SILAC AA, referred to as tsmSILACAA).

2.2.7 Food indices

Mice are coprophagic, with approximately one-third of their dietary intake being faeces (181), which is a good source for a range of nutrients but primarily for vitamins B and K (177). However as murine faeces is composed of ~1% protein (177) and protein is the limiting macronutrient in our diets, the effects of coprophagy on our food and protein index evaluation are considered negligible. Therefore, the Food Efficiency Ratio (FER) was defined as previously reported (182): FER = 100*cumulative body mass gain / cumulative protein intake). Protein Efficiency Ratio (PER) was also defined as previously reported (51): PER = cumulative body mass gain / cumulative protein intake). Both were calculated after 20 weeks of post-weaning diet treatment (N = 20). Net protein utilization (NPU) was defined as the ratio of utilized to supplied nitrogen: NPU = 2 x amount of nitrogen utilized in 24 hours (mg) / amount of nitrogen excreted in 24 hours (mg). NPU can be affected by proteostatic processes (e.g. protein turnover), but is primarily determined by the level of limiting amino acids in a diet (26). As a value, NPU can range from 1 to 0, with a value of 1 indicating 100% utilization of dietary nitrogen as protein and a value of 0 indicating that none of the ingested nitrogen was converted to protein.

2.2.8 Protein extraction

Protein was extracted from females treated in the respective diets for 20 weeks (approximately 23-24 weeks of age). Harvested and flash-frozen 15-20mg mouse liver pieces were homogenized in 2ml of the RIPA buffer with use of IKA T10 basic ULTRA-TURRAX homogenizer and centrifuged for 1h at 13,000 rpm (4°C). The supernatant was collected as the protein fraction.

2.2.9 Protein and DNA extraction from the same samples

To obtain the protein:DNA ratio, a single tissue sample was utilized for protein and DNA extraction with the use of AllPrep DNA/RNA/Protein Mini Kit (Qiagen, 80004). Subsequently, the BCA assay and DNA concentration measurement (Nanodrop 2000 Spectrophotometer) were performed.

2.2.10 Metabolic rate, energy expenditure, and activity

Eight animals per group were used to measure energy homeostasis in metabolic cages (PhenoMaster, TSE Systems). For the metabolic measurements, the mice were housed singly and supplied with a constant flow of air. Due to their metabolism, mice consume O_2 and produce CO_2. During indirect calorimetry, the difference of O_2 and CO_2 content of the air before entering the cage and after leaving the cage is measured. With this measured difference, the O_2 consumption, CO_2 production, the respiratory exchange ratio and the basal energy rate were calculated. Moreover, the activity of the mice was recorded by laser-beam obstruction. During the monitoring of mice in metabolic cages, food and water intake were also measured with automatic food and water sensors.

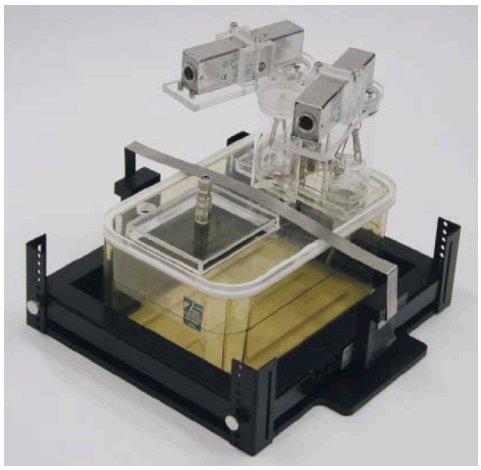


Figure 2.1: metabolic cage (Phenomaster, TSE Systems)

Before starting the measurements, mice were trained in training cages. During this time the mice were acclimatized to single housing, hanging water bottles, special drinking nipples, and hanging food baskets. Mice were intensively checked for their drinking and eating behavior to ensure they accepted the new drinking and feeding system. After the time in the training cages, the mice are acclimatized for 24 hours in the metabolic cages. Data was collected for 48 hours.

Body surface temperature was assessed by infra-red (IR) thermometry applied on the ear canal at 23 weeks of age (20 weeks of treatment) using a standard infrared thermometer (Thermoscan, Braun). IR thermometry is a clinically reliable indicator of body core temperature due to its location near the hypothalamus (the body's temperature regulator) and is typically applied to mice.

2.2.11 Blood metabolic profile

Blood glucose and insulin levels were monitored at week 23. All animals were fasted for 3 hours and blood samples were collected after this fasting period.

Blood was extracted from tail-tip clippings and used to determine blood glucose levels, as well as insulin levels. Each clipping aimed to collect ~5μl of blood for the glucose measurement and ~5μl of blood for the insulin measurement. Blood insulin and leptin levels were determined using commercially available enzyme-linked immunosorbent assays (ELISA) ALPCO 80-INSMSU-E01, E10 (insulin) and 22-LEPMS-E01 (leptin).
For the ketone assay, mice were fasted over night, blood was collected by tail clippings, and β-hydroxybutyrate was quantified using Precision Xtra ® test strips.

2.2.12 Glucose tolerance test

To analyze glucose homeostasis, glucose tolerance tests (GTT) were performed at 23 weeks of age. GTT analyzes how efficiently the body metabolizes glucose. After an approximately 16 hour-fasting period (GTT mice were not fed a morning 1g food aliquot, unlike mice subjected to insulin tests, see below) with free access to drinking water mice were intraperitoneally injected with 2 g of glucose per kg body weight. The injected volume of glucose solution is 10 µl per g body weight. Blood glucose was measured before, 15, 30, 60 and 120 minutes after glucose injection.

2.2.13 Insulin tolerance test

The insulin tolerance test (ITT) measures the acute reaction to exogenous-applied insulin and the resulting blood glucose-lowering effect. The decline of blood glucose after insulin application is a direct measurement of insulin sensitivity and a standardized method for the analysis of glucose homeostasis in mice. ITT was performed at 24 weeks of age. Mice were intraperitoneally injected with 0.75 units of insulin (in 0.9% NaCl) per kg body weight. The injected volume of insulin solution was 10 µl per g body weight. Blood glucose was measured before, 15, 30 and 60 minutes after insulin injection. ITT mice were fed a small meal of 1 gram per mouse approximately 2 hours prior to being tested.

2.2.14 Fat content characterisation

Body fat content was determined by *in vivo* magnetic resonance tomography imaging (time domain nuclear magnetic resonance or TD-NMR) at weeks 3, 13 and 23 of age (0, 10, and 20 weeks of treatment respectively). This non-invasive procedure was applied to all 20 animals/group to measure the effects of dietary amino acid nutrition upon fat deposition and fat metabolism. The fat content measured by TD-NMR included all types of fat in the body and the lean content included all kind of muscle tissue and free fluids in the body (e.g. water in the ankles and in the brain; but not blood). Typically, the fat, lean and free fluid content amounted to ~92-96% of the total body mass. Absolute fat and lean mass (g) were calculated by:

$$\text{Fat mass (g)} = \text{fat \%} \times \text{total body mass}$$
$$\text{Lean mass (g)} = \text{lean \%} \times \text{total body mass}$$

2.2.15 Urea excretion and nitrogen balance

When protein is metabolized, about 90% of the protein nitrogen is excreted in the urine in the form of urea, uric acid, creatinine and other nitrogen end-products, allowing the accurate characterisation of nitrogen-balance. The

remaining 10% of the nitrogen is eliminated in faeces. The total nitrogen excreted is compared to the total N content consumed, to estimate nitrogen balance. To establish nitrogen losses, urine urea concentration was determined by a coupled enzyme reaction (phenylalanine assay) at 570 nm using a commercial kit (Sigma-Aldrich MAK006). Urine samples were collected following 4 hours of starvation and normalized to body mass and urine loss rate (N=10).
In addition, nitrogen balance studies were carried out singly-housed animals (N=6) in specifically designed metabolic cages (Tecniplast, see Figure 2.2) that eliminate the possibility of coprophagy, collect all excreta (solids and fluids), and contain custom-made feeding inserts to accurately measure feed intake. Animals were first acclimatised for 24 hours in these cages, with the measurement spanning an additional 24 hours approximately at 23 weeks of age. Within these cages, food is supplied as powder in a container that ensures no food losses. It should be noted that non-solid pellet foods in rodents can exacerbate obesity and weight gain due to less work required for the digestion of the food (183).

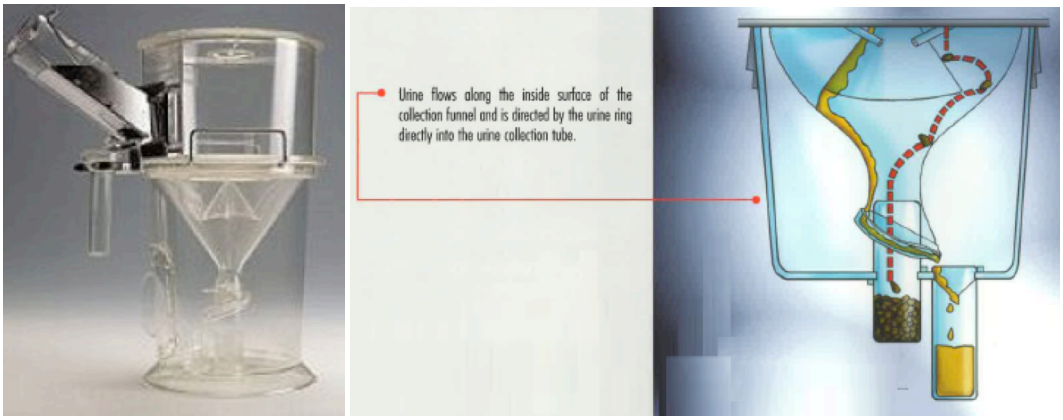


Figure 2.2: Tecniplast metabolic cages for excreta collection used in N-balance assessment.

2.2.16 Organ mass and tissue harvest

After the end of the 20-week treatment period (age 23 weeks), all mice were euthanised by gassing CO_2 (spinal displacement inappropriate due to skeletal size measurement) and organs were harvested. Wet organ masses were recorded immediately, including: brain, right quadricep, fat pads, kidney, liver, heart, spleen, thymus, and thyroid. All organs were then flash-frozen. All mice were fed a small meal of 1 gram per mouse approximately 2 hours prior to their euthanisation – tissues were harvested immediately after this.

2.2.17 Characterisation of skeletal growth and bone mineral parameters by μCT

The C57BL/6J strain is an ideal model for assessing BMD effects (184). The age at which bone parameters were assessed (23 weeks) is close to the peak of bone mass accretion after development (around 18-20 weeks), so any effects of dietary nutrients upon bone tissue development should be

detectable at this age (185). After the end-point of the study (week 23), seven right femur bones from seven animals of each group were harvested, placed in 10% PBS saline solution, and stored at -80°C. Before scanning, samples were thawed and hydrated overnight in a saline solution at 5°C. Femurs were scanned with a high resolution µCT scanner (SkyScan 1176, Bruker, Belgium) with an isotropic voxel size of 8.8 μm^3. The x-ray settings for each scan were 50 kV and 500 µA using a 0.5 mm aluminum filter. All scans were performed over 360 degrees with a rotation step of 0.3 degrees and a frame averaging of 1. Images were reconstructed and analyzed using NRecon and CTAn software, respectively (Bruker, Belgium). Trabecular and cortical bone regions of distal femurs were selected with reference to the growth plate (0.44-2.2 and 2.2-2.64 mm from growth plate for group 1, respectively. Size and position of bone regions were adjusted for the other groups depending on femur length). Bone mineral density was determined based on calibration with two phantoms of known density (Bruker, Belgium), which were scanned under the same conditions as the bone samples. Skeletal growth was also analysed by manually measuring the height (skeletal length) of all 120 main cohort mice (from nose tip to tail base) immediately after mice were killed.

2.2.18 Histopathology

Formalin-fixed specimens from the liver, kidneys, skeletal muscles, skin, adipose tissue, sternum and intestines of 80 mice were used for histopathologic evaluation. Tissues were trimmed, processed, blocked, sectioned, stained with H&E and examined microscopically. All slides were examined unbiased for the presence of histopathologic lesions. Severity score of all recorded lesions was semi-quantitatively assessed in all organs on a scale of 0 to 5 with 0.5 interval. Scores were given as absent (0), subtle (1), mild (2), moderate (3), severe (4), and marked (5) for each criteria. Representative digital images to the recorded lesions were taken for each analysis. The histopathologic examination was done unbiased without previous information to the sample examined. Total skin thickness (including epidermis, dermis, subcutaneous fat, and panniculus carnosus) and thickness of subcutaneous fat were measured using arbitrary line option of the LabSense 1.1 image analysis software (Olympus soft imaging solutions).

2.2.19 Portal vein plasma metabolomics analysis

Portal vein blood samples were harvested from pair-fed female C3B6F1/J mice at 23 weeks of age. Each animal was fed a 1 g meal approximately 1 hour prior to sample harvest. Mice were euthanized by CO_2 gassing, dissected, and portal vein blood collected in EDTA tubes, and span for 15 minutes at 1,200 rpm (4°C). Isolated plasma samples were flash-frozen in liquid nitrogen and thawed once for an LC-MS-based metabolomics analysis performed by the Finnish Institute for Molecular Medicine (FIMM).

2.2.20 Power analysis

To determine the number of animals used in our mouse growth analysis, a power analysis was carried out. This analysis was based on data obtained from our collaborator, Professor Stephen Simpson (University of Sydney, Australia). The growth data (body mass in grams) for the first 10 weeks in these diets are shown on Table 2.2:

Table 2.2: Body mass data (in grams) for 5% crude protein diets.

	Body mass (grams)				
Post-weaning week	**0**	**2**	**6**	**10**	**Growth rate**
DIET 1 (5% protein)	11,94	13,72	15,91	18	0,6
DIET 3 (5% protein)	12,79	13,52	16	17,8	0,5
DIET 6 (5% protein)	12,28	13,77	16,78	18,73	0,64
Mean	**12,34**	**13,67**	**16,23**	**18,18**	**0,58**

* Growth rate at week 10.1 is taken as the difference in body mass between weeks 10.1 and 0, divided by the number of weeks (10.1).

Previous data from our lab (by Dr. Sebastian Groenke) were used to determine standard deviation (S.D.) in 5 randomly selected female C3B6F1/J animals (F1 hybrid annotation) for the same time point (13 weeks of age). These are shown in Table 2.3, below.

Table 2.3: Body mass data for 5 randomly selected animals, with cage number, ear annotation, and body mass (in grams).

	Post wean age	**Week 10**
Ad libitum fed group	**Animal age**	**Week 13**
Cage number	**Ear number**	**Body mass (grams)**
988	22	27.6
214	19	29.7
1004	56	27.4
201	39	27.3
996	27	26.8
	Mean	**27.8**
STDEV (range of values)	**STDEV**	**1.12**
SQRT (square Root of the population size, number)	**SQRT**	**2.24**
	SEM (STDEV/SQRT)	**0.50**

From these two data sets (week 10 growth data of Prof. Simpson's 5%P diets, and week 10 standard deviation data from Dr. Groenke's study) we performed a standard power analysis. In the basis of this power analysis we projected the numbers needed at week 20 to give an 80% power to detect a difference of 1.19 grams for n=15:

- a maximum difference of 5.84g between the groups, (Mean growth rate, Table 2.2)
- a power of 80%
- a SD of 1.12 (STDEV)
- 15 animals per group required to detect a minimum mass difference of 1.19 grams between groups in a statistically significant manner for the first 12 weeks post-weaning.

To account for the event of smaller statistical differences and to ensure a high resolution to further secure the detection of any growth depression effects, we used 20 animals per group. We determined this number satisfactory to detect statistical differences in our mouse growth experiments.
As the phenotypes (excluding lifespan and food consumption) tested in the lifespan experiment compare mice across a wider range of ages (from 0 to 24 months of age), the statistical difference across those time-points and

between the groups were projected to be more significant for all the tested phenotypes.

Chapter 3: Exome-matching predicts the limiting EAA in flies and mice

3.1 Abstract

Defining the identity and degree of a limitation or excess in the intake of an AA is dubious as many past attempts to determine a reliable measure for dietary AA requirements have had limited success. A possible general predictor of the AA requirement of an organism could be its genome, potentially allowing a rational design for a balanced dietary AA ratio. Here it is shown that exome-matching correctly predicts the identity of the limiting EAA for female fly fecundity, and that it can be used to accurately estimate the degree of that limitation. Moreover, dietary manipulations in the amount of the limiting EAA in the fly diet result in predictable modulations of the fly's fecundity. In mice, a comparison of EAA-limiting diets to the mouse exome AA usage also reveals that exome-matching is a valid measure for the prediction of the identity of the limiting EAA. Therefore, these results suggest that the exome composition of an organism may provide a suitable template for its AA requirements for at least some anabolic traits.

3.2 Introduction

Historically, many approaches have been used to approximate dietary AA requirements. Qualitative and quantitative determinations of the metabolic fate of dietary nitrogen (186), or of stable AA isotopes of carbon (187), hydrogen (56), or sulphur (188), can indicate the relative AA requirements of an organism by revealing how ingested AAs are utilized. The response of plasma AAs to the intake of different AA sources can also be used to estimate AA needs (4). Alternatively, the body AA approach is based on the assumption that an organism requires AAs in a proportion that reflects the accrued whole body AA profile (187). The method has been used in mice (153), rats (189), pigs (190), cows (191), sheep (192), fish (193), and humans (187, 191). For growing mammals, the AA composition of maternal milk is also thought to match AA requirements (47, 191, 193). However, there are severe limitations in each of these approaches, discussed elsewhere in detail (4, 25, 186, 187, 194-197).

A hypothesis that was developed before I joined the laboratory by Dr. Matthew D. Piper relied on the assumption that an alternative possible general predictor of the AA requirement of a healthy organism could be its genome, potentially allowing a rational design for a balanced dietary AA ratio. Therefore, using flies and mice we asked if the biological demand of an organism for AAs is best reflected by its exome. We translated all predicted protein-coding genes *in silico* and derived a proportional representation of each AA in the predicted exome. We thus tested our hypothesis by designing diets that contain AAs in the proportions defined by the exome, for both flies and mice, and then assessing both production traits and ageing.

Drosophila melanogaster is a particularly useful model in studying how dietary manipulations impact on production traits and ageing. Recently, we reported the development of a chemically defined medium for *Drosophila*, which supports development, fecundity, and long-term maintenance (29). This holidic medium was first used to define the identity of the limiting EAA in various diets with different AA compositions, and then to make quantitative predictions on the effects of limiting EAAs upon production traits. Preliminary results showed that the exome composition of an organism may provide a suitable template for its AA requirements. Exome-matched supply of dietary AAs promoted egg production in flies and did so in a manner that successfully identified the identity of the limiting EAA. Moreover, several rodent studies were identified in the literature where the identity of the limiting EAA in diets with specific AA compositions has been experimentally verified. We therefore tested the accuracy of using the exome-matched AA profile in predicting the identity of the limiting EAA in those diets. These preliminary results showed that exome-matching is a reliable measure for predicting the identity of the limiting EAA in a range of rodent diets, each with different limiting EAAs. Moreover, other previous measures or estimating EAA requirements failed to correctly identify the limiting EAA. Thus, our preliminary results suggested that exome-matching can allow modeling of the requirement for all EAAs.

3.3 Results

3.3.1 Exome-matching predicts the limiting AA for Drosophila fecundity

It was hypothesized that the EAA requirements of an organism for anabolic traits match the mean AA usage by its exome. Thus, to predict the requirement of the fruit fly *Drosophila melanogaster* for EAAs, we translated *in silico* the 19,736 predicted protein-coding genes from the whole genome sequence, and calculated the proportional representation of each amino acid (FlyAA) (Figure 3.1A). If FlyAA accurately predicts the requirement of the fly for EAAs, then we can identify the limiting EAA in any diet as the one with value *r*, where $r = \min_i, d_i/e_i$ (for EAA*i*, $\min_i$ is the minimum, d_i and e_i are the relative concentration in the diet and in the exome respectively). This methodology is based on the chemical score calculation for different protein sources(198). Restricting dietary protein reduces fly fecundity (2) but for flies kept on a laboratory yeast-based diet the limiting EAA for egg-laying is methionine (8). Compared to the yeast proportional AA composition (YAA), FlyAA shows several differences (Figure 3.1B), but correctly predicts the limiting EAA in YAA as methionine (Figure 3.1C, left panel).

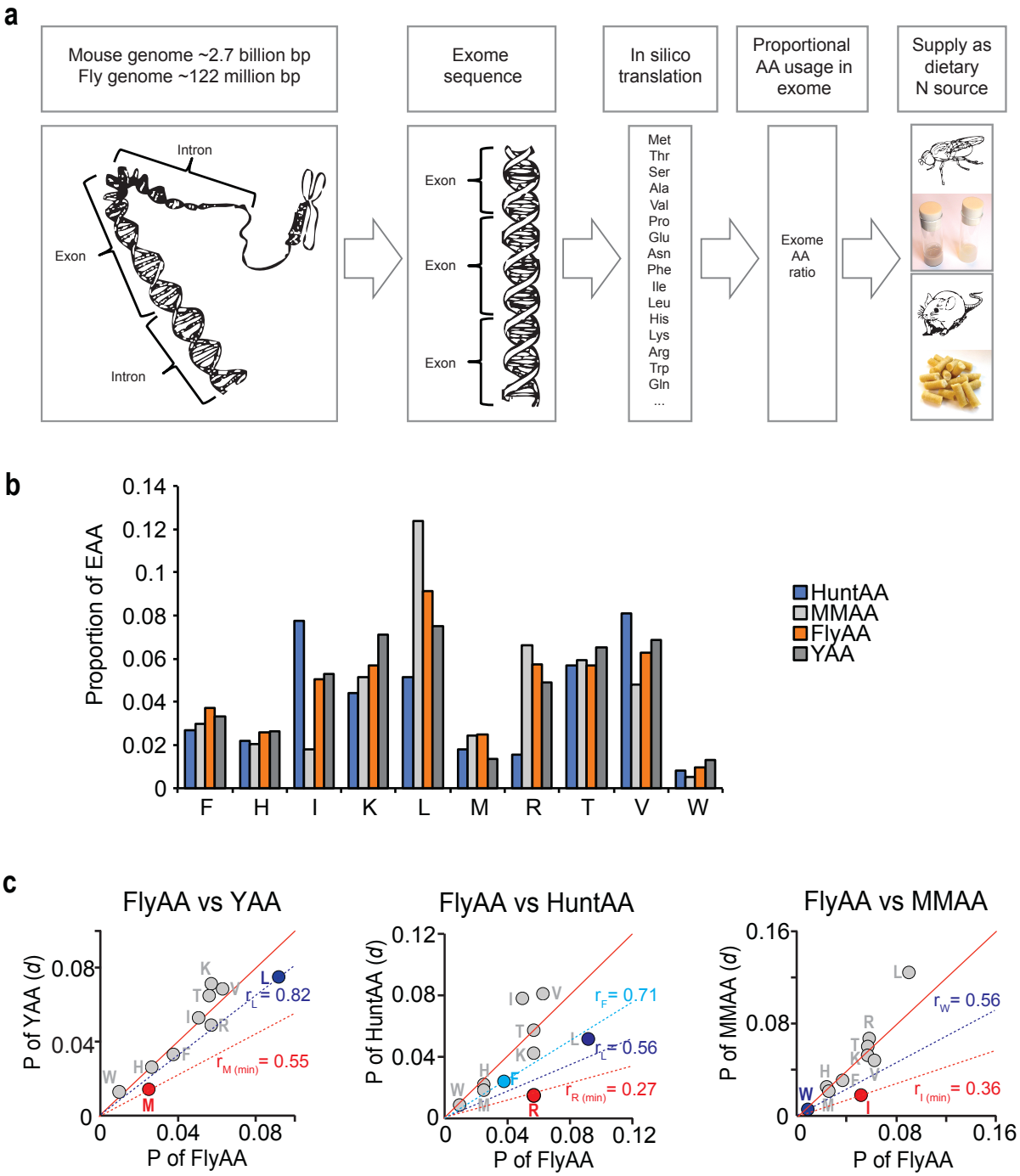


Figure 3.1

a. Overview of exome matching in *Mus Musculus* and *Drosophila melanogaster*. The entire mouse genome (2.7 Gb) or fly genome (122 Mb) (left panel) are trimmed to their respective gene-coding exome sequence (second panel). This sequence is then translated *in silico* (third panel), and the genome-wide proportional representation of each amino acid is defined as the proportional amino acid usage in the exome (fourth panel). This amino acid profile is then added to chemically defined diets for flies and mice respectively (right panels).

b. Comparison of dietary AA ratios used in our Drosophila melanogaster experiments. The EAA proportions in the fly exome ratio (FlyAA) are compared against those in HuntAA, MMAA, and YAA. Each bar represents the molar proportion for each EAA in each ratio, and isonitrogenous amounts of total EAAs are compared between ratios. FlyAA, HuntAA, and MMAA data by Dr. Matthew D. Piper and Dr. Eric Blanc.

c. Calculation of the most limiting dietary EAA(s) for each exome-mismatched ratio compared to the fly exome ratio (FlyAA) using $r = \min_i, d_i/e_i$ (for EAA*i*, $\min_i$ is the minimum, d_i and e_i are the relative concentration in the diet and in the exome respectively). Arginine (R) is the most limiting EAA in HuntAA (aR (min) = 0.27, or 27% of the proportional exome requirement for R), while methionine (M) is the most limiting EAA in Yaa (aM (min) = 0.55, or 55% of the proportional exome requirement for M). All EAAs above the solid red diagonal (vectors 1,1) are predicted to be in excess in the exome-mismatched ratios compared to the exome ratio. FlyAA, HuntAA, and MMAA data by Dr. Matthew D. Piper and Dr. Eric Blanc.

HuntAA (Figure 3.1B), an AA ratio previously reported to be adequate for *Drosophila* growth and development (199), was recently used to develop a holidic food medium that supports multi-generational development, adequate fecundity, and a healthy lifespan (29). FlyAA is substantially different from HuntAA, with arginine (R), an EAA in *Drosophila* (57), predicted to be the limiting EAA in HuntAA, and leucine (L) the next most limiting (Figure 3.1C, middle panel). In our equation, because r will predict the limiting EAA for egg laying on the current diet (diet 1), the proportional change in egg laying when flies feed on a diet with a different amino acid ratio (diet 2) should also be predictable according to r_1/r_2. We therefore determined whether exome-matching could quantitatively predict the response of female egg-laying on HuntAA to alterations in arginine or leucine contents, using the same holidic medium. Increasing or reducing arginine concentration alone in HuntAA caused egg-production to increase or to decrease to the same extent as when all amino acids were altered by the same amount (Figure 3.2A). Considering the second most limiting EAA in HuntAA, methionine, we predicted that restoring arginine by 50% would increase fecundity by the same degree (~50%), but restoring arginine to the exome-required proportion (by increasing by 270%) would roughly double egg-laying ($r_{R(min)}/r_M = 0.58$). Indeed the results confirmed this prediction accurately (Figure 3.2B).

The effect of the dietary EAA ratio on egg-laying could be attributable to one or more of the EAAs acting as a signal, rather than to the efficiency of their use in anabolism. To probe this possibility, we used another EAA ratio also predicted to be MisMatched (MMAA) to FlyAA but with a different proportion of all 20 AAs (Figure 3.1B) and with isoleucine predicted to be the limiting EAA (Figure 3.1C, right panel). Confirming this prediction, increasing or reducing isoleucine concentration alone in MMAA by 50% caused egg-production to increase or to decrease to the same extent as when all amino acids were altered by the same amount (Figure 3.2C). Considering the second most limiting EAA in MMAA, tryptophan, we correctly predicted that restoring isoleucine to the exome-required dietary proportion would not significantly raise fecundity compared to MMAA + 50% I (Figure 3.2C).

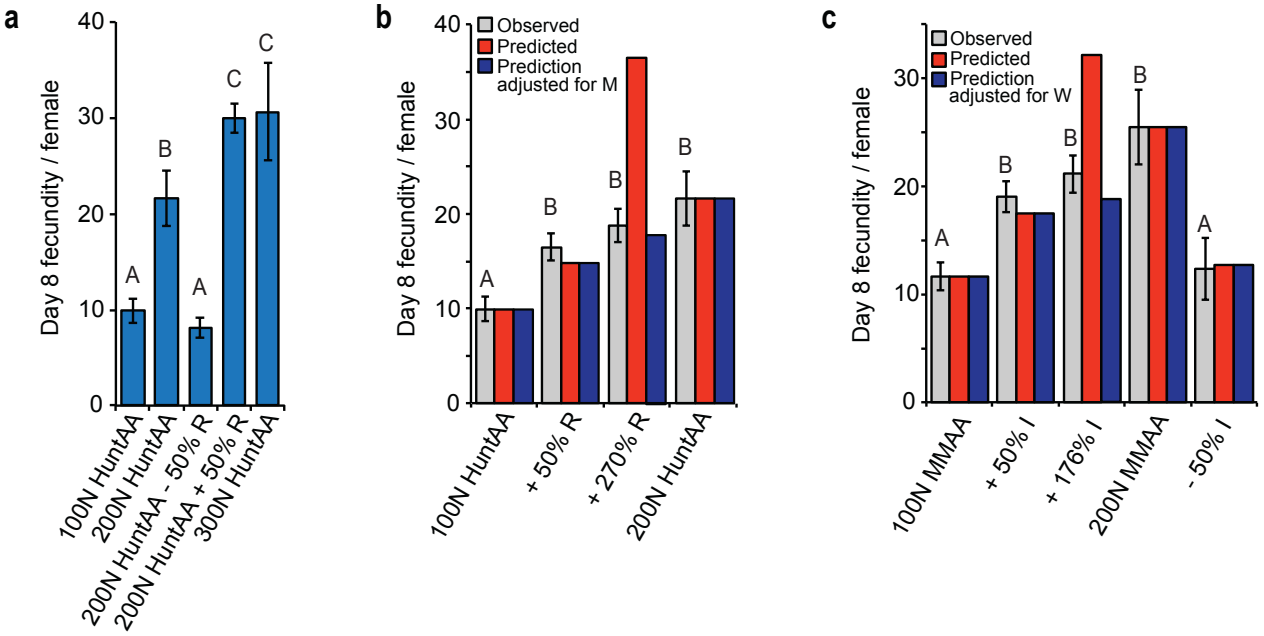


Figure 3.2

a. Egg laying prediction in *Drosophila* females fed HuntAA diets. Observed egg laying for increase in total AAs increases or decreases in the same proportion as when adjusting Arginine in the medium (Arginine addition or reduction by 50%). Each bar represents 2-9 trials, each trial using (per treatment) 6 biological replicates of 10 flies. Error bars represent SD, letters indicate statistically significant differences ($p<0.05$) between pair-wise comparisons (Student's t-test).

b. Observed egg laying (grey bars) increases or decreases by arginine adjustment in the medium (arginine addition or reduction), matching the predicted response (red bars) based on the identification of arginine as the limiting EAA, and methionine the second most limiting EAA, in HuntAA compared to the exome ratio (FlyAA). Restoring arginine to the exome-required proportion (by increasing it by 270% on its original HuntAA level so that $r_R = 1$) roughly doubles egg-laying as predicted from $r_{R(min)}/r_M = 0.58$. Thus exome-matching accurately predicts the fecundity effects from adjustments in the limiting amino acid, Arginine, the next most limiting EAA (methionine). Each bar represents 2-9 trials, each trial using 6 biological replicates of 10 flies per treatment. Error bars represent SD, letters indicate statistically significant differences ($p<0.05$) between pair-wise comparisons (Student's t-test).

c. Observed egg laying (grey bars) increases or decreases by isoleucine adjustment in MMAA (isoleucine addition or reduction), matching the predicted response (red bars) based on the identification of isoleucine as the limiting EAA, and tryptophan the second most limiting EAA, in MMAA compared to the exome ratio (FlyAA). Considering the second most limiting EAA in MMAA, tryptophan, we predicted that restoring isoleucine to the exome-required dietary proportion by increasing it by 176% on its original MMAA level (so that $r_I = 1$) would not significantly raise fecundity compared to MMAA + 50% I. When leucine is added to this diet (100N MMAA + 176% isoleucine), egg laying is further increased to a level predicted by the next limiting EAA, tryptophan (W). Each bar represents 2-9 trials, each trial using 6 biological replicates of 10 flies per treatment. Error bars represent SD, letters indicate statistically significant differences ($p<0.05$) between pair-wise comparisons (Student's t-test).

3.3.2 Exome-matching predicts the limiting AA for mouse growth

Because exome-matching is a genome-based approach, it can be applicable to any organism whose genome sequence is known. Indeed, we found several published mammalian diets designed to be limiting for single EAAs, with the identity of the limiting EAA verified experimentally. In each case, the exome successfully identified the growth-limiting amino acid: threonine (T) for rats fed a threonine-basal diet (200) (Figure 3.3A), leucine (L) for rats fed a leucine-basal diet (201) (Figure 3.3B), arginine (R) for rats fed an arginine-limited diet, (Figure 3.3C) (202) and methionine (M) for mice fed a 0.15% Met diet (203) (Figure 3.3D). In contrast, we found instances where other measures of dietary AA requirement, such as the body AA composition, fail to correctly predict the limiting AA (Figure 3.3E). (Data for Figure 3.s 3A-B and D by Dr. Matthew D. Piper and Dr. Eric Blanc).

The AA profile of dietary proteins has long been implicated in healthspan and lifespan. A good example of this is plant proteins such as soy protein, which has been reported to be beneficial for a range of physiometabolic parameters in rodents and humans (23, 24, 138, 204). A comparison of the AA profile of soy protein showed that its proportional EAA composition is highly similar to MouseAA ($r_{T\ (min)}$ = 0.75 ; Figure 3.3F) and to the human exome profile (HumanAA; $r_{T\ (min)}$ = 0.77 ; Figure 3.3G). Thus the AA profile of a dietary protein source reported to be beneficial across mammalian species highly resembles the exome-predicted requirement. Moreover, compared to the human or mouse exome, the two most limiting AAs in soy protein are methionine and leucine (Figure 3.s 3F-G). Interestingly, both AAs are implicated in lifespan modulation, as methionine restriction extends lifespan, while leucine activates the TOR pathway, whose down-regulation is also linked to lifespan extension (113). Moreover, the human exome requirement also appears enriched in tryptophan, an AA whose restriction also extends lifespan, when compared to the AA profile recommended by the WHO (25) (Figure 3.3I). More experimental work will firmly establish whether a link exists between such single AA differences in these protein sources and the modulation of anabolic traits and lifespan. Therefore in light of all these observations the exome-matching approach appears as a potentially useful tool in resolving some of the issues around AA ingestion, anabolic traits, and life history traits.

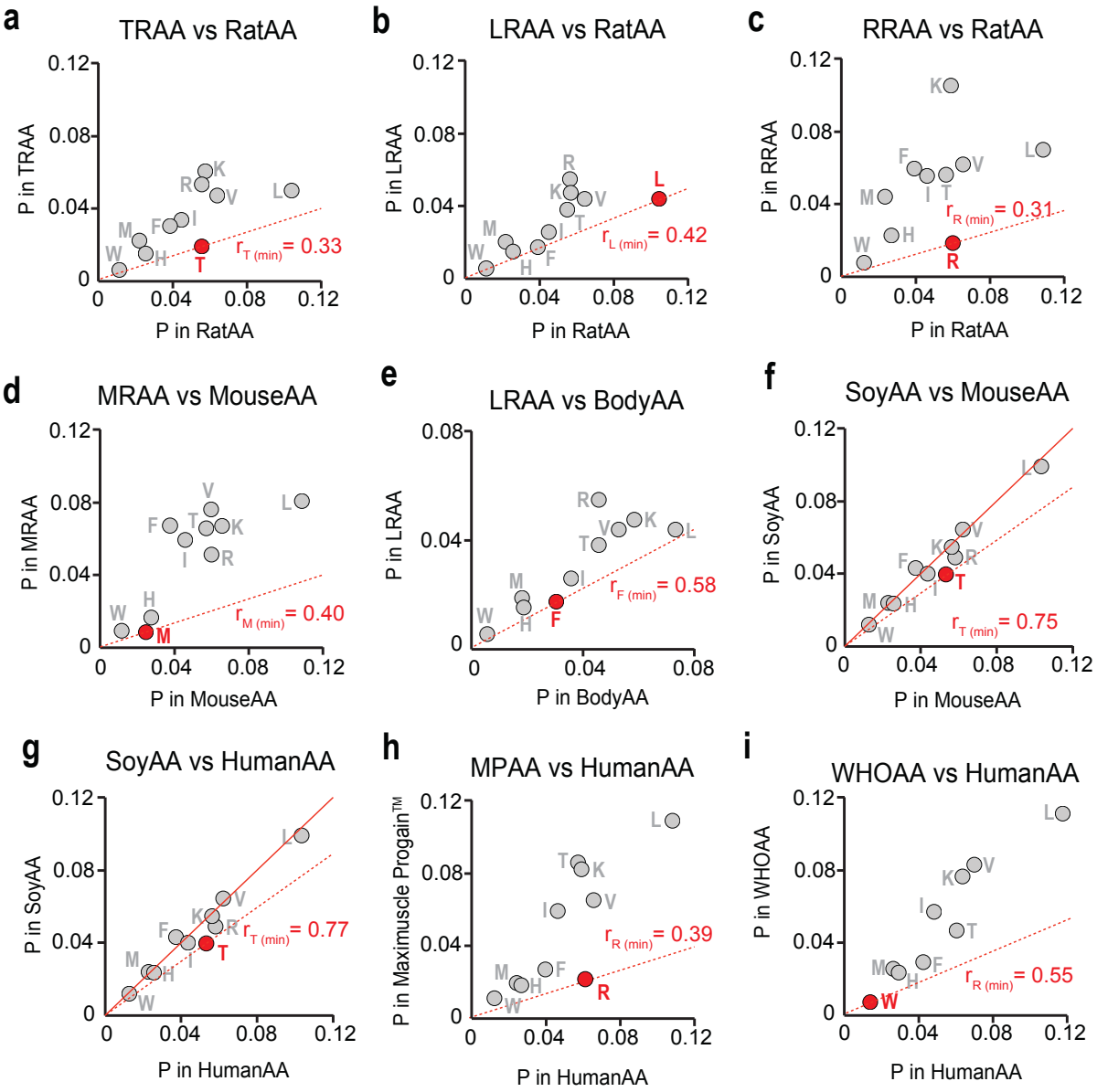


Figure 3.3

a. The exome AA usage of rats (RatAA) correctly identified threonine as the limiting AA in a diet previously shown experimentally to be threonine-restricted (TRAA) ($r_{T(min)}$ = 0.33). See text for more details. (Data by Dr. Matthew D. Piper).

b. The exome AA usage of rats (RatAA) correctly identified leucine as the limiting AA in a diet previously shown experimentally to be leucine-restricted (LRAA) ($r_{L(min)}$ = 0.42) (53)(201). See text for more details. (Data by Dr. Matthew D. Piper).

c. The exome AA usage of rats (RatAA) correctly identified arginine as the limiting AA in a diet previously shown experimentally to be arginine-restricted (RRAA) ($r_{L(min)}$ = 0.42). See text for more details.

d. The exome AA usage of mice (MouseAA) correctly identified methionine as the limiting AA in a diet previously shown experimentally to be methionine-restricted (MRAA) ($r_{M(min)}$ = 0.40). See text for more details. (MouseAA data by Dr. Eric Blanc).

e. The body AA composition of rats (BodyAA; Kremen et al. 2003) incorrectly identified phenyalanine as the limiting AA in a diet previously shown experimentally to be leucine-limiting ($r_{F(min)}$ = 0.58).

f. The exome AA usage of mice (MouseAA) identifies threonine as the limiting AA in an AA profile reflecting soy protein (SoyAA). However, the degree limitation of the most limiting AA in soy ($r_{T(min)}$ = 0.75) is only 25%, indicating that SoyAA shares a high degree of AA contents to MouseAA.

g. The exome AA usage of *Homo Sapiens* (HumanAA) identifies threonine as the limiting AA in an AA profile reflecting soy protein (SoyAA). However, the degree limitation of the most limiting AA in soy ($r_{T(min)}$ = 0.77) is only 23%, indicating that SoyAA shares a high degree of AA contents to HumanAA. (HumanAA data by Dr. Eric Blanc).

h. The exome AA usage of *Homo Sapiens* (HumanAA) identifies arginine as the limiting AA in an AA profile reflecting Maximuscle protein supplement (MPAA). Thus, the exome AA usage can potentially be used to improve the design of AA supplementation for anabolic traits, avoiding adverse effects of imbalanced AA ingestion.

i. Comparison of the AA profile recommended by the World Health Organization compared to the human exome AA requirement. The WHO recommendation provides 55% of the exome requirement, although is very close to the exome requirement for most other EAAs.

3.3.5 Individual exons and their dissimilarity to the mean exome AA usage

Individual exons (especially oligo-peptides) can be dissimilar to the whole-exome AA usage, as highly non-random AA sequences are abundant in living systems (205). Thus, an averaged exome or proteome AA profile may not equally facilitate the transcription and translation of all exons, which can have complex and unforeseeable metabolic consequences depending on what exons are affected most. In this respect, it was hypothesized that larger proteins, consisting of a greater number of AAs, would be less likely to be dissimilar to the mean exome AA usage. Indeed, analysis of the $\log_2$ distance (wd^2) of each exon to the mean exome AA usage revealed a strong negative correlation ($R^2 = 0.55$) of this measure to the exon length (Figure 3.4). This finding supported the logical assumption that longer exons are less likely to be highly dissimilar to the mean exome AA usage (FlyAA) while, in contrast, shorter protein-coding exons are more likely to be dissimilar to the mean exome AA usage (FlyAA). Thus, the synthesis of large macromolecular structures consisting of long peptides appears to be constrained towards employing an AA sequence that resembles the mean AA usage of the entire exome.

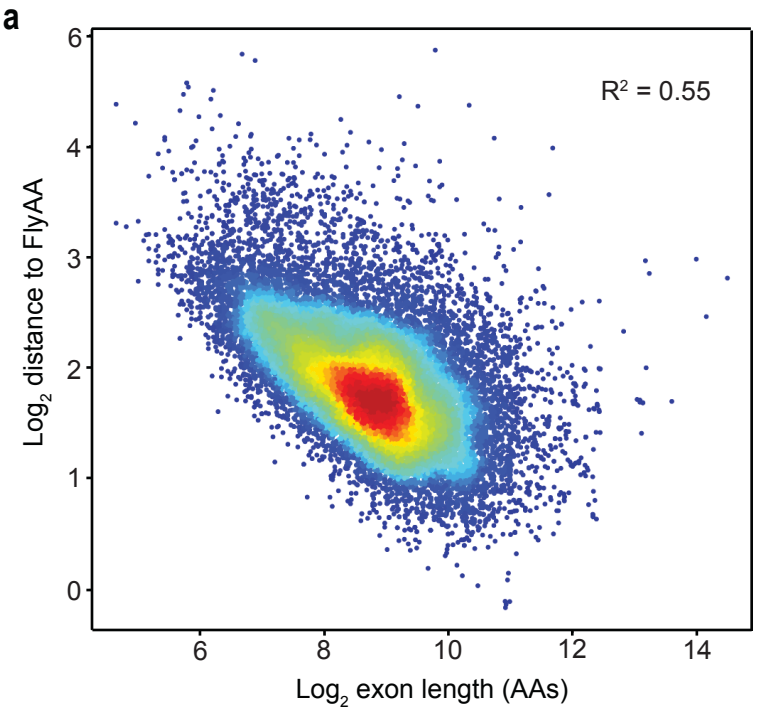


Figure 3.4

a. Analysis of the $\log_2$ distance (wd^2) of each of the 19,736 predicted protein-coding exons to the mean exome AA usage (FlyAA) reveals a strong negative correlation ($R^2 = 0.55$) of this

measure to the exon length (log_2 of the number of AAs). See text for more details. (Data by Dr. Ilian Atanassov).

3.4 Discussion

Despite the complexity of the multitude of factors that can alter AA usage, nutrigenomics may be the most promising approach for defining AA requirements. The genetic composition of a species may affect the response to ingested AAs and contribute to the diversity of protein sources consumed across species. In humans, recent nutrigenomics methods employ the use of genetic information on inborn errors of metabolism to direct the use of dietary AAs in clinical research (206). Yet, the advance of multiple –omics technologies has opened the possibility of evaluating dietary AA requirements across species through novel approaches. Here, a genome-based technique for designing an enhanced quality protein was tested. The preliminary results herein showed that the exome is a powerful measure for defining AA requirements in vertebrates and invertebrates. Because this model is driven by the genome sequence of the organism in question, the principle can be applied to any organism whose genome sequence is known.

The preliminary results presented in this chapter strongly suggested that exome-matching can be used to successfully predict the identity of the limiting EAA. The principle of the minimum poses that the most limiting nutrient in a diet can prevent successful utilization of other, non-limiting nutrients that are, as a result, acquired in excess (31). Accordingly, it is shown here that exome-defined limiting EAAs prevent the usage of non-limiting EAAs for egg-laying in a predictable manner. These observations provided supporting evidence to the proposed exome-based approach for designing enhanced protein quality in fly diets by improving female fecundity. Therefore it was decided to further test exome-matching by focusing on other anabolic and physiometabolic traits in *Drosophila*. This work is described in the following chapter.

Chapter 4: Exome-matching of essential amino acids in the fly diet maximizes anabolic traits

4.1 Abstract

In the previous chapter, it was shown that exome-matching can accurately predict the identity of the limiting EAA in various dietary AA sources in both flies and mice. In this chapter a comprehensive analysis of how dietary AA imbalances, rather than limitations, affect fly metabolism is carried out. Exome-matching is found to promote anabolic traits including growth and fecundity more than do amino acid profiles found in commonly used protein sources including yeast. The metabolic fate of ingested AAs is characterized and exome-matching is shown to promote anabolism by increasing dietary nitrogen utilization. Moreover exome-matching reduces lipid storage, whilst increasing respiration in the fat body. Such biological responses to ingested AA profiles may be critical in the determination of healthspan and lifespan. Therefore the amino acid profile of a food source is an important factor when considering the usage of dietary nitrogen, anabolic traits, and health. Finally, exome-matching is compared to proteome-matching, and the role of the identity of individual limiting AAs is also highlighted.

4.2 Introduction

Across species, dietary AAs modulate key life history traits such as growth, reproduction, and lifespan (5, 26). Consequently dietary protein sources with distinct AA proportions can largely affect growth signaling. A balanced dietary AA ratio is typically considered to reflect the organism's AA requirements, thereby promoting growth. In contrast, imbalanced AAs subtly (imbalance) or grossly (restriction, deficiency, or toxicity) misrepresent an organism's AA requirements (26). The preliminary results described in the previous chapter suggest that the exome can be a reliable predictor of key life history traits including fecundity in flies, as well as growth in mice (26). Yet, the molecular mechanisms mediating the responses to the ingestion of exome-matched AAs are as yet uncharacterized. Thus, work in this chapter aims to further characterize such physiometabolic responses by focusing on flies. *Drosophila melanogaster* is a particularly useful model in studying how dietary manipulations impact on production traits and ageing. Recently, the development of a chemically defined medium for *Drosophila*, which supports development, fecundity, and long-term maintenance (29), enabled the study of individual dietary AA modulations. Here this holidic medium is used to define the identity of the limiting EAA in various diets with different AA compositions, and to make quantitative predictions on the effects of limiting EAAs upon production traits.

The effects of ingesting exome-imbalanced AA profiles are assessed through several physiometabolic responses. Dietary amino acids modulate key life history traits including development, fecundity, and lifespan. Restriction of individual essential amino acids (EAAs), or an imbalanced proportion between some EAAs, also affects lifespan. In this chapter, it is shown that an exome-matched supply of dietary AAs promotes production traits in flies more than do other commonly used dietary AA sources, including AAs provided in the proportion found in yeast. Furthermore, a comprehensive phenotypic characterization is carried out focusing on fecundity, development, lipostasis, proteostasis, nitrogen utilization, feeding behavior, AA bioavailability and sensing, glucose homeostasis, energy homeostasis, and ageing. Results show that exome-matching promotes fecundity and development, decreases fat storage, mitochondrial activity, and starvation resistance, increases proteasomal activity and nitrogen utilization, diminishes nitrogen losses, and defines feeding behaviour. These findings in *Drosophila* suggest that the exome composition of an organism may provide a suitable template for its AA requirements. Furthermore, dietary AA balance appears critical for the modulation of key life history traits, including growth, reproduction, and ageing.

4.3 Results

4.3.1 Fecundity predictions

After establishing the identity of the limiting EAA in HuntAA and MMAA (Chapter 3), a direct comparison of the fecundity of flies on HuntAA, MMAA and FlyAA showed that exome-matching quantitatively predicted egg-laying (Figure 4.1A), suggesting that the nutritional, rather than signaling, properties of the EAAs are important in determining their effect on egg production. The effect persisted in 65 day old females, where the three-fold increase of FlyAA over MMAA was preserved (Figure 4.1B). To test if the arginine limitation in HuntAA affects fecundity independently of dietary nitrogen levels we repeated fecundity tests on diets with arginine modulations across various dietary N levels (25N-300N). Interestingly, although predictions were accurately confirmed at lower dietary nitrogen levels (Figure 4.1C), this was not the case at higher dietary N levels (300N), where the observed responses of dietary arginine modulations were less severe than the predicted ones (Figure 4.1D). Further analysis of the relationship between observed vs. predicted fecundity across dietary nitrogen levels showed that the exome-predicted response overestimated the observed response at nitrogen levels of >100N against both HuntAA and MMAA (Figure 4.1E). Examination of the interaction between dietary nitrogen and the AA ratio showed that increases in fecundity were linearly correlated to the dietary nitrogen content (expressed as mM of N) for HuntAA and MMAA, but this was not the case for FlyAA, which was instead best described by a logarithmic fit (Figure 4.1F). This implied that the physiological limit for egg production is approximated by FlyAA when dietary N levels are high. Indeed, although increasing dietary yeast, the fly's protein source in a common sugar-yeast diet, increases fecundity (Figure 4.1G) flies fed a high nitrogen FlyAA diet were no more fecund than flies fed live yeast, which maximizes egg laying (Figure 4.1H-I). Therefore, the difference between the predicted and observed values in high nitrogen diets (Figure 4.1E) is the result of the divergence of the linear (HuntAA and MMAA) vs. logarithmic (FlyAA) fecundity increases as dietary nitrogen is raised (Figure 4.1F). Combined these observations indicate that exome-matching increases the utilization of dietary AAs to maximize egg-laying, and that increasing dietary nitrogen alleviates the fecundity-reducing effect of a limiting EAA in the diet. Therefore the response of fecundity depends on the dietary nitrogen levels, with fecundity modulations accurately predicted at low but not high levels.

The finding that exome information, unweighted by transcriptional or translational information, is such an accurate predictor of egg laying was unexpected, so the ovarian accrued proteome was analyzed to see whether its AA abundance is uniquely identical to the mean AA usage in the exome. However, this was not the case as AA usage did not vary considerably between the fly ovarian and other tissue-specific proteomes (Figure 4.2A; data by Dr. Ilian Atanassov). Thus, the possibility that the ovarian expressed transcriptome uses AAs in a manner that is representative of the whole genome was investigated. To do this, all *in-silico* translated genes were

ranked on the basis of how similar their predicted AA usage is compared with that of the median usage for the whole exome. From the most predominantly abundant egg proteins, the three highly-expressed yolk proteins ranked in the top 25% of genes most similar to the exome ratio (Figure 4.2B; data by Dr. Matthew D. Piper, Dr. Eric Blanc, and Dr. Ilian Atanassov), while the average ranking of the ovarian-expressed transcripts (207) was smaller than expected by chance when compared with randomly selected gene lists of the same size (P=0.09, CATMAP (208); Figure 4.2C; data by Dr. Matthew D. Piper, Dr. Eric Blanc), which was not the case for other tissues. Thus, the ovarian transcriptome appears constrained towards encoding AAs in exome-like proportions to maximize future biomass production.

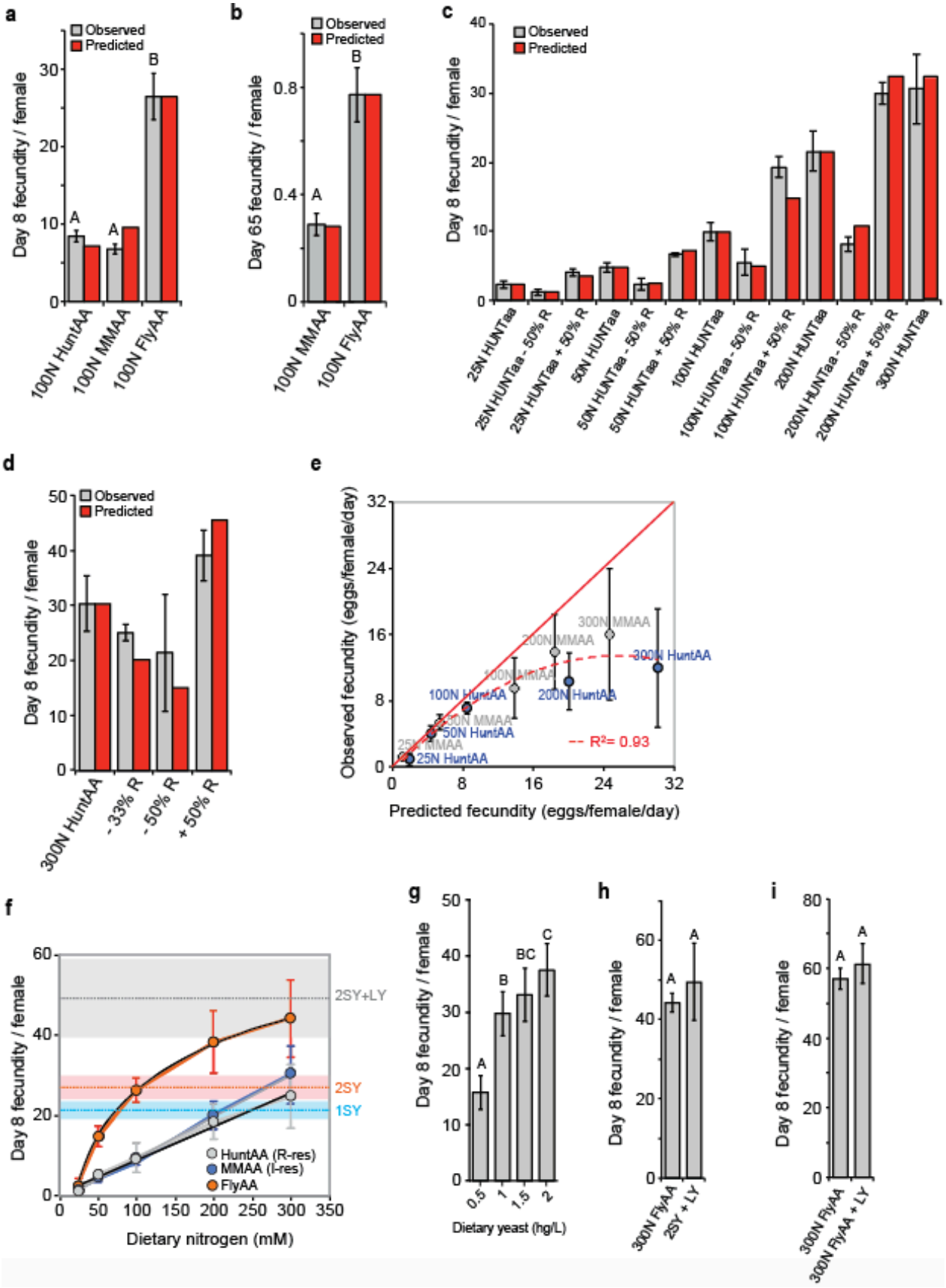


Figure 4.1

a. Egg laying predictions for *Drosophila* females fed MMAA and HuntAA diets at 100N dietary nitrogen (N) levels. Observed egg laying (grey bars) for both MMAA and HuntAA supplied at

100N dietary nitrogen levels matches the predictions made by exome matching (red bars) for each ratio. N = 6-14 trials per treatment, with each trial using (per treatment) 6 biological replicates of 10 flies. Error bars represent SD, letters indicate statistically significant differences ($p<0.05$) between pair-wise comparisons (Student's t-test).

b. The predicted fecundity differences between FlyAA and MMAA match the observed fecundity responses even at older ages (65 day-old females). Each bar represents 3 trials, each trial using 6 biological replicates of 10 flies per treatment. Error bars represent SD, letters indicate statistically significant differences ($p<0.05$) between pair-wise comparisons (Student's t-test).

c. Day 8 fecundity for dahomey females across dietary nitrogen levels, expressed as mM concentration of biologically available nitrogen in the diet. Modulations include changes in total AAs (25N-300N) or changes in the limiting AA arginine (+/- R). Each bar represents >2 trials, each trial using 60 flies per treatment. Error bars represent SD (1-way ANOVA analysis with Dunnet posthoc test, F ratio = 10.48; P value < 0.0001 for multiple comparisons to 25N HuntAA control treatment).

d. Effect of high dietary nitrogen on single EAA manipulations at high dietary nitrogen levels. The observed fecundity response to increases or decreases in Arginine is less potent than the predicted predicted response when dietary nitrogen is at the higher level of 300N. At this level, both the increase or decrease of fecundity as a response to dietary Arginine adjustment reach approximately half of the predicted extent. Each bar represents 2-7 trials, each trial using (per treatment) 6 biological replicates of 10 flies. Error bars represent SD (1-way ANOVA analysis with Dunnet posthoc test, F ratio = 0.8700; P value = 0.4884).

e. Observed versus predicted fecundity for HuntAA and MMAA across dietary nitrogen (N) levels. The red diagonal starting from the origin of (*x,y*) indicates the predicted relationship between dietary N and fecundity when fecundity is independent of dietary N. However the observed values indicate that the accuracy of fecundity predictions depends on dietary N levels as the predicted response overestimates the observed response for both HuntAA and MMAA at >100N levels, where the exome-based predictions gradually start to fail. At 300N, a reduction of 33% Arginine reduced fecundity by only 20%, while a reduction of dietary Arginine by 50% reduced fecundity by 34%. Moreover, an increase of 50% Arginine increases fecundity only by 25%. Each bar represents 4-16 trials, each trial using (per treatment) 6 biological replicates of 10 flies. Error bars represent SD.

f. Observed fecundity for FlyAA, HuntAA, and MMAA across dietary nitrogen levels (25N-300N). The fecundity increases for HuntAA and MMAA are linearly correlated to the increasing intake of nitrogen (in the form or amino acids) and are best described by a liner fit (R^2 values for HuntAA and MMAA are 0.998 and 0.986 respectively). In contrast, the fecundity increase for FlyAA shows a negative acceleration phase, so the curve is best described by a logarithmic fit ($R^2 = 0.993$). Each bar represents 4-16 trials, each trial using (per treatment) 6 biological replicates of 10 flies. Error bars represent SEM (2-way ANOVA analysis with Bonferonni posthoc test, HuntAA vs. FlyAA F ratio = 1.099; MMAA vs. FlyAA F ratio = 1.172; for both comparisons, N level-AA ratio interaction P value > 0.05, and both N level and AA ratio P value < 0,0001).

g. Observed fecundity for a range of sugar-yeast diets across a range of yeast dilutions. Each bar represents (per treatment) 6 biological replicates of 10 flies. Error bars represent SEM, letters indicate statistically significant differences ($p<0.05$) between pair-wise comparisons (Student's t-test).

h/i. Day 8 fecundity for dahomey females fed the exome-matched diet (FlyAA) at high dietary nitrogen levels (300N) compared with females fed (d) a high sugar-yeast diets (2SY) supplemented with live yeast (LY), or (e) a 300N/FlyAA diet supplemented with LY. Each bar represents >3 trials, each trial using 60 flies per treatment. Error bars represent SD. Statistical analysis uses Student's T-test for pair-wise comparisons.

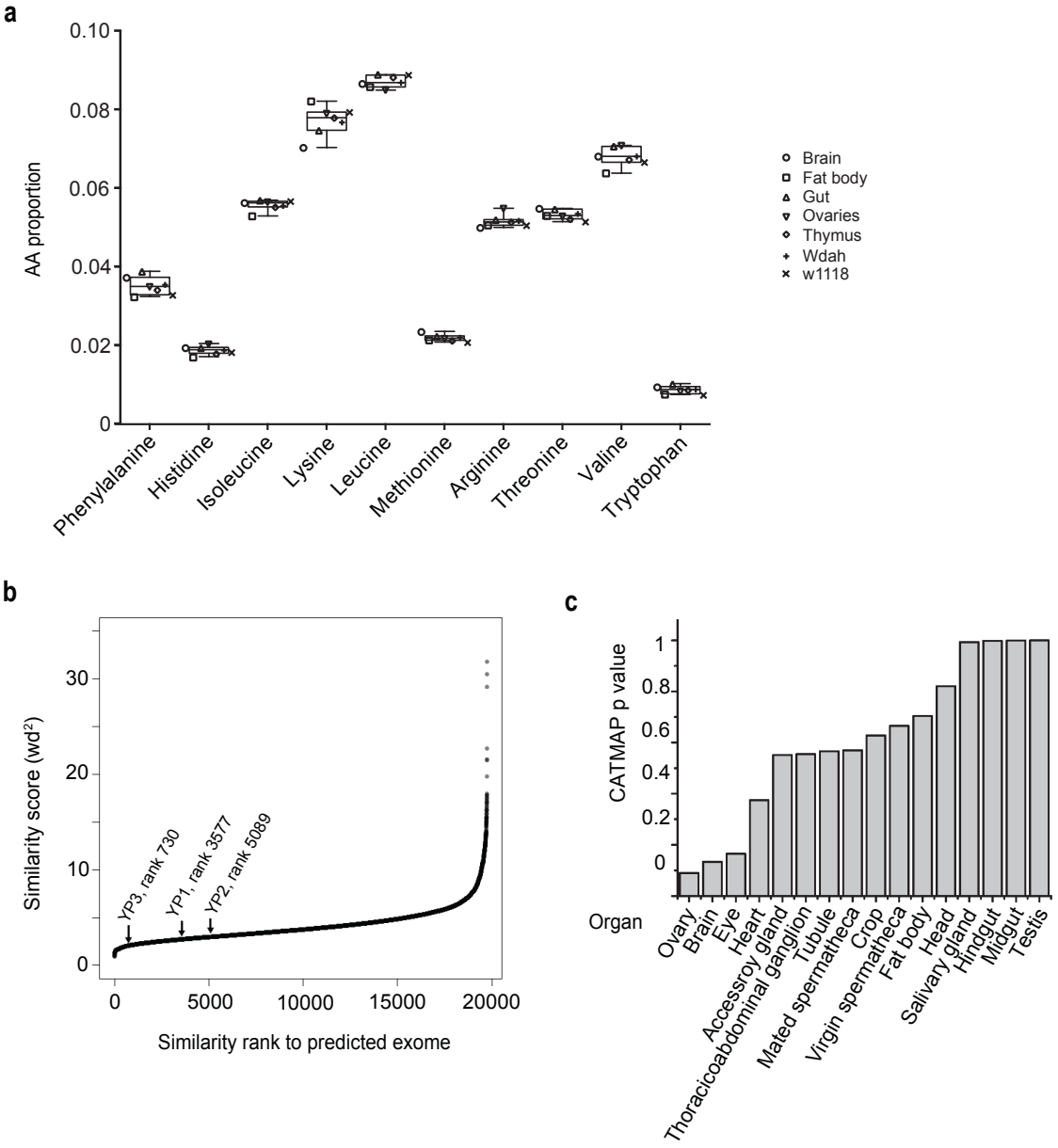


Figure 4.2

a. Proportional AA usage in the tissue-specific proteome in Wdah flies, as determined by SILAC protein quantification. The AA content of ovaries is within the 95% CI of the mean AA usage from all other tissues, indicating that it is very similar in AA usage to other tissues. The AA usage of w1118 flies is also similar to that of Wdah flies, indicating the genetic background differences between the two strains has no effect upon AA usage. Data provided by Dr. Ilian Atanassov.

b. Ovarian expressed genes use amino acids in a ratio representative of that of the whole exome. All in silico translated genes were ranked by how similar their proportional amino acid usage is compared with that of the mean AA usage for all genes in the predicted fly exome. Genes were ranked from most to least similar according to a distance measure: wd2 = sqrt (sum over all amino acids of((ratio in protein – mean ratio in proteome)/sd ratio in exome)^2). The three yolk proteins (YP) ranked in the top 25% of genes. Data by Dr. Matthew D. Piper, Dr. Eric Blanc, and Dr. Ilian Atanassov.

c. We interrogated how likely it is that the average rank of tissue-specific subsets of genes, identified in FlyAtlas (207), could be found by chance. This revealed that the ovarian expressed genes may have been specifically constrained towards using amino acids in the same proportion as that of the whole genome (P=0.09, CATMAP). Data by Dr. Matthew D. Piper and Dr. Eric Blanc.

To check if the effects of MMAA and HuntAA on fecundity depended on insulin signaling, the fecundity response of females that do not secrete insulin-like peptides 2-3, and 5 due to a knock-out mutation (dilp2-3,5) (154) was tested. Although dilp2-3,5 knock-out females showed much lower levels of fecundity than dahomey females in the holidic medium, the fecundity differences observed between AA ratios in dahomey flies were also seen in dilp2-3,5 flies (Figure 4.3A). This suggested that the effects of MMAA and HuntAA on fecundity were at least partially independent of the presence of dILPs 2-3,5. Similarly, to establish if the effects of HuntAA on fecundity depended on regulation of the target of rapamycin (TOR), the fecundity levels of females on HuntAA and FlyAA upon inhibition of TOR by rapamycin was determined. Rapamycin decreased fecundity regardless of the dietary AA ratio, but fecundity differences between the two ratios were still preserved (Figure 4.3B). These observations suggest that the effects of exome-predicted limiting dietary AAs upon fecundity are partially independent of suppression in IIS or TOR signaling.

However, despite the fact that the observed fecundity effects were very close to the predicted values (Figure 4.1A), some discrepancies between the observed and predicted fecundity effects were also seen. Therefore, MMAA had a marginally lower mean fecundity than that predicted, while an HuntAA had a marginally higher mean fecundity than that predicted (Figure 4.1A). To establish whether this discrepancy may reflect a biological effect stemming from the identity of the limiting EAA in these exome-mismatched dietary AA profiles, the fecundity of females fed the exome AA profile (FlyAA) but with an 80% reduction in individual EAAs was assessed. Restricting individual AAs had a diverse effect on fecundity, with isoleucine restriction reducing day 8 egg-laying more than did arginine, histidine, or methionine restriction (Figure 4.3C). Moreover, an 80% restriction of histidine or methionine resulted in a negligible suppression of fecundity across dietary nitrogen levels, whereas 80% restriction of all other EAAs significantly suppressed fecundity (Figure 4.3D). In addition, upon deprivation of each single EAA in a high nitrogen diet (300N HuntAAA), only histidine and methionine deprivations failed to immediately suppress fecundity (Figure 4.3E), resulting in significantly higher levels of lifetime fecundity (Figure 4.3F).

As discussed in Chapter 3 (Figure 4.s 1A-B), yeast, the common protein source of laboratory flies, is methionine-limiting (8). Therefore it was tested whether exome-based fecundity predictions are accurate upon a dietary AA profile matching that of yeast (YAA). The observed day 8 fecundity for flies fed YAA grossly surpassed the predicted value and matched that observed in flies fed FlyAA (Figure 4.3G), suggesting that the exome-based fecundity prediction is not accurate when the limiting EAA is methionine. Moreover, there where no significant differences between YAA and FlyAA across dietary nitrogen levels (Figure 4.3H), but when the prediction was adjusted to the next

most limiting EAA (leucine) it accurately reflected the observed values (Figure 4.3I). Similarly in the HuntAA ratio, considering the second predicted most limiting EAA methionine was a less accurate predictor of fecundity that considering the third predicted most limiting EAA leucine (Figure 4.3J). All these observations supported a replenishable role of methionine for *Drosophila* fecundity.

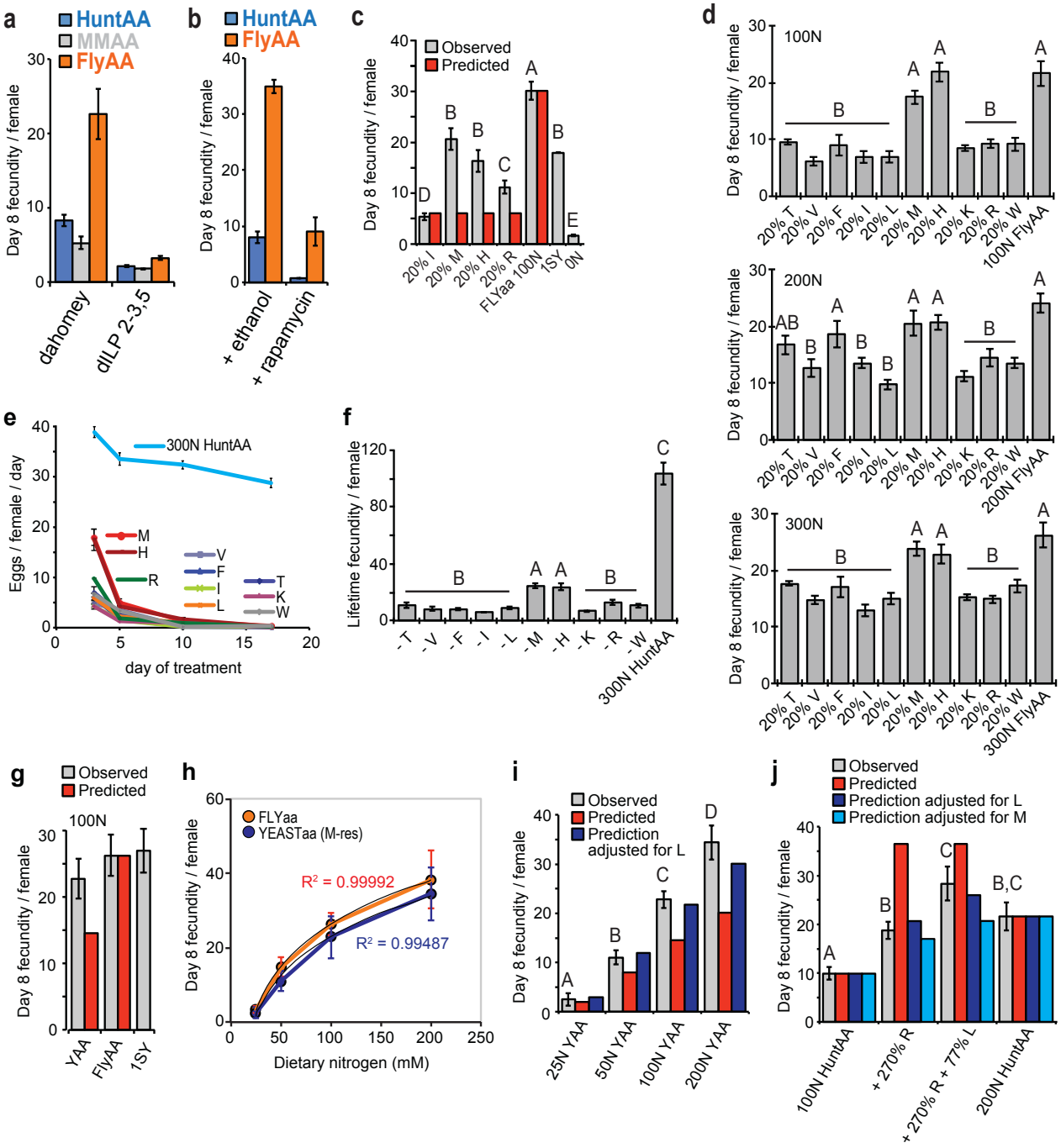


Figure 4.3

a. Day 8 fecundity for dahomey females and knock-out females lacking dILPs 2-3,5. The effects of dietary AA ratios on fecundity appear to be at least partially independent of the presence of dILPs 2-3,5 as the fecundity differences observed across AA ratios in dahomey females were also seen in flies lacking dILPs 2-3,5. Each bar represents two trials, each trial

using 60 flies per treatment. Error bars represent SD (1-way ANOVA analysis with Dunnet posthoc test, F ratio = 4.276; P value = 0.0097).

b. Day 8 fecundity for dahomey females with and without rapamycin (5μM). The effects of dietary AA ratios on fecundity appear to be at least partially independent of the presence of dILPs 2-3,5 as the fecundity differences observed across AA ratios in dahomey females were also seen in flies lacking dILPs 2-3,5. Each bar represents two trials, each trial using 60 flies per treatment. Error bars represent SD (1-way ANOVA analysis with Dunnet posthoc test, F ratio = 3.571; P value = 0.0036).

c. Observed and predicted fecundity responses for a range of 80% restrictions of selected EAAs (100N). Each bar represents (per treatment) three experiments, each with 6 biological replicates of 10 flies. Error bars represent SEM. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

d. Observed fecundity for a range of 80% restrictions of all EAAs, and across a range of dietary nitrogen (100N, 200N, 300N for the top, middle, and bottom panels respectively). Each bar represents (per treatment) 6 biological replicates of 10 flies. Error bars represent SEM. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

e-f. Observed fecundity for complete EAA deprivations in a HuntAA 300N diet. Except for the EAA noted as deprived with a minus sign, all others are at the concentration found in the HuntAA 300N diet. Each bar represents (per treatment) 6 biological replicates of 10 flies. Error bars represent SEM. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

g. Observed and predicted fecundity responses for YAA and FlyAA at 100N. Each bar represents 6 or more trials, each using (per treatment) 6 biological replicates of 10 flies. Error bars represent SEM. (1-way ANOVA analysis with Dunnet posthoc test, F ratio = 1.036; P value > 0.05).

h. Observed fecundity for YAA and FlyAA across dietary nitrogen levels (25-200N). Each bar represents 3 or more trials, each using (per treatment) 6 biological replicates of 10 flies. Error bars represent SEM. (2-way ANOVA analysis with Bonferonni posthoc test, P value = 0.0376 for the N level-AA ratio interaction, F ratio = 2.683; N-level factor P value < 0,0001, AA ratio P value = 0.2427).

i. Observed versus predicted fecundity for YAA and FlyAA across dietary nitrogen levels (25-200N). Each bar represents 3 or more trials, each using (per treatment) 6 biological replicates of 10 flies. Error bars represent SEM. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

j. Observed versus predicted fecundity for HuntAA with additions of the most limiting EAA (arginine), the second most limiting EAA (leucine), and the third most limiting EAA (methionine). Each bar represents 2 or more trials, each using (per treatment) 6 biological replicates of 10 flies. Error bars represent SEM. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

4.3.2 Amino acid sensing and perception

As FlyAA promoted key production traits in flies including fecundity, and fly fecundity is known to be modulated by the fly's fat body, sensing of the different AA sources could involve changes in AA-sensing pathways. A key protein involved in satiety induced by protein consumption is the target or rapamycin (TOR), which mediates AA sensing (77). S6K is one of the main downstream effectors of the target of rapamycin (TOR), which in turn is critical for AA sensing (77) and whose inhibition extends fly and rodent lifespan (113). Upon assessing S6K levels and activation in exome-matched and exome-mismatched diets, an increase in S6K activation in the heads of females fed the exome-matched (FlyAA) diet was observed, compared to exome-mismatched diets (HuntAA, MMAA) (Figure 4.4A).

To test if an exome-matched diet is perceived by flies as a more potent source of protein the feeding rates and food intake of flies kept at the different diets was quantified. Surprisingly, similar feeding rates and food intake across ratios and nitrogen levels were observed (Figure 4.s 4B-C). Perception of satiety by looking at food choice behavior was also tested. A feeding assay was developed to investigate the food preference behavior of *Drosophila*, whose method is described in detail in Chapter 2 (Methods). Flies were maintained for 3 days on holidic media lacking all AAs (0N), and then were presented with a choice of FlyAA or an exome-mismatched diet to assess their preference for HuntAA, MMAA, or FlyAA. Flies starved of all AAs for 3 days consistently showed a dietary preference for FlyAA over HuntAA or MMAA, but no preference for FlyAA against FlyAA, a trend that persisted across dietary nitrogen levels (Figure 4.4D). Combined, these data are consistent with exome-matching defining a perceptibly enhanced quality of protein to the adult fly.

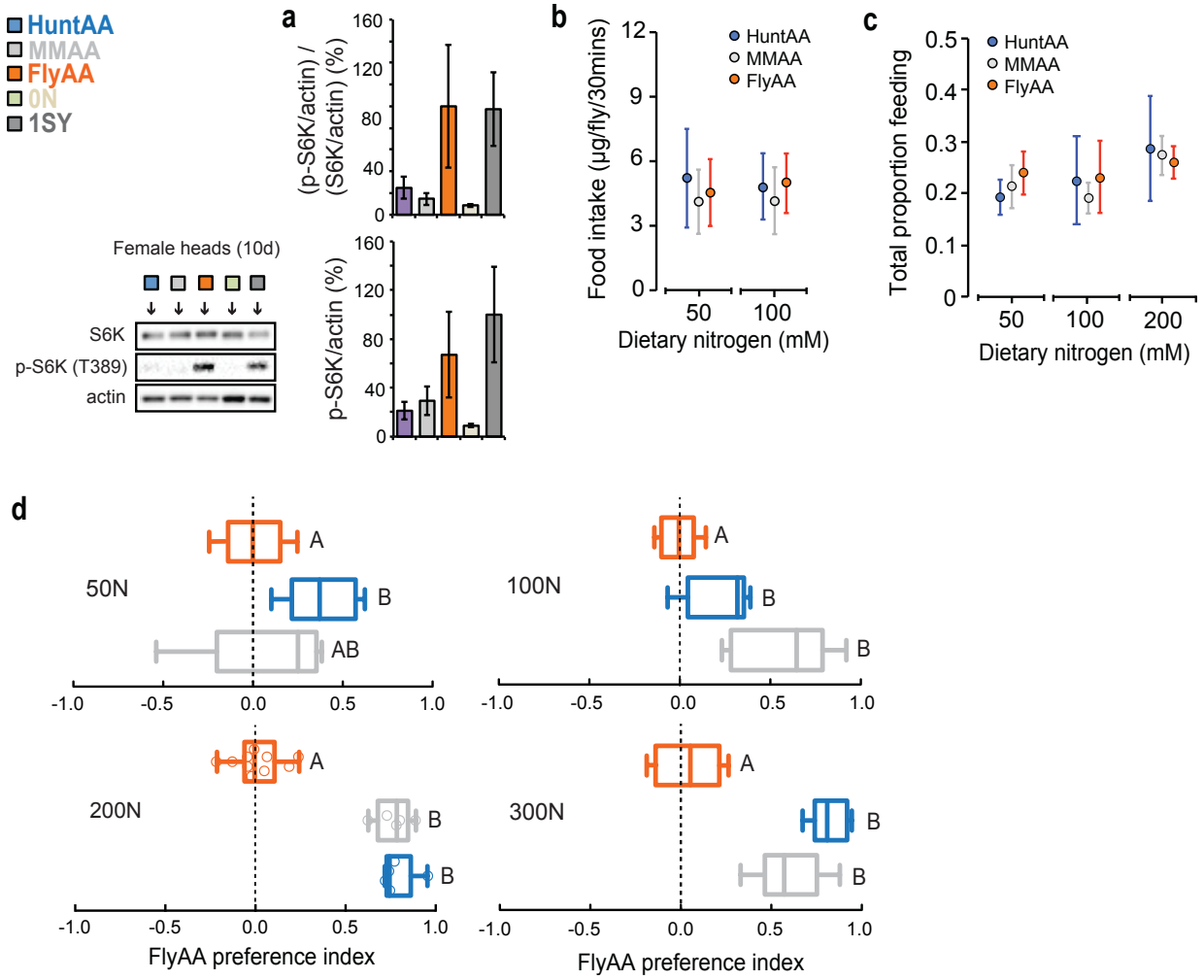


Figure 4.4

a. S6 kinase (S6K) protein and phosphorylation (T389) levels in the heads of 10-day old females across different dietary AA profiles. For quantification, each bar shown represents 4-5 biological replicates, each replicate using a protein extract from 20 heads per treatment. Error bars represent SEM.

b. Effects of dietary AA ratio on food intake. Food intake across the dietary AA ratios was quantified using blue-stained holidic media as previously described (169). For each assay, 50 mated female flies at 10 days of age (following 2 days mating in 1SY media and 8 days on the respective holidic diet) were placed in vials of 10 flies for each treatment. Each data point represents the mean of 5 trials (N=5), and error bars represent inter-trial SEM (2-way ANOVA analysis with Bonferonni posthoc test, P value > 0.05).

c. Proboscis Extension Behaviour (PEB) shows the feeding response of dahomey females to each ratio as previously described (169). Assays recorded the proportion of 2-day-mated females extending their proboscis to the medium at 3-10 days of age (3-4 every-other-day measurements per trial). Each trial used 5 or 10 females per vial, and 10 replicate vials per treatment (i.e. 50-100 flies per treatment per trial). For 50N and 100N treatments 5 trials were carried out (N = 5), while 3 trials were performed for 200N treatments (N = 3). Error bars represent inter-trial SD (2-way ANOVA analysis with Bonferonni posthoc test, P value > 0.05).

d. Dietary preference index. Colour of box indicates the AA profile offered to flies against FlyAA in a two-choice assay (see methods). Pre-starving flies of amino acids flies for 3 days resulted in an increased dietary preference for FlyAA over HuntAA or MMAA, but not over FlyAA, confirming flies perceived the exome ratio as a more attractive dietary AA source. Each bar represents 5-10 biological replicates, with 40 females per replicate. Error bars represent SD, letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data for Figure 4.4D by Hanna Salmonowicz and George Soultoukis.

4.3.3 Development

Development time is a valid indicator of *Drosophila*'s dietary AA requirements as the proportion of dietary AAs greatly impacts upon development rates (57). Moreover, protein-restricted diets also delay or inhibit growth both in flies (Figure 4.5A) and in rodents (28). As exome matching improves dietary AA utilization for fecundity, it was next tested whether the exome also defines diets to enhance development, because a large proportion of the genome is likely to be involved in the egg's transformation to an adult. *Drosophila* larvae were reared on each of the three AA ratios and measured egg to eclosion time. Confirming expectations, FlyAA supported faster development than either HuntAA or MMAA, especially at lower dietary nitrogen levels (Figure 4.5B). Interestingly, we observed the same trends for developmental survival, as larvae reared on FlyAA showed a significantly enhanced viability compared to larvae reared on HuntAA or MMAA, an effect that was more significant at lower levels of dietary nitrogen (Figure 4.5C). We also assessed the possibility that total biomass synthesis during development is affected by an exome-balanced dietary AA ratio supply. Comparison of female adult body masses upon eclosion showed a significantly increased body mass when dietary AAs were exome-matched, again more prominently at lower dietary nitrogen levels (Figure 4.5D). This effect was consistent in male flies (Figure 4.5E), indicating that AA usage for anabolic processes is not strictly affecting egg-production or ovarian-related processes, but is rather linked to how utilizable an EAA profile is towards whole-body biomass synthesis. Combined, these observations indicate that matching the dietary AA supply to the exome requirement enhances AA utilization for biomass synthesis, both in developing larvae and adult flies, especially at lower dietary nitrogen levels.

The endosymbiont bacterium Wolbachia can be essential for insect growth and development due to its provision of cofactors including B vitamins to the host (209), while in some cases endosymbionts may even produce and supply AAs to the host and accelerate development (210). Therefore it was tested whether the presence of Wolbachia affects development in the same conditions. The effects of dietary AA ratios on development time, viability, and body mass were independent of the presence of Wolbachia in both genders, as well as of fly strain (Figure 4.6A-D). In addition, the effects of dietary AA ratios on developmental time and viability were at least partially independent of the presence of dILPs 2,3-5 as differences for both traits observed across AA ratios in dahomey flies were also seen in flies lacking dILPs 2,3-5 (Figure 4.6E-F). Thus the developmental effects of FlyAA were independent of Wolbachia status or of the secretion of dILPs 2,3-5.

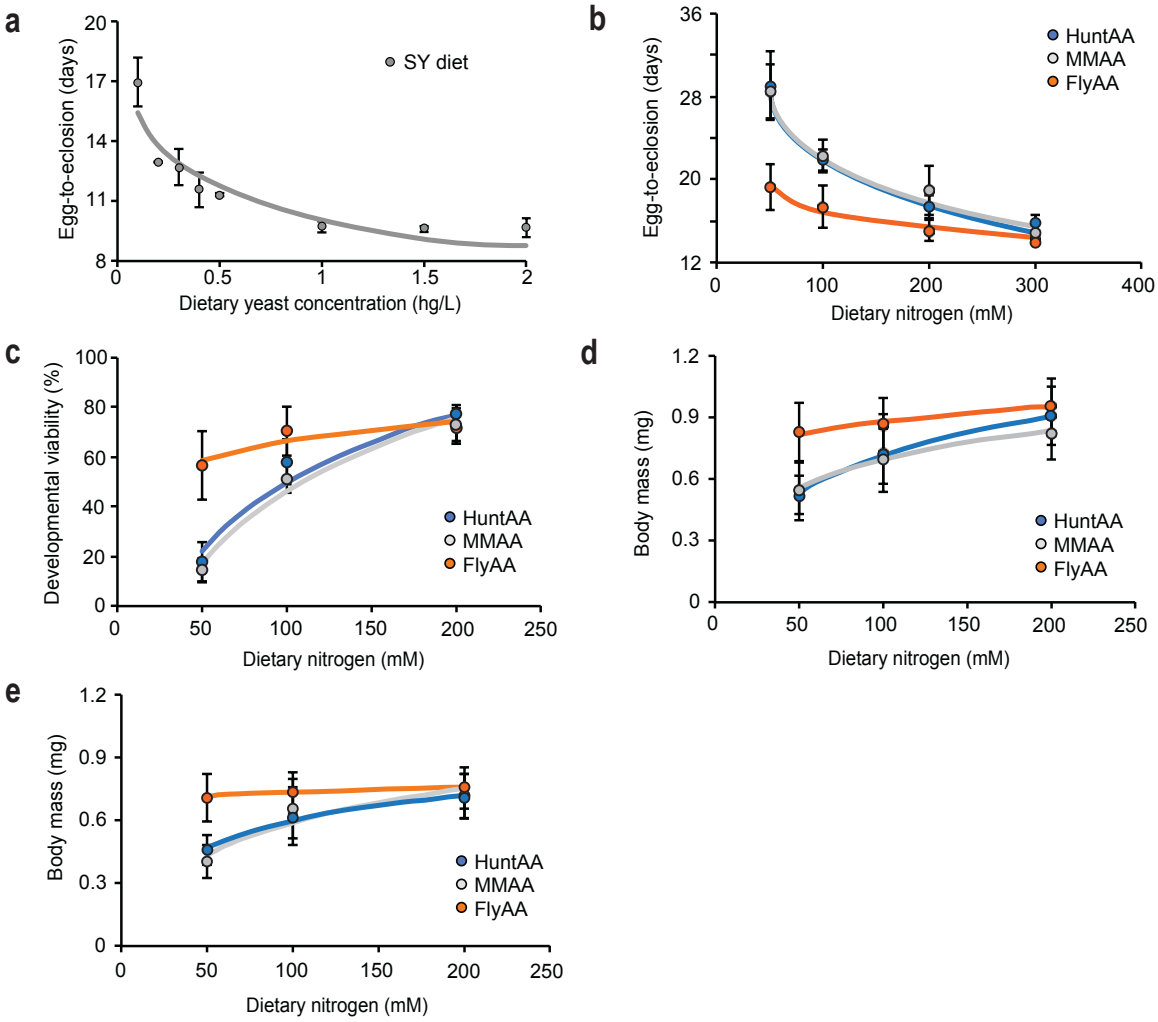


Figure 4.5

a. Development time as a function of dietary yeast concentration. Development is delayed when the supply of dietary yeast, the only protein source in the standard SY diet, is decreased. Each date point represents a trial using (per treatment) 5 biological replicates of 20 flies. Error bars represent SEM.

b. Effects of dietary AA ratio on developmental time across dietary nitrogen levels. Developmental delay for both male and female flies, seen as prolonged egg-to-eclosion time (days), occurred in both exome-mismatched ratios (HuntAA and MMAA) compared to the exome-balanced ratio (FlyAA). However, the effect is prominent at low dietary N levels (<200N) and dissipates as dietary N increases. Error bars shown represent SD between trials, N = 5-10 trials per treatment, with 125 eggs per trial per treatment (2-way ANOVA analysis with Bonferonni posthoc test, HuntAA vs. FlyAA P value = 0.0153 for the time-AA ratio interaction, F ratio = 3.567; MMaa vs. FlyAA P value = 0.0231 for the time-AA ratio interaction, F ratio = 2.3565; for both comparisons, both viability and AA ratio P value < 0,0001).

c. Effects of dietary AA ratio on developmental viability across dietary nitrogen levels. Developmental survival, seen as proportion of eggs eclosing, is decreased in both exome-mismatched ratios (HuntAA and MMAA) compared to the exome-matched ratio (FlyAA). However, as for developmental timing, the effect is clear at low dietary N levels (<200N) but wanes as dietary N increases. Error bars shown represent SD between trials, N = 5-10 trials per treatment, with 125 eggs per trial per treatment (2-way ANOVA analysis with Bonferonni posthoc test, HuntAA vs. FlyAA P value < 0.0001 for the viability-AA ratio interaction, F ratio =

10.28; MMaa vs. FlyAA P value < 0.0001 for the viability-AA ratio interaction, F ratio = 17.54; for both comparisons, both viability and AA ratio P value < 0,0001).

d. Female body mass upon eclosion across dietary AA ratios and dietary nitrogen (N) levels. Upon eclosion (day 1) female body mass is decreased for flies reared in HuntAA or MMAA compared to flies reared in the exome ratio (FlyAA). However, as for developmental timing, the effect is strong at low dietary N levels (<200N) but less so as dietary N increases. Error bars represent SD between trials, N = 5-10 trials per treatment, with 125 eggs per trial per treatment (2-way ANOVA analysis with Bonferonni posthoc test, HuntAA vs. FlyAA P value < 0.0001 for the mass-AA ratio interaction, F ratio = 15.31; MMaa vs. FlyAA P value = 0.0057 for the mass-AA ratio interaction, F ratio = 5.24; for both comparisons, both mass and AA ratio P value < 0,0001).

e. Male body mass upon eclosion across dietary AA ratios and dietary nitrogen (N) levels. Upon eclosion (day 1) male body mass is decreased for flies reared in HuntAA or MMAA compared to flies reared in the exome ratio (FlyAA). However, as for developmental timing, the effect is strong at low dietary N levels (<200N) but less so as dietary N increases. Error bars represent SD between trials, N = 5-10 trials per treatment, with 125 eggs per trial per treatment (2-way ANOVA analysis with Bonferonni posthoc test, HuntAA vs. FlyAA P value < 0.0001 for the mass-AA ratio interaction, F ratio = 14.93; MMaa vs. FlyAA P value < 0.0001 for the mass-AA ratio interaction, F ratio = 29.44; for both comparisons, both mass and AA ratio P value < 0,0001).

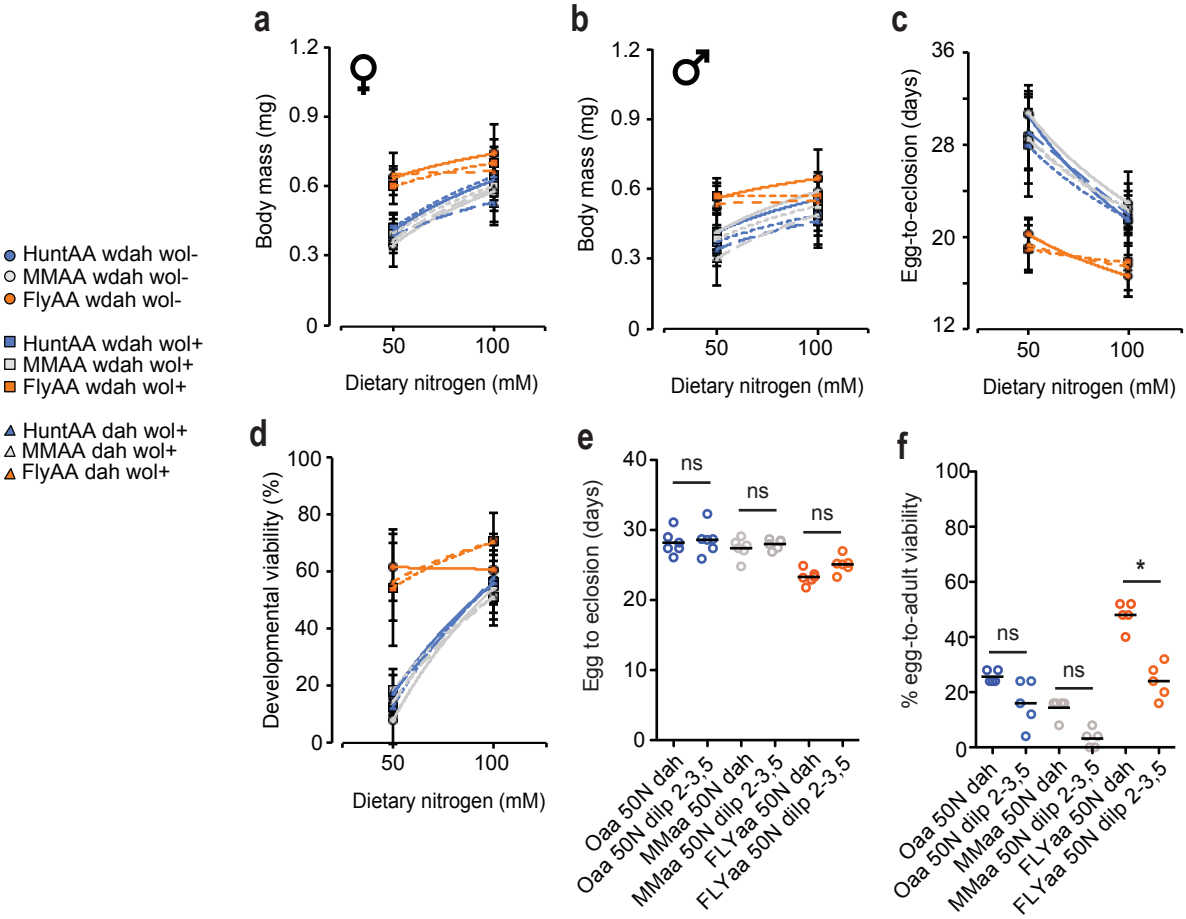


Figure 4.6

a/b. Female (a) and male (b) body mass upon eclosion across dietary AA ratios and dietary nitrogen (N) levels of dahomey (Wolbachia +), and white dahomey (Wolbachia+ or Wolbachia-). Upon eclosion (day 1) both female and male body mass is decreased for flies reared in HuntAA or MMAA compared to flies reared in the exome ratio (FlyAA), and this is independent of the presence of Wolbachia (+ vs. -) or the fly strain (dahomey vs. white dahomey). Moreover, the effect is strong at low dietary N levels (<200N) but less so as dietary nitrogen (N) increases. Error bars shown represent SD, while N = 5 biological replicates of 25 eggs per treatment (2-way ANOVA analysis with Bonferonni posthoc test, HuntAA vs. FlyAA P value < 0.05 for the N level-AA ratio interaction, F ratio = 3.12; MMaa vs. FlyAA P value < 0.05 for the N level-AA ratio interaction, F ratio = 2.67; for both comparisons, both N level and AA ratio P value < 0,0001).

c. Effects of dietary AA ratio on developmental time across dietary nitrogen levels of dahomey (Wolbachia +), and white dahomey (Wolbachia+ or Wolbachia-). Developmental delay for both male and female flies, seen as prolonged egg-to-eclosion time (days), occurred in both exome-mismatched ratios (HuntAA and MMAA) compared to the exome-balanced ratio (FlyAA) and this is independent of the presence of Wolbachia (+ vs. -) or the fly strain (dahomey vs. white dahomey). However, the effect is prominent at low dietary N levels (<200N) and dissipates as dietary N increases. Error bars shown represent SD, while N = 5 biological replicates of 25 eggs per treatment (2-way ANOVA analysis with Bonferonni posthoc test, HuntAA vs. FlyAA P value < 0.05 for the N level-AA ratio interaction, F ratio = 8.941; MMaa vs. FlyAA P value < 0.05 for the N level-AA ratio interaction, F ratio = 6.54; for both comparisons, both N level and AA ratio P value < 0,05).

d. Effects of dietary AA ratio on developmental viability across dietary nitrogen levels of dahomey (Wolbachia +), and white dahomey (Wolbachia+ or Wolbachia-). Developmental

survival, seen as proportion of eggs eclosing, is decreased in both exome-mismatched ratios (HuntAA and MMAA) compared to the exome-matched ratio (FlyAA) and this is independent of the presence of Wolbachia (+ vs. -) or the fly strain (dahomey vs. white dahomey). However, as for developmental timing, the effect is clear at low dietary N levels (<200N) but wanes as dietary N increases. Error bars shown represent SD, while N = 5 biological replicates of 25 eggs per treatment (2-way ANOVA analysis with Bonferonni posthoc test, HuntAA vs. FlyAA P value < 0.05 for the N level-AA ratio interaction, F ratio = 3.12; MMaa vs. FlyAA P value < 0.0001 for the N level-AA ratio interaction, F ratio = 1.02; for both comparisons, both N level and AA ratio P value < 0,0001).

e. Effects of dietary AA ratio on developmental time across dietary nitrogen levels of dahomey females, and of females that do not secrete insulin-like peptides 2-3, and 5 due to a knock-out mutation (dilp2-3,5) (42)(154). Developmental delay for both male and female flies, seen as prolonged egg-to-eclosion time (days), occurred in both exome-mismatched ratios (HuntAA and MMAA) compared to the exome-balanced ratio (FlyAA) and this is independent of dilps2-3,5. N = 5 biological replicates of 25 eggs per treatment. Statistical analysis uses Student's T-test for pair-wise comparisons.

f. Effects of dietary AA ratio on developmental viability across dietary nitrogen levels of dahomey females, and of females that do not secrete insulin-like peptides 2-3, and 5 due to a knock-out mutation (dilp2-3,5) (42)(154). Developmental survival, seen as proportion of eggs eclosing, is decreased in both exome-mismatched ratios (HuntAA and MMAA) compared to the exome-matched ratio (FlyAA) and this is independent of dilps2-3,5. N = 5 biological replicates of 25 eggs per treatment. Statistical analysis uses Student's T-test for pair-wise comparisons.

4.3.4 The metabolic fate of ingested AAs

Having established that exome-matching can promote anabolic traits, other aspects of AA metabolism were next considered. There are common processing steps that all AAs go through during absorption, metabolism, and excretion. Ingested AAs first encounter intestinal cells and gut bacteria, where some AAs are terminally metabolized. Next, AAs pass through the rest of the splanchnic bed, where more AAs are metabolized *in situ*. In the liver, AAs can have a rather diverse metabolic fate. They can be directly utilized for protein synthesis, with the rate of protein synthesis and degradation determining proteostasis. Alternatively, they can be broken down to their carbon skeletons and amino groups. Although amino groups are typically excreted, carbon skeletons can be used for lipogenesis, respiration, or glucogenesis. The balance between lipogenesis and lipolysis determines lipostasis, while cellular respiration also determines energy homeostasis. Finally, glucose and glycogen synthesis determine glucose homeostasis. To compare imbalanced dietary AA ratios to the exome-matched ratio, focus was given on the multiple downstream biochemical pathways involved in AA processing, metabolism, and excretion. A series of phenotypes was assessed linked to such metabolic phenomena including nitrogen metabolism, lipostasis, respiration, energy homeostasis, glucose homeostasis, and proteostasis (Figure 4.7A).

4.3.5 Nitrogen losses

Degradation of AAs results in amino groups that are ultimately secreted by the fly as uric acid (Figure 4.7A). Comparing nitrogen losses to nitrogen intake provides an estimation of nitrogen balance, a measure of the difference between anabolism and catabolism of biologically available nitrogen (here, in the form of amino acids) (186). Differences in nitrogen balance therefore indicate how utilizable a nitrogenous source is (186). Therefore, to assess how utilizable the exome-matched (FlyAA) profile is compared to exome-mismatched (HuntAA, MMAA) profiles, uric acid excretion in *ad-libitum* fed adult females was quantified across AA ratios and dietary nitrogen levels. In all tested conditions, FlyAA consistently reduced the excretion of uric acid compared to HuntAA or MMAA (Figure 4.7B), indicating that FlyAA is a more bio-utilizable AA source. Moreover, we observed a negative correlation between day 8 fecundity and day 8 uric acid excretion (Figure 4.7C; R^2=0.54), suggesting that the higher usability of AAs in FlyAA contributes towards egg biosynthesis. In rodents, nitrogen utilization prior to the onset of a starvation period positively correlates to excretion of nitrogen during this starvation period, as animals on a positive nitrogen balance in the period before the onset of starvation secrete more nitrogen after the onset of starvation (211). Therefore we asked whether this positive correlation is also seen in flies in order to confirm the higher usability of FlyAA. We checked whether flies previously maintained on the more usable FlyAA can, upon starvation, catabolize and excrete more uric acid than flies previously fed on a less utilizable AA ratio (HuntAA, MMAA). During 16 hours of starvation, secretion of uric acid in adult females was the highest in females previously kept in 50-100N FlyAA (Figure 4.7D), reversing the trend observed in *ad libitum*-fed flies

(Figure 4.7B). Combined these results suggest that under normal feeding conditions, flies fed FlyAA utilize more of the ingested AAs compared to flies fed HuntAA or MMAA, and that upon starvation flies fed FlyAA catabolize more of their utilized nitrogen.

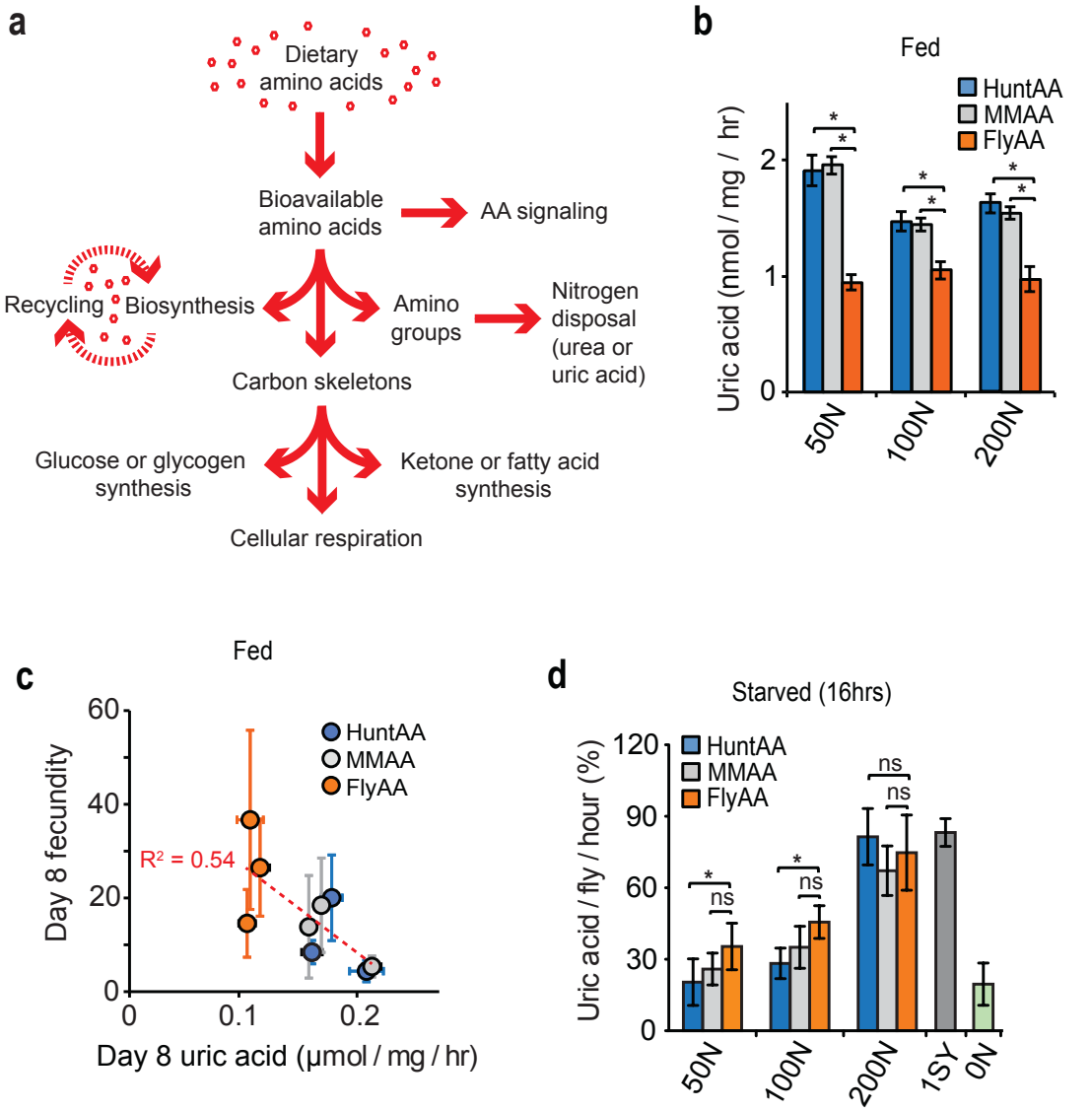


Figure 4.7

a. The metabolic fate of ingested amino acids. Modified from (69). See text for details.

b. Uric acid excretion of 10-day old *ad libitum*-fed females across different dietary AA profiles. For quantification, each bar shown represents 4 biological replicates, each replicate representing the uric acid excreted by 10 flies per treatment. Error bars represent SD, stars indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

c. Correlation of uric acid excretion of 10-day old *ad libitum*-fed females with day 8 fecundity across different dietary AA profiles. For quantification, each point represents 4 biological replicates on the x axis (uric acid concentration), and 4-16 biological replicates on the y axis (day 8 fecundity). For uric acid, each replicate representing the uric acid excreted by 10 flies per treatment, whereas for fecundity each trial used (per treatment) 6 biological replicates of 10 flies. Error bars represent SD.

d. Uric acid excretion of 10-day old 16-hour starved females across different dietary AA profiles. For quantification, each bar shown represents 3-4 trials, each trial representing 6 biological replicates, each replicate representing the uric acid excreted by 10 flies per treatment. Error bars represent inter-trial SD, stars indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

4.3.6 Lipid storage and starvation resistance

As discussed above, exome-matching increases body mass in adult male and female flies compared to exome-imbalanced dietary AA ratios. To establish if such body mass changes are due to changes in body composition, fat storage in adult females was examined. Upon analysis of the effect of dietary nitrogen in triacylglyceride (TAG) levels, an inverse relationship was observed between dietary nitrogen and TAGs in flies kept in YAA (Figure 4.8A). Moreover, flies with higher TAG levels survived longer upon starvation (Figure 4.8B), with a strong correlation between the two traits (R^2=0.93; Figure 4.8C). These results suggest that a high utilization of dietary AAs decreases fat storage and starvation resistance in flies. We therefore tested whether manipulating the AA source, rather than the overall AA intake, also has similar effects upon fat storage and starvation resistance. Interestingly, we saw decreased TAG storage in flies fed FlyAA (Figure 4.8D), and this was accompanied by a reduced starvation resistance across dietary nitrogen levels (Figure 4.8E-F). Analysis of this TAG storage and starvation resistance data also revealed a strong correlation between the two variables (Figure 4.8G). Therefore, an increase utilization of ingested AAs leads to leaner animals, which is detrimental for starvation resistance. Importantly, this leanness can be conferred by either an increase in the total dietary AA levels, or by a change in the profile of the ingested AAs. Interestingly, the decrease in TAG levels was not seen at lower dietary nitrogen levels (50N), as flies fed FlyAA were able to maintain their adipocity.

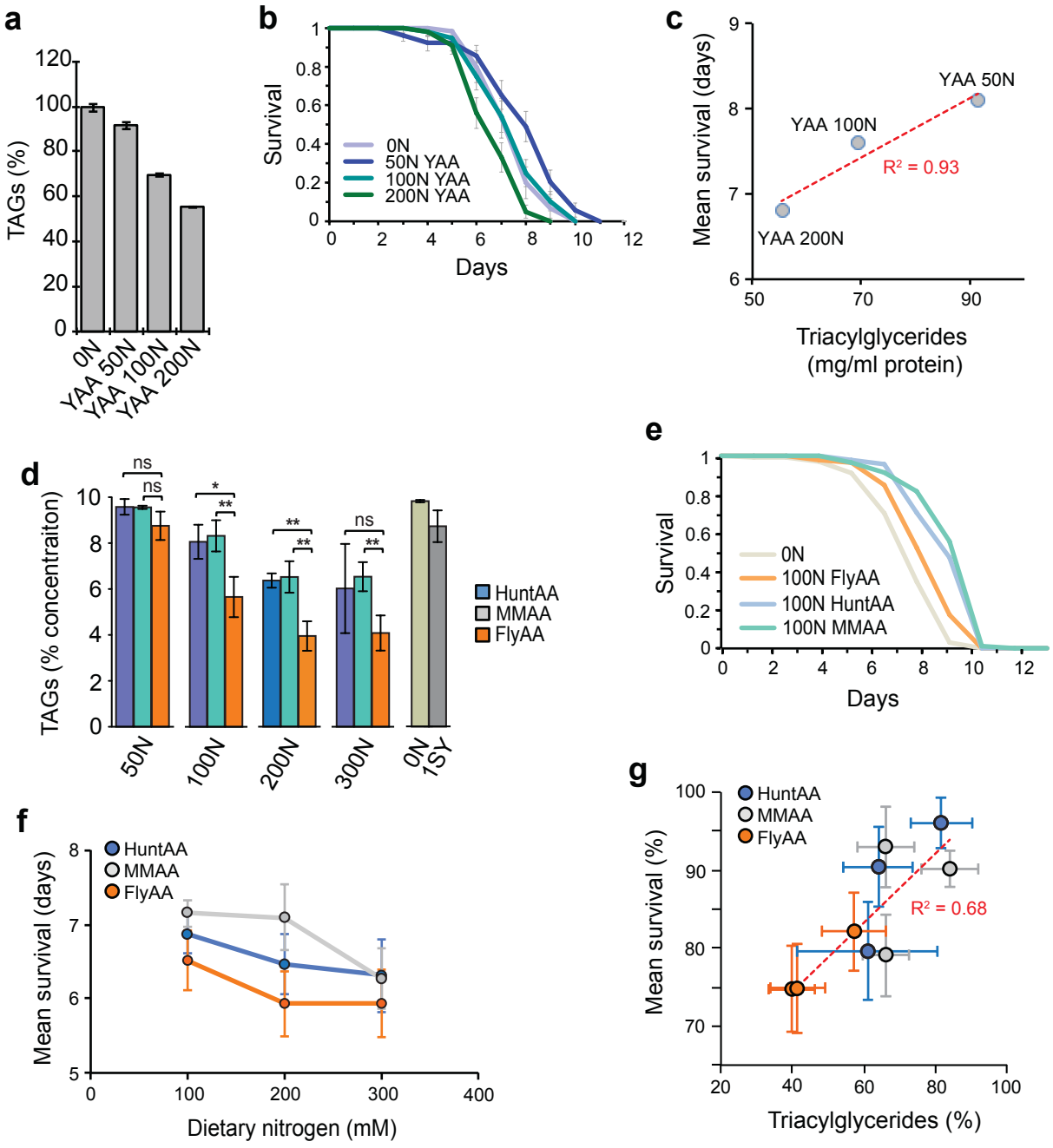


Figure 4.8

a. Triacylglyceride levels shown as % across dietary nitrogen levels (0N-200N) for flies kept in an AA ratio matching that of yeast (YAA). For quantification, each bar shown represents 2-5 trials, each trial representing 5 biological replicates, each replicate representing 5 flies per treatment. Error bars represent inter-trial SD, stars indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

b. Starvation resistance for 10-day old females on an agar-only medium. Flies were previously kept for 8 days across different dietary AA profiles as indicated in the panel. For each treatment N=100.

c. The amount of TAGs stored over 8 days of treatment in different dietary nitrogen levels (50N-200N) by flies kept in an AA ratio matching that of yeast (YAA) was positively correlated to the amount of uric acid excreted (R^2 = 0.93). Data points as described in Figure 4.s 8A-B.

d. Triacylglyceride levels across dietary nitrogen levels (0N-300N) for FlyAA, HuntAA, and MMAA in 10-day old females. Whole-body TAG levels were measured in adult flies (10 days) and normalized to total protein levels (25 flies per group per trial). For quantification, each bar shown represents 3-6 trials, each trial representing 5 biological replicates, each replicate representing 5 flies per treatment. Error bars represent inter-trial SD, stars indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

e. Starvation resistance for 10-day old females on an agar-only medium. Flies were previously kept for 8 days across different dietary AA profiles as indicated in the panel. For each treatment N=100.

f. Starvation resistance on an agar-only medium for 10-day old females. Flies were previously kept for 8 days across different dietary AA profiles as indicated in the panel. Values shown represent the mean from 2-4 independent trials, each trial using per treatment N=100.

g. The amount of TAGs stored over 8 days of treatment in different dietary AA profiles across nitrogen levels (50N-200N) was positively correlated to the mean survival upon starvation for flies pre-treated on the respective diet (R^2 = 0.68). Data points as described in Figure 4.s 8D-F.

4.3.7 Organ masses and protein content

Apart from the effects of exome-matching upon body composition (TAG contents), body masses of adult (10-day-old) females reared in standard sugar-yeast media and kept for 8 days in holidic diets of the respective treatments also increased significantly with FlyAA (Figure 4.9A). Moreover, higher gut dry weights were seen in females fed FlyAA (Figure 4.9B), but no differences were observed in the dry weight of whole heads (Figure 4.9C). To confirm the increased lean mass of flies fed FlyAA, the protein content in flies maintained in different dietary AA ratios was next assessed. Flies kept on FlyAA had significantly increased protein contents, both in terms of protein content per fly (Figure 4.9D) and of protein content per mg of fly tissue (Figure 4.9E; data for Figure 4.s 9B-E by Hanna Salmonowicz, Chirag Jain, and George Soultoukis).

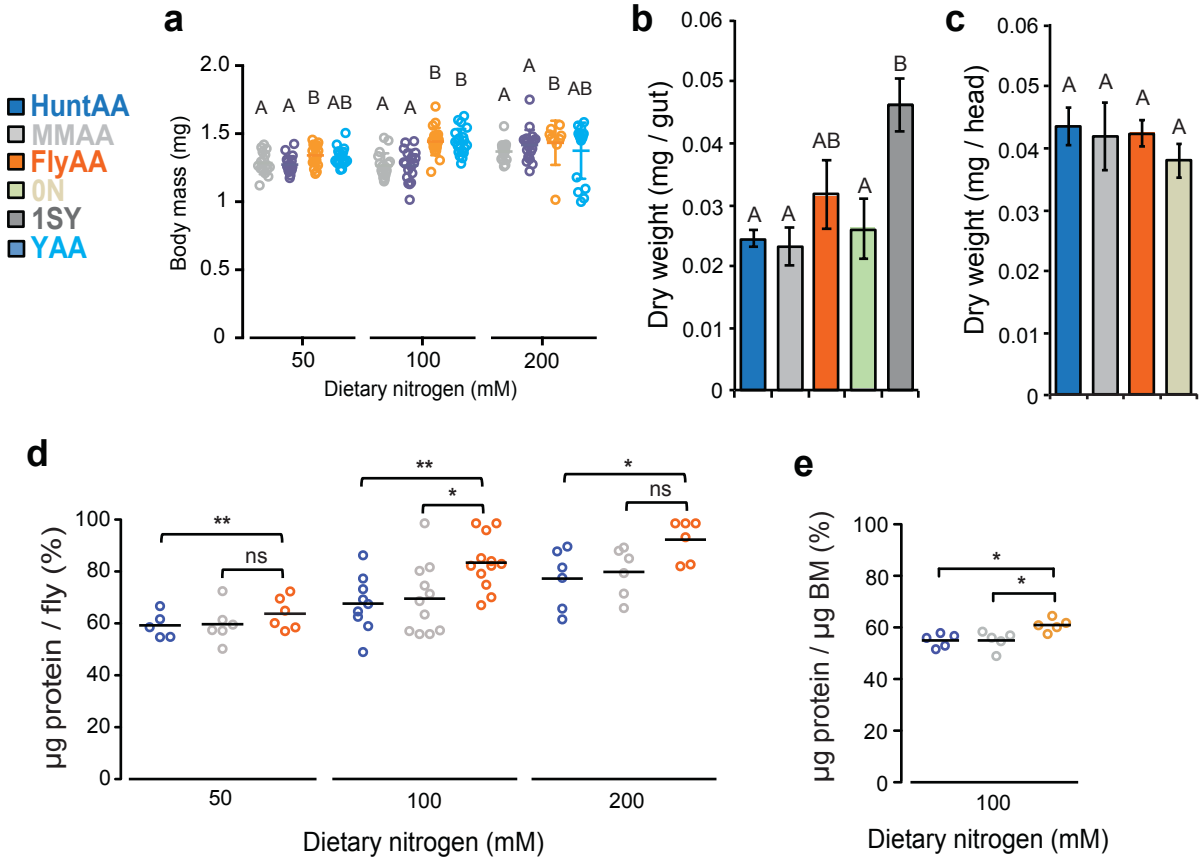


Figure 4.9

a. Effects of dietary AA ratio on adult female body mass across dietary nitrogen (N) levels. Day 10 female body mass is decreased for flies reared in both exome-mismatched ratios compared to flies reared in the exome-balanced ratio. However, as for day 1 body masses, the effect is strong at low dietary N levels (<200N) but less so as dietary N increases. Error bars shown represent SD between pairs of females weighed, N = 10-20 pairs of females weighed per treatment across 2 trials, letters indicate statistically significant differences ($p<0.05$) for pair-wise comparisons (Student's t-test) in one dietary N level.

b. The dry weight of guts for 10-day old females kept in 100N FlyAA, HuntAA, or MMAA. Each bar represents 4 biological replicates, each replicate using 20 guts per treatment. Error bars represent SD, letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

c. The dry weight of heads for 10-day old females kept in 100N FlyAA, HuntAA, or MMAA. Each bar represents 4 biological replicates, each replicate using 15 heads per treatment. Error bars represent SD, letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

d. Protein content in the whole body of 10-day old females kept in 50-200N FlyAA, HuntAA, or MMAA. Each data point represents 5 biological replicates, each replicate using 5 flies per treatment. Error bars represent SD, stars indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

e. Protein content in the whole body of 10-day old females kept in 100N FlyAA, HuntAA, or MMAA. Each data point represents 5 biological replicates, each replicate using 5 flies per treatment. Error bars represent SD, stars indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

4.3.8 Mitochondrial respiration

To assess the possibility that the ingested AA profile can induce changes in mitochondrial biogenesis, whole body levels of the voltage-dependent anion channel (VDAC), which is ubiquitously expressed and located in the outer mitochondrial membrane and is used as a marker of mitochondrial abundance and biogenesis, were first quantified. No differences in VDAC protein levels were seen between FlyAA and HuntAA or MMAA in whole female flies or in the head and thorax specifically (Figure 4.10A. The respiration rate of female flies across diets in a tissue-specific manner was next assessed. Analysis of oxygen consumption in the head (Figure 4.10B) and gut (Figure 4.10C) revealed no significant differences between females fed on FlyAA, MMAA, or HuntAA. However, oxygen consumption in the thorax (Figure 4.10D) and fat body (Figure 4.10E) of females fed FlyAA was significantly increased across all respiratory substrates, indicating that animals fed FlyAA exhibit a higher rate of mithochondrial respiration specifically on these two tissues (Figure 4.10 data by Chirag Jain, Hanna Salmonowicz and George Soultoukis).

4.3.9 Glucose homeostasis and glycogen synthesis

Although all of our fly test diets contain standard levels of sucrose (29), sugars and carbohydrates can also be produced from catabolites generated during AA metabolism (Figure 4.7A). Therefore to test if FlyAA, as a more usable AA source, enhances glucose utilization in the fly, whole-body glucose levels were assessed. However, no changes among the diets or across dietary nitrogen levels were observed (Figure 4.10F).

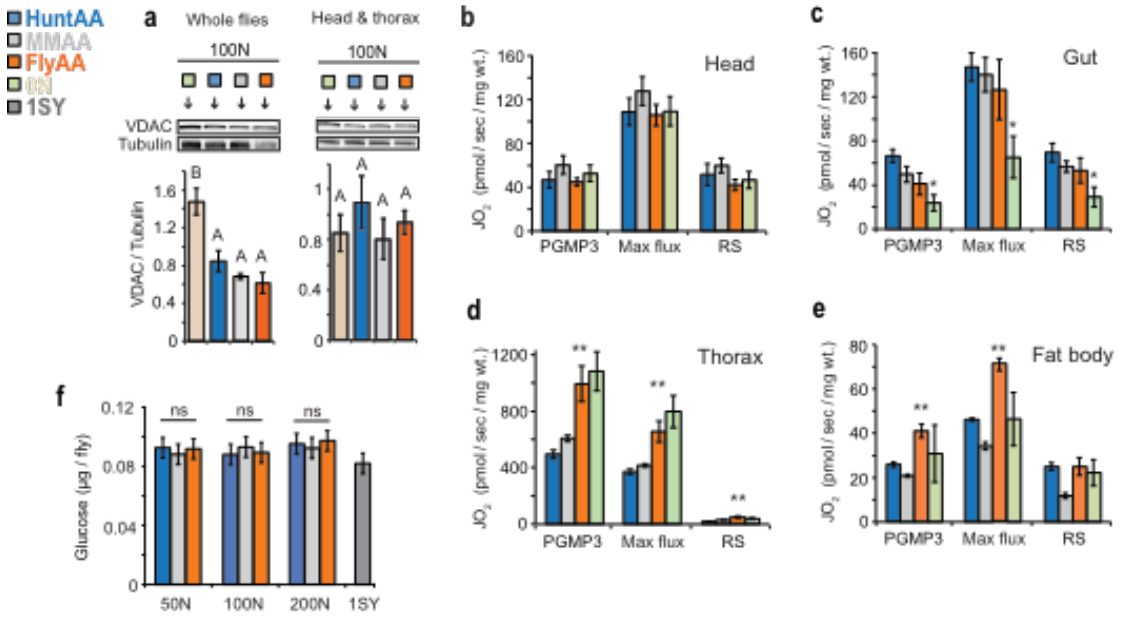


Figure 4.10

a. Protein levels of the voltage-dependent anion channel (VDAC) in the heads and thoraces of 10-day old females across different dietary AA profiles. For quantification, each bar shown represents 3 biological replicates, each replicate using a protein extract from 15 heads and thoraces per treatment. Error bars represent SEM, letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

b. Measurement of mitochondrial oxygen consumption in the head of 10-day old females across different dietary AA profiles. Rate of oxygen consumption normalized to dry weight content in response to different diet regimes. For quantification, each bar shown represents 4 biological replicates, each replicate using an extract from 15 heads per treatment. Error bars represent SD, stars indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

c. Measurement of mitochondrial oxygen consumption in the gut of 10-day old females across different dietary AA profiles. Rate of oxygen consumption normalized to dry weight content in response to different diet regimes. For quantification, each bar shown represents 4 biological replicates, each replicate using an extract from 20 guts per treatment. Error bars represent SD, stars indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

d. Measurement of mitochondrial oxygen consumption in the thorax of 10-day old females across different dietary AA profiles. Rate of oxygen consumption normalized to dry weight content in response to different diet regimes. For quantification, each bar shown represents 4 biological replicates, each replicate using an extract from 2 thoraces per treatment. Error bars represent SD, stars indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

e. Measurement of mitochondrial oxygen consumption in the fat body of 10-day old females across different dietary AA profiles. Rate of oxygen consumption normalized to dry weight content in response to different diet regimes. For quantification, each bar shown represents 4 biological replicates, each replicate using an extract from 15 fat bodies per treatment. Error bars represent SD, stars indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

f. Whole body glucose levels of 10-day old females across dietary nitrogen levels (50N-200N) and ratios, with 1SY as control. Bars represent the mean of 4 biological replicates, each replicate representing 5 flies per treatment. Error bars represent SD, letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

4.3.10 Proteostasis

To test whether exome-matching affects proteostasis in flies, the effects of FlyAA on protein sythesis, proteasome activity, poly-ubiquitination, and autophagy were tested (Figure 4.11 data by Hanna Salmonowicz). Polysome profiling and 35S-methionine incorporation revealed no differences between FlyAA, HuntAA, and MMAA in the protein sythesis rate (Figure 4.11A-D), although total levels of 4E-BP1 were significantly higher for MMAA and 0N, indicating a higher translational repression (Figure 4.11E). Proteasomal degradation was increased by FlyAA in both genders (Figure 4.11F-G), and in females this effect was not restricted to the germ-line but was also seen in somatic tissues (Figure 4.11H). Ubiquitination status, measured by Western blot analysis with a K48-linkage specific poly-ubiquitin antibody, was highly elevated by FlyAA (Figure 4.11I). Measures of autophagy were not altered between the three dietary AA ratios (Figure 4.11J-K). A decreased survival upon proteasome inhibition on FlyAA was also observed (Figure 4.11L), suggesting a reliance on proteasomal activity to cope with proteotoxic stress load.

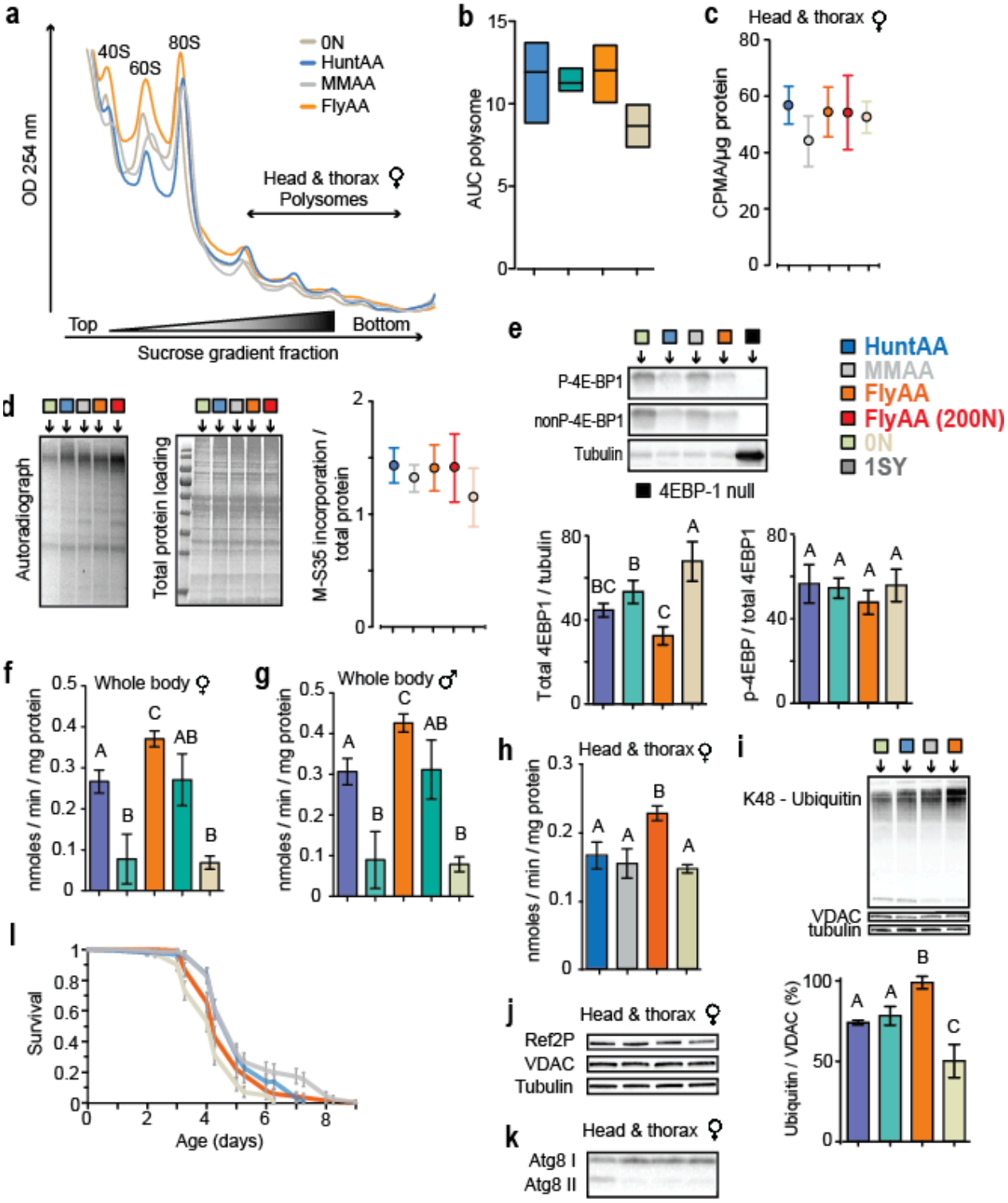


Figure 4.11

(data by Hanna Salmonowicz)

a. Polysome profiling showing similar patterns across different dietary AA ratios. Curves represent the ribosomal/polysomal fractions across a sucrose gradient. Each curve shown represents a single representative biological replicate (out of 3) of 60-80 heads and thoraxes.

b. Quantification of the AUC (area under curve) in the polysome profiles across different dietary AA ratios. No significant shift was detected between the different dietary AA ratios (P value > 0.05, one-way ANOVA, and Dunnet post-hoc test). Each box plot represents three biological replicates, each using 60-80 heads and thoraxes.

c. The effect of the dietary AA ratio on 35S-methionine incorporation. Bars represent mean scintillation counts per minute normalized to protein content (in µg), for 5 trials, each using 5 heads and thoraxes per dietary AA profile. Error bars represent SEM, and letters indicate no statistically significant differences (P value > 0.05, one-way ANOVA, and Dunnet post-hoc test).

d. Autoradiograph (left panel) showing the amount of the 35S-methionine incorporated into the proteins of dissected heads and thoraces under equal protein loading (middle panel). Quantification of the autoradiograph is normalized to the total protein content obtained by Coomassie staining (right panel). Each bar represents 5 trials, each using 5 heads and thoraxes per dietary AA profile. Error bars represent SEM.

e. 4E-binding protein 1 (4E-BP1) protein and phosphorylation levels in the head and thorax of 10-day old females across different dietary AA profiles at 100N. For quantification, each bar shown represents 4-5 biological replicates, each replicate using a protein extract from 15 heads and thoraces per treatment. Error bars represent SEM, letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

f. Proteasome activity of 10-day old females across AAs ratios (HUNTaa, MMaa, FLYaa) at 100N with 1SY and 0N as controls. The chart shows the activity based on the LLE-AMC substrate degradation as both substrates behaved similarly (data not shown). Each bar represents 5 biological replicates of 5 flies each. Error bars represent SEM, letters indicate statistically significant differences (P value < 0.05) as determined by one-way ANOVA, with a Dunnet post hoc test.

g. Proteasome activity of 10-day old males across AAs ratios (HUNTaa, MMaa, FLYaa) at 100N with 1SY and 0N as controls. The chart shows the activity based on the LLE-AMC substrate degradation as both substrates behaved similarly (data not shown). Each bar represents 5 biological replicates of 5 flies each. Error bars represent SEM, letters indicate statistically significant differences (P value < 0.05) as determined by one-way ANOVA, with a Dunnet post hoc test.

h. Proteasome activity of 10-day old female heads and thoraces across AAs ratios (HUNTaa, MMaa, FLYaa) at 100N with 1SY and 0N as controls. The chart shows the activity based on the LLE-AMC substrate degradation as both substrates behaved similarly (data not shown). Each bar represents 5 biological replicates of 15 head and thoraces each. Error bars represent SEM, letters indicate statistically significant differences (P value < 0.05) as determined by one-way ANOVA, with a Dunnet post hoc test.

i. Western blot analysis of ubiquitination status in 10-day old female heads and thoraces across AAs ratios (HUNTaa, MMaa, FLYaa) at 100N with 0N as a control. Quantification performed on the basis on 3 biological replicates expressed as arbitrary units and reproduced >3 times. Error bars represent SEM, letters indicate statistically significant differences (P value < 0.05) as determined by one-way ANOVA, with a Dunnet post hoc test.

j. Western blot analysis of the level of Ref2P across dietary AA ratios. Quantification performed on 9 biological replicates, each using 15 heads and thoraces, and expressed as arbitrary units. Error bars represent SEM and statistical comparison was performed using one-way ANOVA, and Dunnet post hoc test.

k. Western blot analysis of the level of Atg8 across AA ratios. Quantification performed on 9 biological replicates, each using 15 heads and thoraces, and expressed as arbitrary units. Error bars represent SEM and statistical comparison was performed using one-way ANOVA, and Dunnet post hoc test.

I. Proteotoxic stress resistance in 10-day old females kept in 100N FlyAA, HuntAA, or MMAA, as assessed by Bortezomib addition to the media and kept at 29°C. Chi squared p values for all the treatments differed significantly between all treatments except HuntAA vs. MMAA.

4.3.11 Proteasome activity, biogenesis, and fecundity

To further establish if proteasome activity can be dictated by modulating the most limiting EAA, arginine was modulated in HuntAA and it was observed that increasing or decreasing arginine alone augmented or reduced whole body proteasome activity (Figure 4.12A). Furthermore, introducing an 80% arginine or isoleucine restriction in FlyAA suppressed whole body proteasome activity (Figure 4.12B), reflecting the proteasome activities of exome-mismatched ratios with arginine (HuntAA) or isoleucine (MMAA) limitations respectively (Figure 4.11F). However, a correlation between proteasome activity and fecundity rate in these diets was observed (Figure 4.12C), so proteasome activity was next tested when methionine, earlier seen to be replenishable for fecundity, was restricted. Although an 80% restriction of Leucine significantly suppressed both proteasomal activity (Figure 4.12D) and fecundity (Figure 4.12E), restriction of Methionine by 80% significantly decreased whole body proteasome activity (Figure 4.12D) but not fecundity (Figure 4.12E). These results suggest that fecundity and proteasome activities are not necessarily coupled, as methionine restriction is conditionally essential for fecundity but limits *in vivo* proteasome activity.

To further probe into why restricting isoleucine reduced proteasome activity more than restricting arginine, we considered proteasome AA usage. We hypothesized that if the composition of the proteasome's proteome is enriched in isoleucine (the limiting EAA in MMAA), and not in arginine (the limiting EAA in HuntAA), dietary isoleucine limitations will reduce proteasome biogenesis to a greater extent than will dietary arginine limitations. However, the proteasome's proteome (GO:0000502) has a proportional requirement for isoleucine that is similar to that for arginine (Figure 4.12F). We next considered the proteasome's core complex 20S pros-α subunit (P12881, Uniprot), for which a commercial antibody is available. Analysis of the proportional AA usage in the 20S pros-α subunit showed a similar proportional requirement for arginine and isoleucine (Figure 4.12F), and western blot analysis of the subunit's protein levels showed similar levels in MMAA and HuntAA (Figure 4.12G).

We decided to experimentally test the principle of minimum at the molecular level by assessing the effect of single EAA restrictions upon pros-α subunit biosynthesis. Tryptophan usage in the exome is 62% higher compared to that in pros-α (Figure 4.12F), so we tested whether a 62% restriction of Tryptophan in FlyAA ratio reduces pros-α subunit biosynthesis. In contrast, threonine, isoleucine, and arginine usages are 32%, 14%, and 19% higher in the pros-α subunit compared to FlyAA respectively (Figure 4.12F), so we tested whether reducing these three AAs in a FlyAA diet by 62% limits pros-α subunit biosynthesis. Pros-alpha subunit levels and proteasome activities were reduced in all four single dietary AA-restrictions, including that of tryptophan (Figure 4.12H-I). Therefore no correlation was observed between the degree of a dietary limitation in an EAA and usage of that EAA towards pros-α biosynthesis. However, pros-α subunit levels matched the observed proteasome activity in these conditions (Figure 4.12H-I), suggesting that by

restricting these individual AAs in FlyAA, proteasome subunit biogenesis and proteasomal activity are both similarly altered.

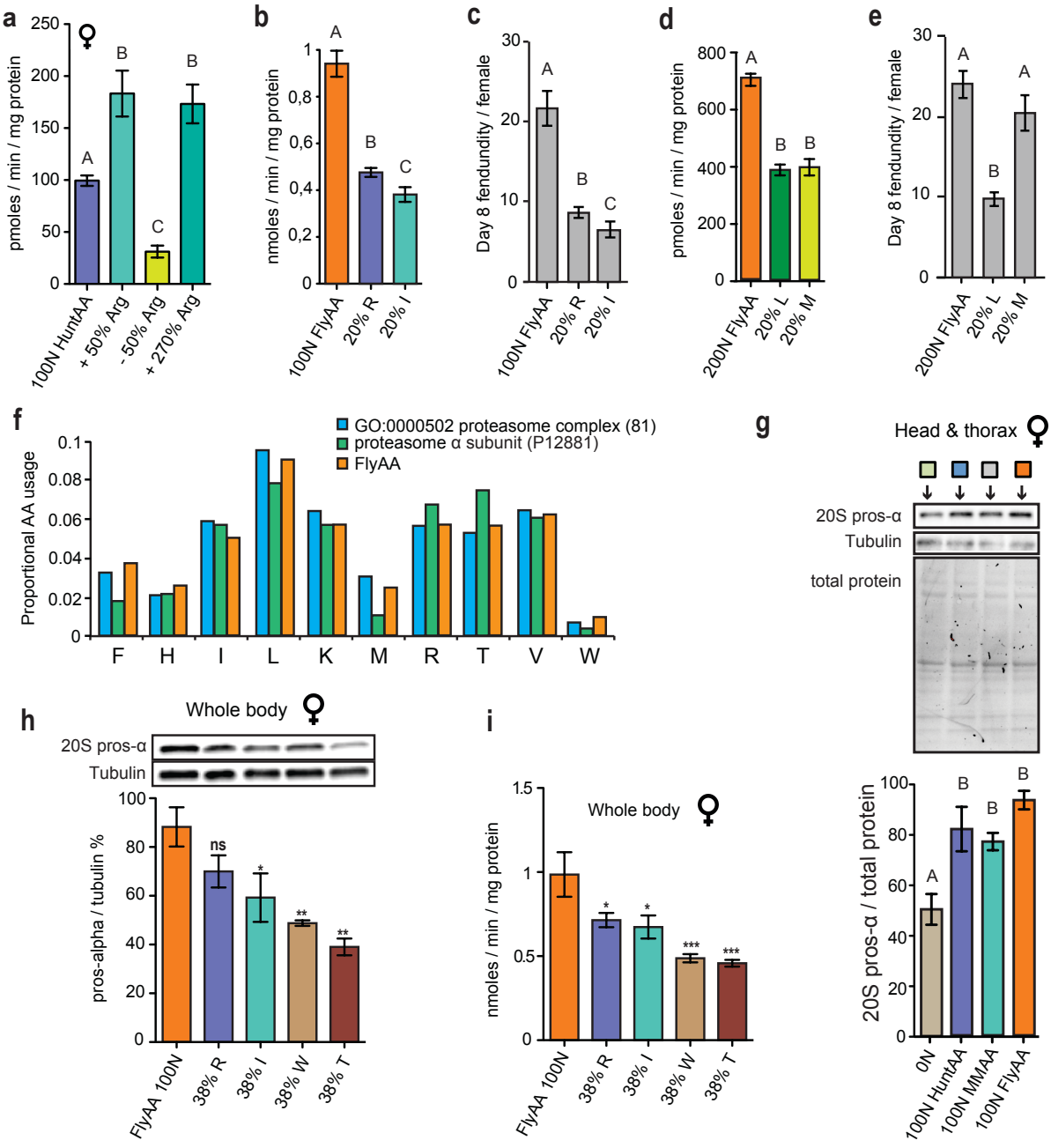


Figure 4.12

(data by Dr. Ilian Atanassov, Hanna Salmonowicz, and George Soultoukis)

a. Whole body proteasome activity of 10-day old females fed HuntAA at 100N, but with manipulations in Arginine, the most limiting AA. The chart shows the activity based on the LLE-AMC substrate degradation as both substrates behaved similarly (data not shown). Each bar represents 5 biological replicates of 10 flies each. Error bars represent SEM, letters indicate statistically significant differences (P value < 0.05) as determined by one-way ANOVA, with a Dunnet post hoc test.

b. Whole body proteasome activity of 10-day old females fed FlyAA at 100N, but with manipulations in Arginine, the most limiting AA in HuntAA, or Isoleucine, the most limiting AA in MMAA. The chart shows the activity based on the LLE-AMC substrate degradation as both

substrates behaved similarly (data not shown). Each bar represents 5 biological replicates of 10 flies each. Error bars represent SEM, letters indicate statistically significant differences (P value < 0.05) as determined by one-way ANOVA, with a Dunnet post hoc test.

c. Observed fecundity for FlyAA, against FlyAA with 80% restrictions in Arginine (20%R) or Isoleucine (20%I). The fecundity decrease for the 20%I treatment is more severe than it is for the 20%R treatment. Each bar represents 3-4 trials, each trial using (per treatment) 6 biological replicates of 10 flies. Error bars represent SEM.

d. Whole body proteasome activity of 10-day old females fed FlyAA at 100N, but with manipulations in Leucine or Methionine. The chart shows the activity based on the LLE-AMC substrate degradation as both substrates behaved similarly (data not shown). Each bar represents 5 biological replicates of 10 flies each. Error bars represent SEM, letters indicate statistically significant differences (P value < 0.05) as determined by one-way ANOVA, with a Dunnet post hoc test.

e. Observed fecundity for the treatments shown in d. Each bar represents 3-4 trials, each trial using (per treatment) 6 biological replicates of 10 flies. Error bars represent SEM.

f. Proportional weighted AA usage in the proteasome complex (GO:0000502), in the proteasome alpha subunit P12881, and in the exome (FlyAA).

g. Western blot analysis of the level of 20S alpha proteasome subunit in the heads and thoraxes of female flies. The quantification shown in the lower panel represent 3 biological replicates of 5 females each, and is normalized to the total protein staining.

h. Western blot analysis of proteasome alpha subunit upon single AA limitations. The quantification is based on 3 biological replicates consisting of 5 flies each. Statistical analysis was performed with the use of One-way ANOVA, and asterisks indicate $P<0.05$ towards the FlyAA control.

i. Proteasome activity upon single AAs limitations in a 100N FlyAA diet. The trial consisted of 5 biological replicates consisting of 3 flies each. Statistical analysis was performed with the use of One-way ANOVA, and asterisks indicate $P<0.05$ towards the FlyAA control.

4.3.12 Effects of exome-matching on early adult viability

The nutritional efficiency of all three dietary AA ratios was similarly defined by both developmental viability and early adulthood mortality as a strong negative correlation between the two traits was seen across ratios (Figure 4.13A & Appendix I), indicating that diets with lower dietary AAs decrease both developmental and early adulthood viability. This observation is also consistent with the increase in early mortality observed under low protein conditions of restricted dietary yeast (Figure 4.13B), as diet with a lower dietary nitrogen content showed a significantly higher early adulthood mortality (Figure 4.13A).

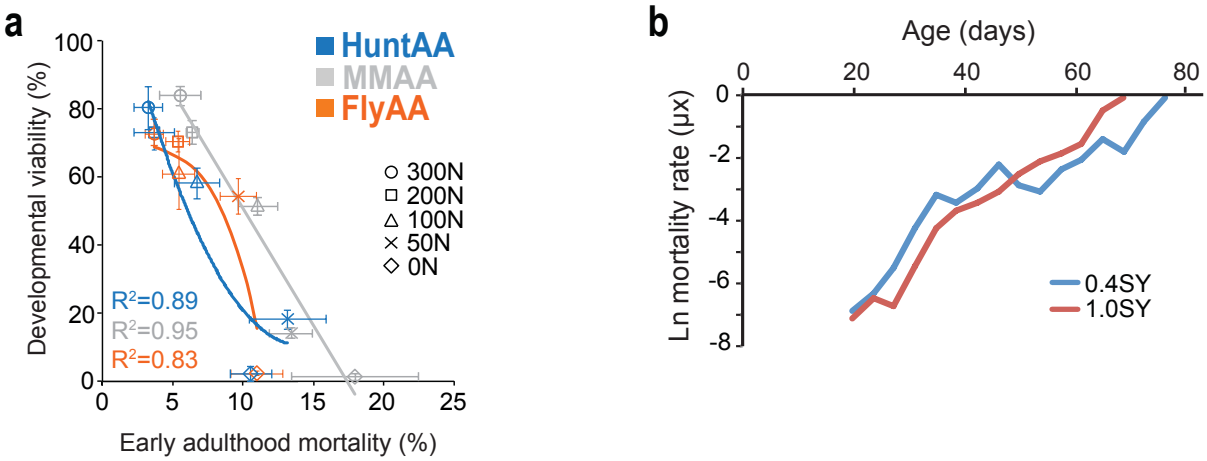


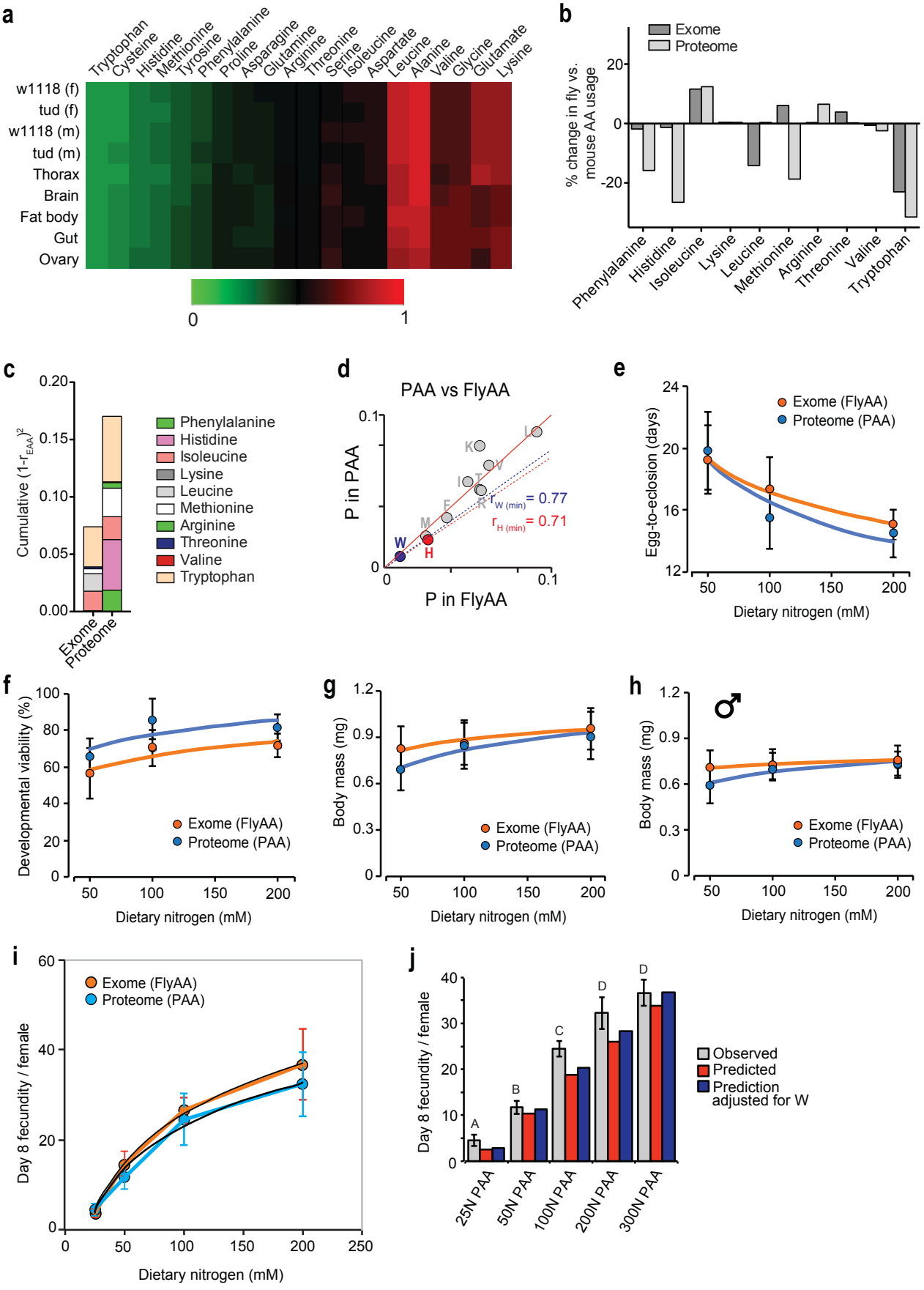
Figure 4.13

a. Developmental viability was negatively correlated to the proportion of early adulthood deaths, and therefore both traits seemed to be equally good predictors of the nutritional efficiency of each dietary AA ratio. The best fit line was described by a polynomial regression fit for HuntAA and FlyAA, and a linear regression fit for MMAA. N = 4-10 trials per treatment for early adult survivorship (see Appendix I).

b. Effect of low protein intake on early survival. When consuming a low yeast diet (0.4SY), an increase in early mortality was observed, compared to a normal yeast diet (1SY); (N = 1000).

4.3.13 Exome-matching vs. proteome-matching

Comparison of the proportional AA representation in the exomes of different organisms reveals considerable differences in AA usage. For example, the human exome shows different AA requirement to that of the fly exome (data not shown). However, exon transcription and translation are also likely to affect AA requirements as they shape the proteome, where most of the ingested AAs are ultimately utilized. The whole-body proteome of both flies and mice, as recently quantified by SILAC techniques (163, 166), allowed us to calculate AA usage in the weighted proteome of both organisms. In the fly, there were no differences between the proteome AA usage between genders (male vs. female), strains (tud vs. w1118), or tissues (Figure 4.14A). However, comparison of the exome and proteome AA usages shows that differences in the proportional AA usage between flies and mice are higher between their accrued proteomes than it is between their exomes (Figure 4.14B-C). This observation suggests that AA usage between organisms may be better reflected by the proteome's, not the exome's AA requirements. Thus, although the exome is an efficient measure for defining AA requirements in the fly, we compared the weighted whole fly proteome AA usage to the exome requirement (Figure 4.14D). The proteome AA ratio (PAA) was in fact very similar to the exome ratio, with the most significant limitation being histidine ($r_{H(min)}$ = 0.71). Although this limitation is relatively small compared to that in HuntAA ($r_{R(min)}$ = 0.27) or MMAA ($r_{I(min)}$ = 0.36), we determined if feeding flies on PAA further boosts anabolic traits such as fecundity and growth compared to FlyAA. However, no significant differences were seen between PAA and FlyAA for traits including development time (Figure 4.14E), developmental viability (Figure 4.14F), or body mass of females (Figure 4.14G) or males (Figure 4.14H) upon eclosion. As for FlyAA, the fecundity increase in higher dietary nitrogen was also best described by a logarithmic fit for PAA (Figure 4.14I). Therefore flies fed either the exome or proteome matched AA profiles performed distinctly to HuntAA or MMAA in terms of anabolic traits and lifespan, suggesting that both exome-matching and proteome-matching are good measures of the fly's AA requirements for egg production. However, exome-matching resulted in an elevated fecundity (Figure 4.14I). Interestingly histidine, whose restriction or deprivation fails to fully suppress fecundity with immediate effect (Figure 4.s 3C-F), is the most limiting EAA in PAA, and exome-matched predictions were more accurately reflected the observed values when adjusted for the second most-limiting EAA, tryptophan (Figure 4.14J). The lifespan of flies fed PAA also did not significantly differ to that of flies fed FlyAA (Appendix II).


a
Tryptophan
Cysteine
Histidine
Methionine
Tyrosine
Phenylalanine
Proline
Asparagine
Glutamine
Arginine
Threonine
Serine
Isoleucine
Aspartate
Leucine
Alanine
Valine
Glycine
Glutamate
Lysine
w1118 (f)
tud (f)
w1118 (m)
tud (m)
Thorax
Brain
Fat body
Gut
Ovary
0
1
b
Exome
Proteome
% change in fly vs. mouse AA usage
20
0
-20
Phenylalanine
Histidine
Isoleucine
Lysine
Leucine
Methionine
Arginine
Threonine
Valine
Tryptophan
c
Cumulative (1-r_EAA)^2
0.20
0.15
0.10
0.05
0.00
Exome
Proteome
Phenylalanine
Histidine
Isoleucine
Lysine
Leucine
Methionine
Arginine
Threonine
Valine
Tryptophan
d
PAA vs FlyAA
P in PAA
P in FlyAA
0.1
0
0.1
W
H
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F
K
I
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V
L
r W (min) = 0.77
r H (min) = 0.71
e
Egg-to-eclosion (days)
24
20
16
12
50
100
150
200
Dietary nitrogen (mM)
Exome (FlyAA)
Proteome (PAA)
f
Developmental viability (%)
100
80
60
40
20
0
50
100
150
200
Dietary nitrogen (mM)
Exome (FlyAA)
Proteome (PAA)
g
Body mass (mg)
1.2
0.9
0.6
0.3
0
50
100
150
200
Dietary nitrogen (mM)
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Body mass (mg)
1.2
0.9
0.6
0.3
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50
100
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Dietary nitrogen (mM)
Exome (FlyAA)
Proteome (PAA)
i
Day 8 fecundity / female
60
40
20
0
0
50
100
150
200
250
Dietary nitrogen (mM)
Exome (FlyAA)
Proteome (PAA)
j
Day 8 fecundity / female
40
30
20
10
0
A
B
C
D
D
25N PAA
50N PAA
100N PAA
200N PAA
300N PAA
Observed
Predicted
Prediction adjusted for W

Figure 4.14

a. The whole-body proteome of flies, as recently quantified by SILAC (163). The calculated AA usage in the weighted proteome reveals no differences between the weighted proteome AA usage between genders (male vs. female), strains (tud vs. w1118), or tissues (wdah; in-house data). Data by Dr. Ilian Atanassov.

b. Comparison of the difference in AA usage between the fly and the mouse. The % change in fly AA usage compared to mouse AA usage is shown, with dark grey bars showing the % change in AA usage on the fly exome over the mouse exome, and light grey bars showing the % change in AA usage on the fly proteome over the mouse proteome. Except for leucine and threonine, differences in AA usage between the two organisms are higher between their proteomes than they are between their exomes.

c. Cumulative difference in AA usage between the fly and the mouse exome compared to the cumulative difference in AA usage between the fly and the mouse proteome. The plot shows that differences in the proportional AA usage between flies and mice are higher between their accrued proteomes than between their exomes. Cumulative distance is the sum of the distance measure: sum over all amino acids of (ratio in protein – mean ratio in exome)^2 .

d. Comparison of the fly's exome (FlyAA) and proteome (PAA) ratios. The two ratios are highly similar, with the most significant Paa limitation being histidine ($r_{H(min)}$ = 0.71). This limitation is relatively small compared to that of HuntAA ($r_{R(min)}$ = 0.27) or MMAA ($r_{I(min)}$ = 0.36), indicating that the proteome profile of 10-day old females does not dramatically differ to the exome-predicted proportional AA requirements.

e. FlyAA and PAA developmental time. Both the proteome and the exome ratio supported similar developmental times ($p>0.05$), indicating both dietary AA sources are utilized by the developing larvae with equal efficiencies. Error bars shown represent SD, N = 5 biological replicates of 125 and 25 eggs per treatment for FlyAA and PAA respectively (2-way ANOVA analysis with Bonferonni posthoc test, PAA vs. FlyAA P value = 0.8636 for the time-AA ratio interaction, F ratio = 0.1479).

f. FlyAA and PAA developmental viability. Although the proteome ratio supported better developmental survival, the difference was not significant ($p>0.05$), indicating that the dietary AA source is utilized by the developing larvae with equal efficiencies. Error bars shown represent SD, N = 5 biological replicates of 125 and 25 eggs per treatment for FLYaa and Paa respectively (2-way ANOVA analysis with Bonferonni posthoc test, P value = 0.9383 for the viability-AA ratio interaction, F ratio = 0.06392; both viability and AA ratio P value > 0.05).

g. Female body mass upon eclosion across exome and proteome ratios and dietary nitrogen (N) levels. Upon eclosion (day 1) female body mass is unchanged between flies reared in the proteome or the exome ratio. Error bars shown represent SD, N = 5 biological replicates of 125 and 25 eggs per treatment for FLYaa and Paa respectively. Body masses were measured for pairs of flies, with the mean for each pair used for analysis (2-way ANOVA analysis with Bonferonni posthoc test, P value = 0.0159 for the mass-AA ratio interaction, F ratio = 4.203).

h. Male body mass upon eclosion across exome and proteome ratios and dietary nitrogen (N) levels. Upon eclosion (day 1) male body mass is unchanged between flies reared in the proteome or the exome ratio. Error bars shown represent SD, N = 5 biological replicates of 125 and 25 eggs per treatment for FLYaa and Paa respectively. Body masses were measured for pairs of flies, with the mean for each pair used for analysis (2-way ANOVA analysis with Bonferonni posthoc test, P value = 0.0225 for the mass-AA ratio interaction, F ratio = 3.849).

i. Fecundity for female flies fed FlyAA and PAA across dietary nitrogen levels. The egg-laying of 8-day-old flies is similar between females maintained in PAA or FlyAA. This suggests that

both ratios are equally bio-utilizable for egg production-related anabolic processes. Each data point represents N=2-16 trials, each trial using 60 flies per treatment. Error bars represent SD. (2-way ANOVA analysis with Bonferonni posthoc test, P value < 0.05 for the fecundity-AA ratio interaction, F ratio = 2.689)

j. Observed versus predicted fecundity for flies fed either the SILAC proteome AA usage (PAA) or flies fed the exome AA usage ratio (FlyAA) across dietary nitrogen levels (25-200N). Each bar represents 3 or more trials, each using (per treatment) 6 biological replicates of 10 flies. Error bars represent SEM, letters indicate statistically significant differences (P value < 0.05) as determined by one-way ANOVA, with a Dunnet post hoc test.

4.3.14 Effects of exome-defined AA restriction or deprivation upon adult survival

Next, it was set out to determine the effects of an 80% limitation in single EAAs compared to the exome requirement (20% of the FlyAA level, or 80% restriction) upon lifespan in flies fed 100N, 200N, and 300N FlyAA diets. The lifespan response was tested where the identity of the limiting AA altered to cover all 10 essential AAs. Results showed that at low dietary nitrogen supply, most AA limitations appeared to be detrimental, but at higher dietary nitrogen supply several AA limitations appeared to be beneficial for lifespan (Figure 4.15A). In addition, despite a similar degree of limitation compared to FlyAA, different AAs caused a different effect upon lifespan. At 100N FlyAA, an 80% restriction of Threonine was more detrimental for survival than an 80% restriction of arginine (log rank test P value <0.05), and similarly an 80% restriction of leucine was more detrimental than phenylalanine (log rank test P value <0.05; Figure 4.15A). These results demonstrated that compared to the exome requirement, and when considering the lifespan response, the identity of the limiting AA should be taken into account even when the degree of limitation is identical between different AAs.

Furthermore, upon analysis of survival at a diet with an ample amount of AAs (300N HuntAA) but 100% deprived of each single EAA separately, the deprivation of some EAAs had a more detrimental effect than that of others, with Isoleucine-deprived flies surviving significantly less compared to flies deprived of Arginine, and Threonine deprivation having a very severe impact on survival (Figure 4.15B). When considering the median survival in the diet with an ample amount of AAs (100N FlyAA) but 80% limited of each single EAA separately (first dataset from Figure 4.15A), there was no correlation between median survival in the 100% deprivation of each single EAA separately (dataset from Figure 4.15B) and median survival in the 80% restriction of each single EAA separately (first dataset from Figure 4.15A), as shown in the comparison between the two survival responses (Figure 4.15C). Thus the survival response to a 100% EAA deprivation is distinct to and does not necessarily correlate with the survival response to an 80% EAA limitation. Moreover, there was no correlation between survival upon deprivation of a single EAA and the usage for that EAA in the whole fly proteome (SILACAA) (Figure 4.15D). Therefore the abundance of an AA in the fly's weighted proteome or exome is not a reliable predictor for survival upon deprivation from that EAA.

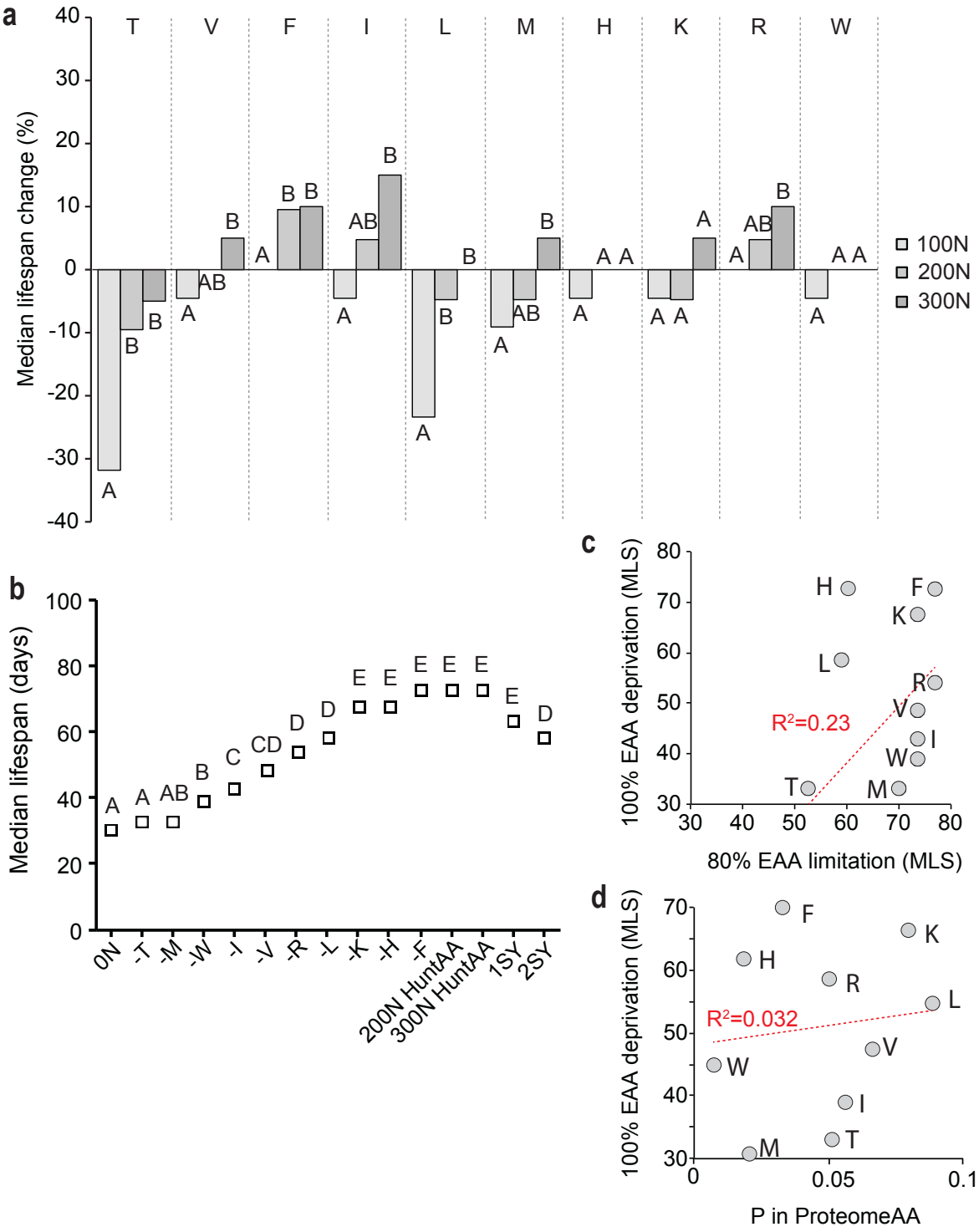


Figure 4.15

a. Survivorship effect of single EAA deprivations in FlyAA 100N, 200N, and 300N. The effect of each deprivation is shown as % to the median survival observed in the respective control (100N, 200N, or 300N FlyAA). At lower dietary nitrogen levels (100N), deprivation of some EAAs (Thr, Met) has a more severe detrimental effect than that of others (Phe, Arg), while at higher dietary nitrogen levels (300N) the deprivation of certain EAAs (Ile, Phe) had a more

positive effect than that of others (Thr, Trp, His). For each treatment, N = 180. The log-rank test was used to compare survivorships in 80% restrictions of a single AA across the 3 dietary nitrogen levels (letters show significance when P value < 0.05). No comparisons of survival in 80% restrictions of different AAs are shown.

b. Survivorship effect of single EAA 80% restrictions in 300N HuntAA. Deprivation of some EAAs (Thr, Met) has a more severe detrimental effect than that of others (Phe, Arg). For each treatment, N = 200. Letters show significance when Log-rank test P value < 0.05.

c. Relationship between median lifespan (MLS) upon a 100% EAA deprivation (Y-axis) and median lifespan (MLS) upon 80% EAA restriction (X-axis) in diets with ample amounts of non-deprived/non-restricted EAAs (300N HuntAA and 100N FlyAA respectively). No correlation was observed between the two survival responses upon linear regression analysis (R^2 value = 0.23).

d. Relationship between median survival upon an EAA deprivation, and the abundance of that EAA in the fly's SILAC proteome (SILACAA). No correlation was observed between the two traits upon linear regression analysis (R^2 value = 0.032).

4.3.15 Effects of larval (L2) free AA-matching

Circulating, intracellular, and protein-bound AA profiles differ significantly, but circulating and intracellular AAs fluctuate more dynamically and play a prominent metabolic role (4). The whole free AA profile of developing *Drosophila* larvae has been reported (162), so it was decided to also test if supplying AAs in a profile that matches the free AAs in the larval body (L2AA) can promote larval development more than FlyAA (Figure 4.16A). Upon comparison, L2AA seemed extremely different to FlyAA, with the most limiting EAA being tryptophan ($r_{W(min)}$ = 0.03), whose limitation was predicted to be very severe. Indeed, upon rearing *Drosophila* eggs in diets containing L2AA at 25N to 300N it was found that development was severely delayed independently of dietary nitrogen levels (Figure 4.16B), and developmental survival was similarly reduced (Figure 4.16C). Confirming the severe deficiency of the Trp limitation in L2AA compared to FlyAA, increasing dietary nitrogen from 25N to 300N resulted in no detectable increase in day 8 fecundity for flies kept in L2AA diets (Figure 4.16D). These observations show that matching the dietary AA profile to the whole body free AA composition of the developing larva is not efficient in meeting the AA requirements of the growing larva.

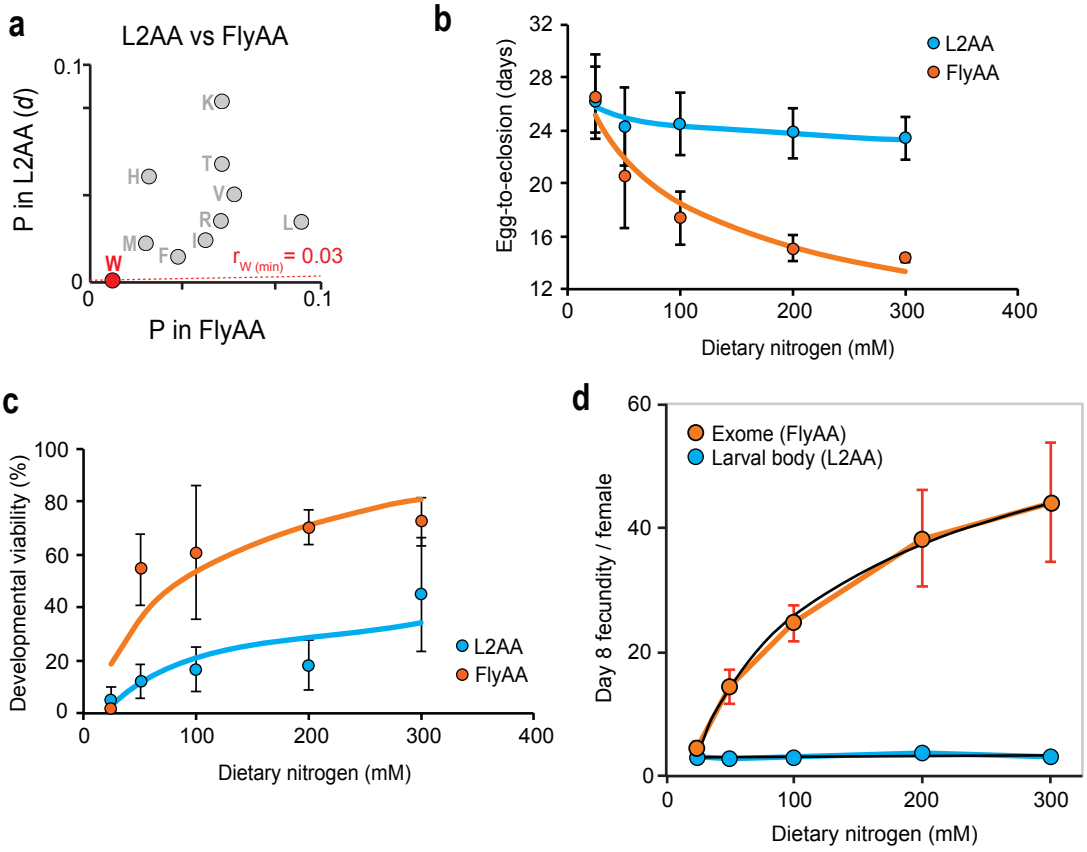


Figure 4.16

a. Comparison of the fly's exome (FLYaa) ratio and the whole larval body (L2) ratio. The two ratios are highly dissimilar, with the most significant L2aa limitation being tryptophan ($r_{W(min)}$ = 0.03). This is a very severe limitation as it is predicted that only 3% of the exome's proportional AA requirement is met by the L2 ratio.

b. FlyAA and L2AA developmental time. The whole larval body ratio severely delayed development ($p<0.05$), indicating that the dietary AA source is utilized by the developing larvae with equal efficiencies. Error bars shown represent SD, N = 5 biological replicates of 125 and 25 eggs per treatment for FlyAA and L2AA respectively (2-way ANOVA analysis with Bonferonni posthoc test, P value = 0.0156 for time-AA ratio interaction, F ratio = 3.643; both time and AA ratio P value < 0,0001).

c. FlyAA and L2AA developmental viability. The whole larval body ratio severely decreased developmental viability ($p<0.05$), indicating that the dietary AA source is utilized by the developing larvae with equal efficiencies. Error bars shown represent SD, N = 5 biological replicates of 125 and 25 eggs per treatment for FlyAA and L2AA respectively (2-way ANOVA analysis with Bonferonni posthoc test, P value = 0.0002 for the viability-AA ratio interaction, F ratio = 8.026; both viability and AA ratio P value < 0,0001).

d. Fecundity for female flies fed FlyAA and L2AA across dietary nitrogen levels. The egg-laying of 8-day-old flies is increasing at higher dietary nitrogen levels for flies fed the FlyAA ratio, but remains at basal low levels for flies fed the whole larval body (L2AA) ratio. This suggests that L2AA is poorly bio-utilizable for egg-production-related anabolic processes. Each data point represents 2-16 trials, each trial using 40-60 flies per treatment. Error bars represent SD (2-way ANOVA analysis with Bonferonni posthoc test, P value < 0.00001 for the AA ratio effect, F ratio = 19.15).

4.4 Discussion

The genetic composition of a species may affect the response to ingested AAs and contribute to the diversity of protein sources consumed across species. In this chapter, a genome-based technique for designing an enhanced quality protein is presented. The results herein show that the exome is a powerful measure for defining AA requirements in flies. Dietary limiting EAAs are accurately predicted by the fly exome, and dietary AA ratios can be qualitatively defined by the degree of their disproportion to the exome AA usage more accurately than when compared to other approaches. For example, HuntAA, an empirical ratio refined to support development time and survival (199), is a less efficient AA source for anabolic traits than the exome usage (FlyAA). Similarly, a dietary AA ratio matching the free AA composition of developing larvae (L2AA) (162) delays development and diminishes fecundity compared to FlyAA. Therefore, exome-matching is an effective measure of determining AA requirements for anabolic life-history traits (i.e. development and fecundity) compared to previously reported measures.

4.4.1 Fly development and fecundity

The principle of the minimum poses that the most limiting nutrient in a diet can prevent successful utilization of other, non-limiting nutrients that are, as a result, acquired in excess (31). Accordingly, we find that exome-defined limiting EAAs prevent the anabolic usage of non-limiting EAAs. In addition, the law of diminishing returns holds that each succeeding increment of the limiting essential nutrient will produce a smaller increment of growth than the preceding increment (31, 190). In agreement, we observed a decrease in the effect of the limiting EAA with incremental increases in its concentration, or in the concentration of dietary nitrogen, upon fly development, growth, and fecundity. At high AA supply, the physiological needs for development were equally met by diverse dietary AA ratios, but at low dietary nitrogen they were adequately met only by the exome-matched diet.

The egg-laying observations described in this chapter are in accord with earlier studies showing that elimination of methionine, arginine, or histidine, even for 8 days, does not completely suppress egg production (212), suggesting that the former can be restored in part by utilization of cysteine, methionine, or sulphur from other molecules, and that arginine and histidine may also be slowly metabolically replenished, as in other organisms. Indeed, methionine salvage in *Drosophila* alleviates the suppression of fecundity by DR (213), so although methionine is an essential AA, being the start codon in approximately 50% of the mammalian exome (214) and its supply is critical for the fly's metabolic homeostasis, when in shortage methionine can be partially obtained through the utilization of other metabolites. Therefore, restricting or depriving methionine or histidine alone does not reduce fecundity to the extend other EAA restrictions or deprivations do, and that fecundity observations are better predicted on the basis of the most limiting AA when these two EAAs are treated as replenishable as previously reported (212). Thus, any predictive model based on the exome AA usage should take into

account the identity of the limiting AA, so that any predictions based on the exome AA usage are accurate and valid. Arginine synthesis via the urea cycle in insects is also unclear, although no evidence is reported in the fly and for other insects.

4.4.2 Fly diet perception

The perception of FlyAA as a higher quality AA source was evident in adult *Drosophila* food choice experiments, where mated females consistently selected FlyAA over any other choice of AAs they were offered. Although the food preference of larvae was not tested, it is likely that FlyAA would also appear attractive to developing larvae as recent studies show that larvae make foraging choices that minimize development time (215).

4.4.3 Fly body composition

Fat storage is a critical component of the lifespan response to DR both in vertebrates and invertebrates (5). In flies, inhibition of fatty acid synthesis or oxidation genes inhibits lifespan extension upon DR (216). In the experiments described above, flies fed higher levels of dietary AAs had lower lipid storage, and in agreement with a higher usage of FlyAA, we also saw reduced lipid storage in flies fed FlyAA, especially at higher dietary nitrogen levels. These changes, however, negatively correlated to the lean mass and protein content, which has highest in FlyAA. As flies consume similar amounts of food on the three tested dietary AA ratios, the greater body size and gut dry weight of flies on FlyAA is likely to be due to the more efficient use of ingested AAs, and due to an increase in nutrient or caloric intake. In addition, the lack of an effect of exome-matching upon the dry weight of heads may be linked to the observation that the fly's brain is well protected from nutritional stress (217). Other insects, such as crickets, also grow bigger on high protein diets, and accumulate greater somatic lipid stores on diets with a low P:C ratio, and greater somatic protein stores on high protein foods (218). Therefore a higher protein content or a more usable AA source both increase lean mass and decrease fat mass in at least some invertebrate models. Importantly the levels of stored TAGs, both as a function of total dietary AA levels and of the dietary AA profile, were correlated to starvation resistance. These results are reminiscent of observations in humans, where individuals with greater fat mass can survive longer upon starvation (219). However, flies fed an AA-depleted diet (0N) died faster despite the highest levels of stored lipids. This is in line with evidence suggesting that although fat storage is a critical determinant in starvation resistance, starved obese humans and animals may die independently of fat storage (220). Moreover, dietary protein can affects a variety of stress responses; for example, a low hypoxic tolerance is observed in flies fed high protein diets (221). Therefore it would be an interesting prospect to further evaluate the effects of ingested AA profiles upon various stress responses in the fly.

4.4.4 Fly nitrogen balance

Uric acid is the main nitrogenous excretory substance in most terrestrial uricotelic insects and constitutes about 80% of the excreted nitrogen in *Drosophila* (222). The uric acid results described above show that an exome-balanced AA ratio increases the utilization of dietary nitrogen (in the form of AAs), thereby decreasing the amount of uric acid excreted and resulting in higher protein contents in these animals. These findings strongly support a higher AA utilization caused by exome-matching towards the promotion of anabolic traits. Moreover, during starvation the depletion of body proteins and urinary nitrogen excretion reflects continuous demands for AA oxidation (220). The results in this chapter suggest that nitrogen catabolism during starvation is higher for animals with larger amounts of stored protein, which may contribute to their lowered starvation resistance.

4.4.5 Fly mitochondria and respiration

Although no overall effect of DR on mitochondrial density is observed, it is difficult to assess mitochondrial functionality *ex vivo* {Magwere:2006tq}. Interestingly, the VDAC-to-total protein ratio was not altered by the dietary AA ratio, suggesting the lack of an effect of the ingested AA profile upon mitochondrial density. Moreover, no differences were observed in oxygen consumption in the head and gut, but there was significantly increased oxygen consumption in the thorax and fat body of females fed the exome-matched diet, suggesting a higher oxidative phosphorylation for those flies. As TAGs are precursors that fuel fatty acid oxidation, a higher oxidative phosphorylation could explain the observed reduction in TAG storage in these flies.

4.4.6 Fly proteostasis and proteasome assembly

Proteasomal degradation was elevated with exome-matching. The increase in both protein content and protein degradation suggested increased protein metabolism rate upon exome-matching. Moreover the increased pS6K levels in flies fed FlyAA are in agreement with cell-based and mouse models with increased TOR activation, where an increased proteasome biogenesis and activity is observed (224). Furthermore, these flies relied on proteasomal function more than flies on exome-mismatched diets, as inhibition of proteasomes resulted in decreased starvation resistance in flies fed FlyAA. Moreover, the results described in this chapter show that the effects of tissue specific AA bioavailability or other transcriptional or translational regulatory mechanisms are critical in establishing biosynthesis of individual peptides, and that proteasome peptide biosynthesis cannot be predicted or modulated simply through dietary AA manipulation on the basis of AA requirement by the proteasome's proteome. These results support a critical role for the identity of the limiting AA in determining proteasome activity. However, the hypothesis of the principle of minimum directly modulating the biosynthesis of single proteasomal subunits was not supported.

4.4.7 Exome-matching versus proteome-matching

The set of encoded proteins alone (exome AA usage) does not take into account the fact that a number of encoded proteins are of very low abundance or are not expressed in an organism or tissue at all. Furthermore, the expressed cellular proteins are not equimolar but instead have very large differences in their cellular copy numbers. However, the exome AA usage can be calculated as either the proportional frequency of an AA across the whole exome, or as the mean of the proportional AA representation in all individual exons. That the two methods give nearly identical values for all 20 AAs (data not shown) means that there is a normal distribution in the variance between single exons and the mean exome AA usage. But can whole body AA usage be skewed (unequally distributed) at a particular moment? To answer this, we would need to sample which exons, and to what extent, are transcribed and translated at any moment, and to calculate real AA usage across different life stages, a technically challenging task. But if the set of exons transcribed and translated at a given moment is sampled, then in statistical terms this sample's mean is likely to be closer to the real mean (the 'population' mean, here represented by the exome AA usage) when the sample size is larger. Indeed, recent evidence from single or multiple point sampling suggests that thousands of exons are transcribed and translated simultaneously both in flies and mice (207, 225). Interestingly, a diet taking into account the cumulative abundance of proteins and the actual AA composition of the fly's proteome ratio (ProteomeAA) promoted anabolic traits to the same extent as the exome-metched diet (FlyAA). The observation that in both flies and mice, the exome AA profile of the organism resembles its weighted proteome AA profile suggests that for these sampled proteomes, the whole body AA usage is equally distributed around the exome AA usage. Therefore, it appears that the exome AA usage represents a good approximation to the AA usage of an organism. However, the time-dependent AA requirements of organisms are also determined by factors such as gender, health state, reproductive status, intra- and inter-species metabolic diversity, tissue-specific AA usage, age, and others. The worm proteome, for example, shows dramatic changes with age (226), which may reflect changes in AA usage and AA requirements. In addition, emerging evidence suggests that many complex elements in the mammalian proteome make it hard to estimate real AA usage by the weighted proteome. Therefore, information of which exons are transcribed and translated may lead to the design of dietary AA ratios that better match AA usage, as calculated by whole-body transcription and translation profiles. Whether matching the dietary AA supply to account for such factors could further benefit anabolic traits would be an interesting challenge to address. Interestingly, the fly SILAC proteome AA profile is very similar to the average AA profile of 550,116 peptide sequence entries from over 250 species (http://web.expasy.org/docs/relnotes/relstat.html; data not shown), perhaps because the biophysical properties of AA side-chains universally apply to protein structure requirements across species, resulting in similar AA usages.

4.4.8 General conclusions and summary and key points

These results demonstrate that small dietary AA modulations can dictate many physiological processes that may ultimately impact on long-term health. It is thus important to understand how modulations in dietary AAs exert their biochemical and physiolometabolic effects. In this chapter, several physiometabolic effects of exome-matching are described. Exome-matching promoted fecundity, enhanced developmental time and viability, resulted in leaner flies and increased mitochondrial respiration specifically in the fat body. Moreover, exome-matching increased nitrogen utilization, lean mass and protein content, and proteasomal function in both somatic and reproductive-tissues. There are ongoing experiments to characterize the effects of exome-matching on longevity.

Moreover, although the degree of limitation of a limiting EAA in the diet is important, these findings also support a key role of the identity of the limiting EAA in mediating the physiological responses to a dietary EAA imbalance. This is in accord with previous studies showing that the identity of the limiting EAA is critical for the effects of protein and single EAA utilization for fly fecundity (212) and development (199). Thus, although a measure to aid the identification of limiting EAAs is of great importance (that measure here defined as the exome), further work is required to resolve the precise biological reasons for such indiscrepancies between different identities of the most limiting EAA.

A complication in the evaluation of the effects of ingested AAs may stem from the fact that dietary-AA dependent processes are not necessarily regulated in an identical way across species. Therefore, in the next chapter the effects of exome-matching upon mouse growth, metabolism, and physiology will be examined.

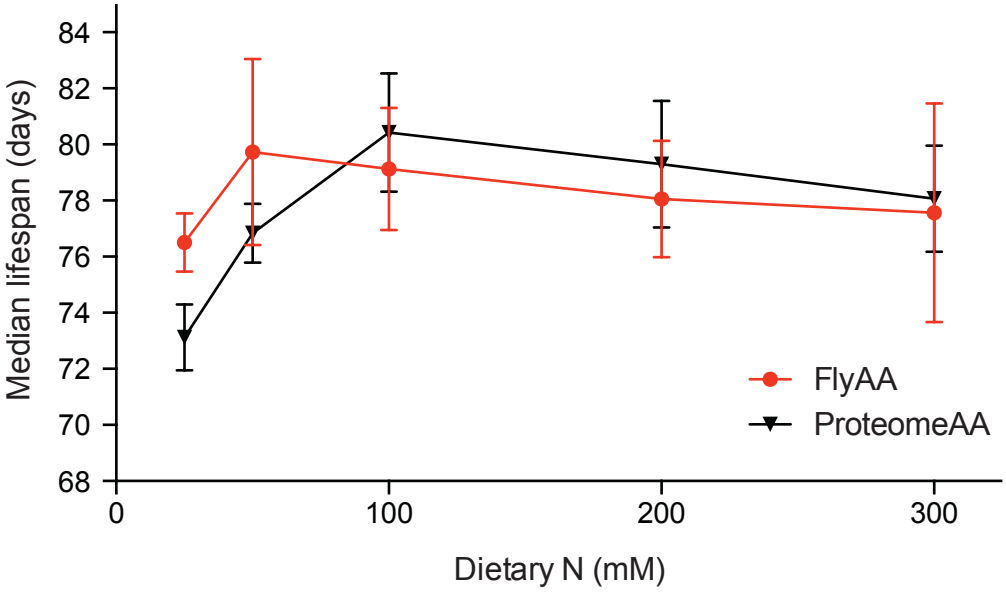
Trial nr	Electronic file name	HuntAA 25N	HuntAA 50N	HuntAA 100N	HuntAA 200N	HuntAA 300N	MMAA 25N	MMAA 50N	MMAA 100N	MMAA 200N	MMAA 300N	FlyAA 25N	FLyAA 50N	FlyAA 100N	FlyAA 200N	FlyAA 300N
Survival at early adulthood (timepoint = 0.5 x median lifespan)																
1	CDM3			0,014	0,016	0,016										
2	CDM4			0,026	0,016	0,020										
3	CDM5				0,027											
4	CDM6				0,010	0,000										
5	CDM7				0,000	0,015										
6	CDM8													0,036		
7	CDM9		0,126	0,096	0,010	0,052		0,150	0,138	0,050	0,041		0,087	0,035	0,066	0,030
8	CDM10	0,118	0,033				0,357	0,157				0,128	0,129	0,123		
9	CDM14ab	0,051	0,136	0,086	0,069		0,125	0,090				0,106	0,143	0,090	0,014	
10	CDM12	0,139			0,150	0,090	0,114			0,077	0,057	0,082			0,080	0,060
11	PJ	0,110	0,170	0,067	0,020	0,044	0,161	0,168	0,072	0,065	0,044	0,204	0,110	0,049	0,070	0,016
12	CDM16ab	0,109	0,192	0,113	0,051	0,025	0,139	0,106	0,119	0,060	0,025	0,125	0,097	0,096	0,035	0,020
13	CDM15								0,113	0,067	0,110					
14	CDM17d													0,053	0,072	0,044
15	PJ 2											0,049	0,040	0,007	0,059	0,058
16	PJ 3											0,082	0,076	0,025	0,073	0,051
17	PJ 4													0,031	0,016	0,016
	Mean	0,105	0,131	0,067	0,037	0,033	0,179	0,134	0,111	0,064	0,055	0,111	0,097	0,055	0,054	0,037
	SD	0,033	0,061	0,039	0,045	0,028	0,101	0,034	0,028	0,010	0,033	0,049	0,035	0,037	0,025	0,019
	n	5	5	6	10	8	5	5	4	5	5	7	7	10	9	8
	SEM	0,015	0,027	0,016	0,014	0,010	0,045	0,015	0,014	0,004	0,015	0,019	0,013	0,012	0,008	0,007

Developmental viability (proportions of eggs eclosing)																
Trial nr	Electronic file name	HuntAA 25N	HuntAA 50N	HuntAA 100N	HuntAA 200N	HuntAA 300N	MMAA 25N	MMAA 50N	MMAA 100N	MMAA 200N	MMAA 300N	FlyAA 25N	FLyAA 50N	FlyAA 100N	FlyAA 200N	FlyAA 300N
1	0213				82,4	93,3		7,2	52,8					12,5	67,3	
2	0913		11,2	51,2				14,4	44,0	66,4			40,0	71,2		
3	0813		29,6	69,6	76,0			13,6	47,2	72,8			52,8	57,6	64	
4	1113		14,4	61,6	70,4			22,4	58,4	82,4			68,8	69,6	81,6	
5	0214		17,6	50,4	64,0		0,8	14,4	54,4	70,4			76,8	68,8	68	
6	0514	0,0	8,8					14,4				4,8	52,8	85,6	71,2	
7	0714		25,6				0,0				78,4		48,0			
8	0714b	0,0				63,2	0,0				84,8	1,6				77,6
9	0914	0,0				85,6	4,0				88,0	1,6				61,6
10	1014	8,8				79,2		12,8				1,6				68,8
11	1114		20,0										41,2			82,4
	Mean	2,2	18,2	58,2	73,2	80,3	1,2	14,2	51,4	73	83,7	2,4	54,3	60,9	70,4	72,6
	SD	4,4	7,53	9,15	7,85	12,8	1,9	4,45	5,75	6,8	4,89	1,6	13,8	25,3	6,75	9,25
	n	4	7	4	4	4	4	7	5	4	3	4	7	6	5	4
	SEM	2,2	2,85	4,58	3,93	6,4	0,95	1,68	2,57	3,4	2,82	0,8	5,2	10,3	3,02	4,62

Appendix I

Raw data used in Figure 4.5e.

* in the "Electronic filename" column, a grey bold font indicates that for these trials, during the preparation of the holidic media, the acetate buffer was added after the autoclaving step (see online supplementary material in (29)).



Trial nr	Electronic file name	ProteomeAA 25N	Proteome 50N	Proteome 100N	Proteome 200N	Proteome 300N	FlyAA 25N	FLyAA 50N	FlyAA 100N	FlyAA 200N	FlyAA 300N
1	CDM10	74,0	76,5						76,5	76,5	74
2	CDM14ab	71,5	76,0	81,0	76,0		76,0	74,0	74,0	76,0	
3	CDM15		76,0	78,0	80,5	76,0					
4	CDM16ab	74,0	78,5	83,0	81,0	78,5	78,5	85,5	83,0	83,0	83,0
5	PJ 2	73,0	77,2	79,7	79,7	79,7	75	79,7	82,5	79,7	79,7
	Mean	73,1	76,8	80,4	79,3	78,1	76,5	79,7	79,0	78,8	78,9
	SD	1,18	1,05	2,11	2,26	1,89	1,80	5,75	4,45	3,24	4,55
	n	4	5	4	4	3	3	3	4	4	3
	SEM	0,59	0,47	1,06	1,13	1,09	1,04	3,32	2,23	1,62	2,63

Appendix II

Lifespan effect of the weighted fly SILAC proteome AA profile (ProteomeAA, also referred to as SILACAA) compared to compared to that of the fly exome AA profile (FlyAA). All trials shown implemented a late addition of the acetate buffer (see online supplementary material in (29)). (2-way ANOVA analysis with Bonferonni posthoc test, P value = 0.2816 for survival-AA ratio interaction, F ratio = 6.343; both time and AA ratio P value > 0.05).

Chapter 5: Exome-matching in the mouse diet promotes growth and determines insulin resistance

5.1 Abstract

In the previous chapters, the effects of exome-matching on *Drosophila*'s anabolic traits, physiology, and metabolism were explored, and dramatic effects were observed upon a range of physiometabolic aspects. To test how transferable these results may be to mammals, this chapter explores the effects of exome-matching upon mouse growth, behavior, physiology, body composition, activity, respiration, glucose homeostasis, and other health features. The results described in this chapter demonstrate a critical role of the ingested dietary AA profile upon all these health aspects. Therefore these findings carry possible implications for interventional applications on the ageing process of mammals, including in the context of human nutrition.

5.2 Introduction

In chapters 3 and 4 we have seen that the effects of exome-matching on *Drosophila*'s anabolic traits, physiology, and metabolism are dramatic. Dietary supply of AAs in the proportions used by the fly exome promoted greatly anabolic traits including fecundity, development time, viability, and body mass growth. Moreover, exome-matching increased TOR activity and resulted in diets that were more attractive to *Drosophila*. In addition, these flies had lower triglyceride levels and starvation resistance, greater mitochondrial respiration in the fat body, augmented levels of dietary AA utilization and protein accretion, and elevated proteasomal activity. Therefore, exome-matching appeared to define several physiometabolic aspects in flies, although whether or not these effects of exome-matching upon metabolism and physiology affect long term health and ageing are currently being explored.

In Chapter 2 and 3 we also saw that exome-matching is a reliable predictor of the limiting EAA in rodent diets experimentally verified to be restricted for specific EAAs. To test how transferable these results may be to mammals, a semi-defined mouse diet was used to assess the effects of exome-matching upon mouse growth. Moreover, given the dramatic effects of exome-matching upon fly physiology, several other mouse phenotypes were assessed. There included feeding behavior, physiology, body composition, activity, respiration, glucose homeostasis, and other health aspects. The results described in this chapter demonstrate the critical role of the ingested dietary AA profile upon all these features in mice. Therefore the effects of exome-matching described in this chapter carry possible implications for interventional applications on the ageing process of mammals, as well as in the context of human nutrition.

5.3 Results

5.3.1 Growth effect of exome-matching against a casein-matched AA supply

To test whether our exome approach is applicable to mammals, we determined if the exome, as a quantitative measure for defining dietary AA requirements, can be used as a reliable predictor of growth in mice. Thus the dietary protein range adequate for the detection of growth effects induced by AA imbalance was first determined. By testing protein levels known to be restricting for mouse growth and in agreement with previous published data (3, 180), it was determined that a CP content level of between 4% and 7% is optimal (Figure 5.1A). Thus the degree of limitation in the most limiting essential AA (EAA) as defined by the exome can be tested as an accurate predictor of growth rates at these low (4-7% CP) dietary nitrogen levels. Therefore, it was first determined whether semi-defined free AA diets promote growth to the same extent as whole protein diets in mice. A diet with almost half of its crude protein (CP) content supplied as free AAs in the proportions found in casein (CaseinAA), the normal protein component of standard rodent chow, promoted mouse growth to the same extent as a diet with a similar CP content containing only whole casein protein (Casein) (Figure 5.1B). As in flies, protein restriction suppresses the growth (28) and reproductive output of rodents (6). Therefore the ability of exome-matching to enhance mouse growth at low dietary nitrogen conditions of 6% CP was next addressed using semi-defined free AA diets.

After translating the mouse (*Mus musculus*) genome *in silico* it was found that the proportional representation (P) of AAs in the exome (MouseAA) was substantially different from that in CaseinAA (Figure 5.1C), with arginine as the exome-predicted limiting AA in CaseinAA ($r_{R\,(min)} = 0.47$) (Figure 5.1D). The fly findings supported a conditionally-essential role of arginine for egg-laying, and in mice arginine is also reported to be conditionally-essential for growth (28). Yet, some studies have found that limitation in dietary arginine can suppress growth (202), while it can also suppress growth when in dietary excess (26). Therefore arginine is reported to be conditionally essential when limiting, having a limited effect upon growth, but also to be antagonistically detrimental for growth when in excess. Moreover, arginine in casein is reported to be only about 70% bioavailable (28). To assess the role of arginine in CaseinAA, its effect upon growth was first assessed when AAs are supplied at low CP levels (4%) in the form of whole casein protein (Casein 4%). Despite a predicted growth improvement of approximately 41% ($r_{W\,(min)} / r_{R\,(min)} = 0.65 / 0.47 = 1.41$), increasing the dietary supply of arginine by approximately 2-fold to restore its levels to the exome requirement in this diet failed to improve growth (Figure 5.1E). Therefore arginine was found to be conditionally essential at the tested CP levels in casein. Therefore the ability of exome-matching to enhance mouse growth at low dietary nitrogen conditions of 6% CP against CaseinAA was next addressed with arginine not treated as an EAA.

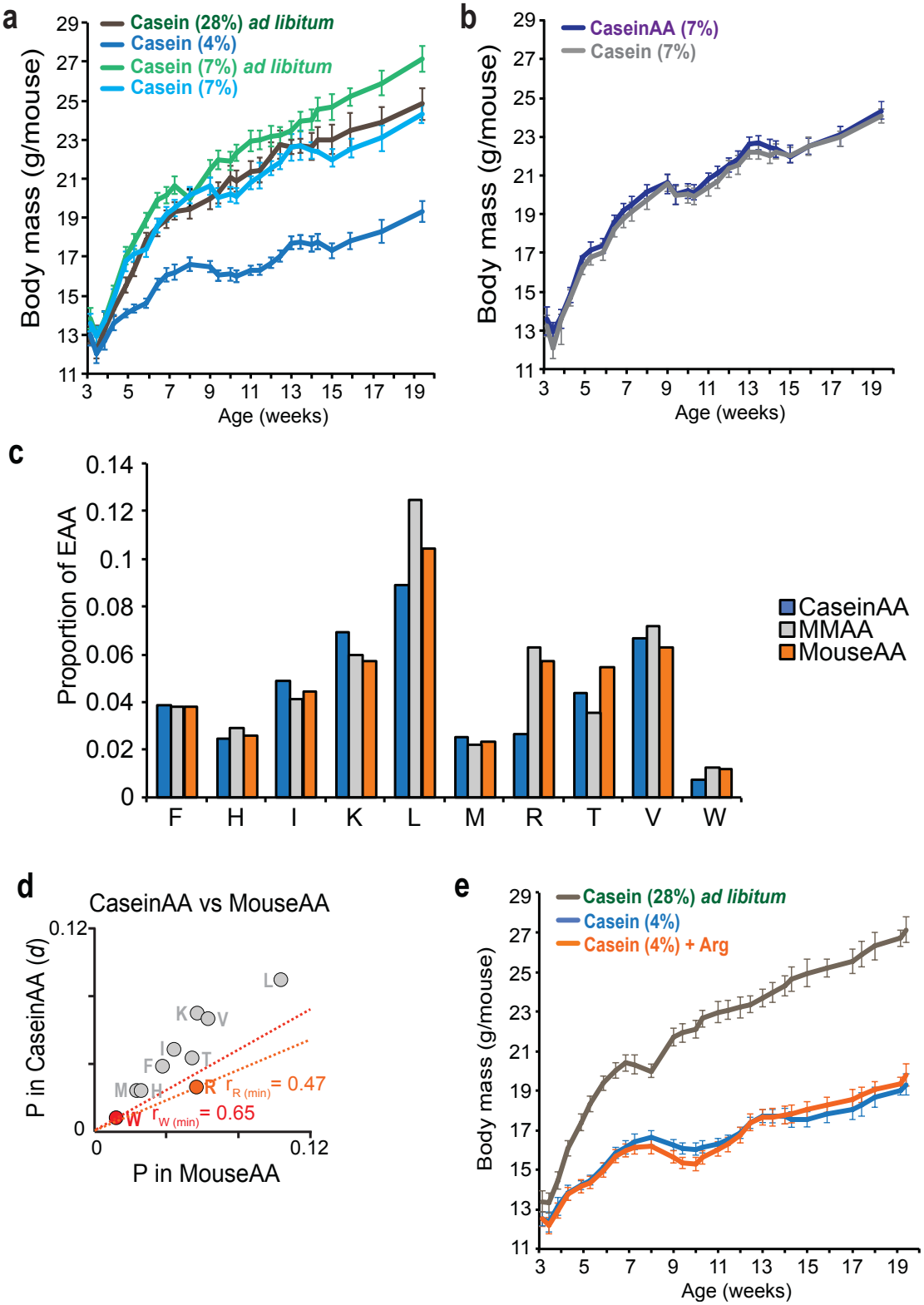


Figure 5.1

a. Mean body mass (grams per mouse) for pair-fed and *ad libitum*-fed diets supplied as whole casein protein across a range of CP contents (4%, 7%, and 28%). The pair-fed range

between 4% and 7% CP was deemed optimal for the testing of AA-imbalanced diets. Growth curves are based on 10 female C3B6F1/J hybrids for each diet (N=10).

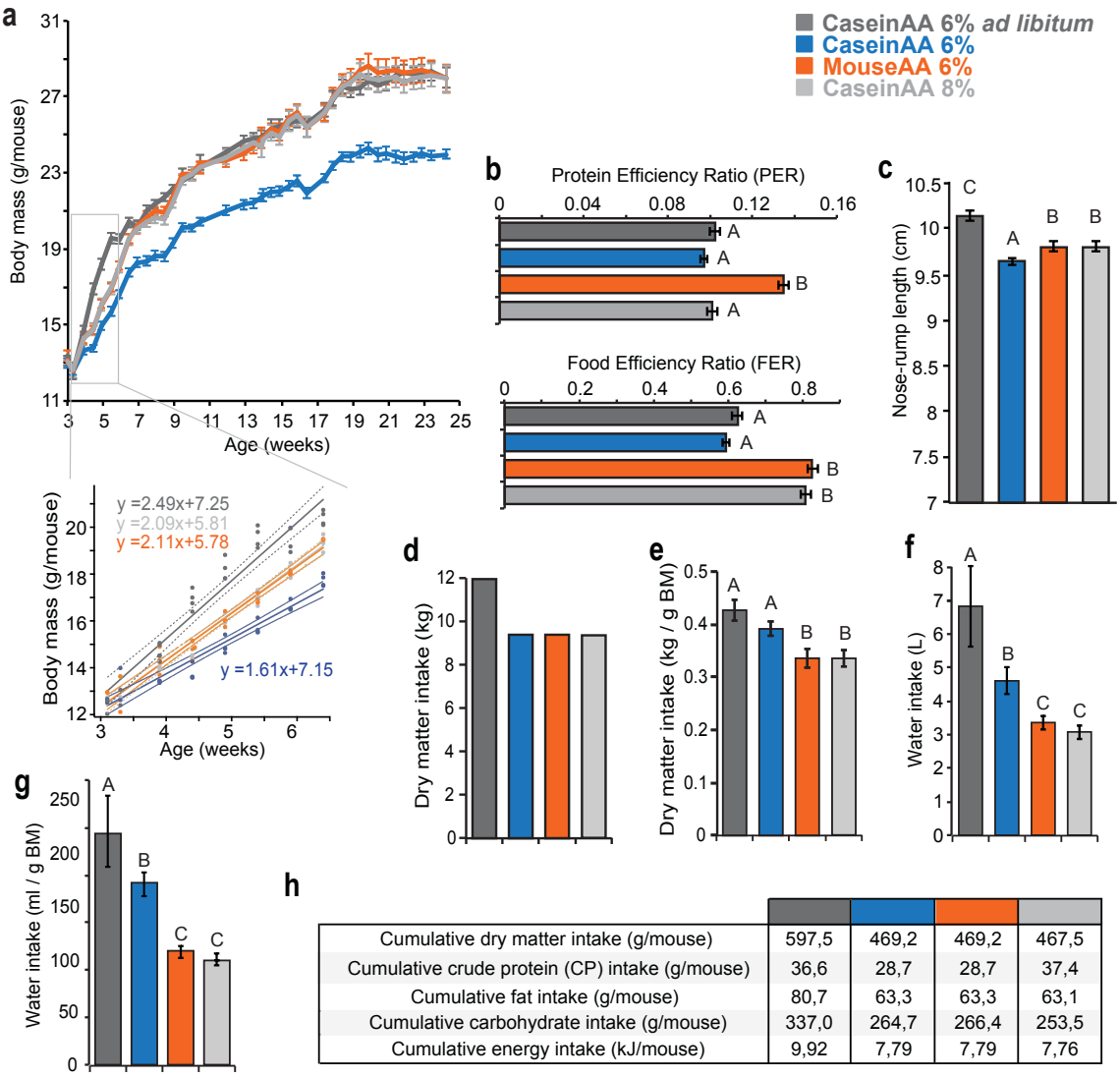
b. Mean body mass (grams per mouse) for pair-fed casein as whole protein (CP content 7%), and a diet comprised of a 5.5:4.5 mix of whole casein:free AAs supplied as CaseinAA. No difference was seen between the two diets, suggesting a similar utilization for the two AA sources (polynomial regression analysis P value > 0.05, N=10).

c. Comparison of CaseinAA, MouseAA, and mMMAA (see text for details). Each bar represents the molar proportional representation of each EAA in the diet.

d. Comparison of casein and mouse exome EAA ratios. In this instance, tryptophan (W) is the most limiting EAA in CaseinAA ($r_{W(min)} = 0.65$), compared to the mouse exome ratio (MouseAA).

e. Mean body mass (grams per mouse) for pair-fed mice on a 4% CP casein diet without (Casein 4%) or with an arginine supplementation (Casein 4% + Arg). Although both groups had restricted growth compared to an *ad libitum* group fed 28% CP casein (Casein 28%), the supplementation of arginine had no effect on growth (polynomial regression analysis P value > 0.05, N=10).

Using this model, mice fed CaseinAA were predicted to have a 59% growth limitation (1/0.65) compared to mice fed a dietary AA ratio matching the exome (MouseAA; Figure 5.1D). In fact, mice fed MouseAA gained 38% more body mass than mice fed CaseinAA (Figure 5.2A). Moreover, animals fed MouseAA at 6% CP (MouseAA 6%) matched the growth rate of animals fed CaseinAA at 8% CP (CaseinAA 8%) when considering the linear part of the growth curve (Figure 5.2A). By 10 weeks of age, animals fed MouseAA 6% also matched the growth rate of animals *ad libitum*-fed CaseinAA 6% (Figure 5.2A). Consequently, the protein efficiency (PER) and food efficiency (FER) ratios were the highest for animals fed MouseAA 6% (Figure 5.2B). Body size (nose-rump length) was also significantly lower in CaseinAA 6% compared to MouseAA 6%, which matched the body size of mice fed CaseinAA 8% (Figure 5.2C). Dry matter intake indicated that the amount of food consumed was equal between the pair-fed groups, but was 25% higher for the CaseinAA 6% *ad libitum* group (Figure 5.2D). When normalized to body mass (BM), mice on CaseinAA 6% consumed 17% more dry matter than mice on MouseAA 6% (Figure 5.2E). Water consumption was 35% higher in CaseinAA 6% compared to the MouseAA 6% (Figure 5.2F), or 45% higher when normalized to BM (Figure 5.2G). The intake for all macronutrients and energy is shown on Figure 5.2H.



Cumulative dry matter intake (g/mouse)	597,5	469,2	469,2	467,5
Cumulative crude protein (CP) intake (g/mouse)	36,6	28,7	28,7	37,4
Cumulative fat intake (g/mouse)	80,7	63,3	63,3	63,1
Cumulative carbohydrate intake (g/mouse)	337,0	264,7	266,4	253,5
Cumulative energy intake (kJ/mouse)	9,92	7,79	7,79	7,76

Figure 5.2

a. Growth rates for females fed *ad libitum* fed CaseinAA 6%, or pair-fed CaseinAA 6%, MouseAA 6%, or CaseinAA 8% (% indicates CP content). The linear part of the growth curves (lower panel) indicates that MouseAA 6% supported a 38% faster growth rate compared to CaseinAA 6% (linear regression analysis P value < 0.0001), based on net mass gain for that period (weeks 3 to 6.4 of age). Growth curves are based on 20 female C3B6F1/J hybrids for each diet (N=20).

b. Effect of dietary AA ratio on protein (upper panel) and food (lower panel) efficiencies. Two common indices of diet efficiency, the protein efficiency ratio (PER) and the food efficiency ratio (FER; see methods), responded according to the exome-based predictions. After 20 weeks of dietary treatment on the different AA ratios, mean PER and FER were the highest in MouseAA (6%), and similarly reduced in *ad libitum*-fed CaseinAA (6%), in pair-fed CaseinAA (6%), and in pair-fed CaseinAA (8%). Neither the feeding mode, nor the increase in total AAs changed the PER of CaseinAA, reflecting the degree of limitation for Trp in CaseinAA ($r_{EAA(min)}$ = 0.65). Bars represent the mean for each group (N = 20).

c. Effect of dietary AA ratio on body size (nose-rump length) after 20 weeks on the respective dietary regime. Body size was the greatest in *ad libitum*-fed CaseinAA (6%), while it was identical between pair-fed MouseAA (6%) and CaseinAA (8%). In contrast, pair-fed CaseinAA 6% had the shortest body size. Bars represent the mean for each group (N = 20), and error

bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

d. Cumulative food intake across dietary regimes. Bars shown represent the cumulative dry matter consumed by 20 females after 20 weeks (age 24 weeks) in the respective dietary regime. As all but one group (*ad libitum*-fed CaseinAA 6%) were pair-fed, there was no cage-to-cage variability, so no error bars are shown for this cumulative measure (N = 1).

e. Food intake normalized to body mass across dietary regimes. Bars shown represent the cumulative dry matter consumed by 20 females after 20 weeks (age 24 weeks) in the respective dietary regime, normalized to the mean body mass for each group (N = 20). Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

f. Water intake across dietary regimes. Figure 5.shows the cumulative water consumed by 20 females after 20 weeks (age 24 weeks) in the respective dietary regime. Bars shown represent the mean water intake between 4 cages (each housing 5 mice) per diet (N = 4). Error bars represent SD, letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

g. Water intake normalized to body mass across dietary regimes. Animals fed CaseinAA (6%) consumed significantly more water independently of the regime (*ad libitum* or pair-fed), compared to animals fed MouseAA (6%) or CaseinAA (8%). Figure 5.shows the cumulative water consumed by 20 females after 20 weeks (age 24 weeks) in the respective dietary regime, normalized to the mean body mass for each group. Bars shown represent the mean water intake between 4 cages (each housing 5 mice) per diet (N = 4). Error bars represent SD, letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

h. Cumulative macronutrient consumption across dietary regimes. Values shown represent the mean amounts consumed by a female after 20 weeks (age 24 weeks) in the respective dietary regime. Crude protein (CP) was calculated as N x 6.25 = CP %. Energy was calculated by normalizing the energy content of the diets (16.6 MJ/kg; all diets were isoenergetic) to the mean dry matter intake per group.

In a repeat experiment at identical dietary nitrogen levels and under a pair-feeding regime ensuring identical food intakes among diets, MouseAA promoted a 32% higher body mass growth than mice fed CaseinAA (Figure 5.3A). Therefore the mean growth improvement from both experiments was 35%. Moreover, when the exome (MouseAA) was replaced as a measure of predicting growth with the weighted mouse SILAC proteome (mSILACAA), CaseinAA was predicted to be only 20% limiting (Figure 5.3B), which was lower than the observed growth suppression, suggesting that MouseAA is a more accurate predictor for growth than mSILACAA.

It was next confirmed whether, similarly to flies, mice also perceive a balanced AA ratio differently. As in the previous experiment, *ad libitum* fed mice given the CaseinAA diet consumed 13% more dry matter than mice fed the MouseAA diet (P value < 0.05, Student's t-test), or 21% more dry matter per gram of body mass (P value < 0.05, Student's t-test; Figure 5.3C). In a separate experiment, mice fed MouseAA *ad libitum* grew significantly more than mice fed CaseinAA *ad libitum* (Figure 5.3D), so the growth promoting effect was independent of the feeding mode as it was observed in both *ad libitum* and pair-fed mice. In addition, in the *ad-libitum* regime animals fed MouseAA consumed less protein than that consumed by mice fed Casein AA (Figure 5.3E), so the enhanced protein and food efficiency ratios observed in the pair-fed animals persisted upon animals fed *ad libitum*. Moreover, mice *ad libitum*-fed MouseAA consumed significantly less water compared to mice *ad libitum*-fed CaseinAA (Figure 5.4F). Combined, these observations demonstrate that the exome-matched diet contains a higher quality protein that is both perceived as more satiating and sustains a higher growth rate even when it results in a lower protein intake.

Finally, to confirm that the growth-limiting effect of CaseinAA compared to MouseAA would be less clear at higher CP levels, we tested the growth effect of the two AA profiles at 7.4% CP and compared it to the effect observed at 6.1% CP. Indeed, the growth difference between CaseinAA vs. MouseAA was diminished when dietary nitrogen was increased to 7.4% CP (Figure 5.3G). This supports the idea that, as in flies, an interaction between dietary nitrogen levels and dietary AA ratios determines AA utilization and growth in mice, with growth rate no longer very limited at higher protein/AA levels, confirming previous reports (180).

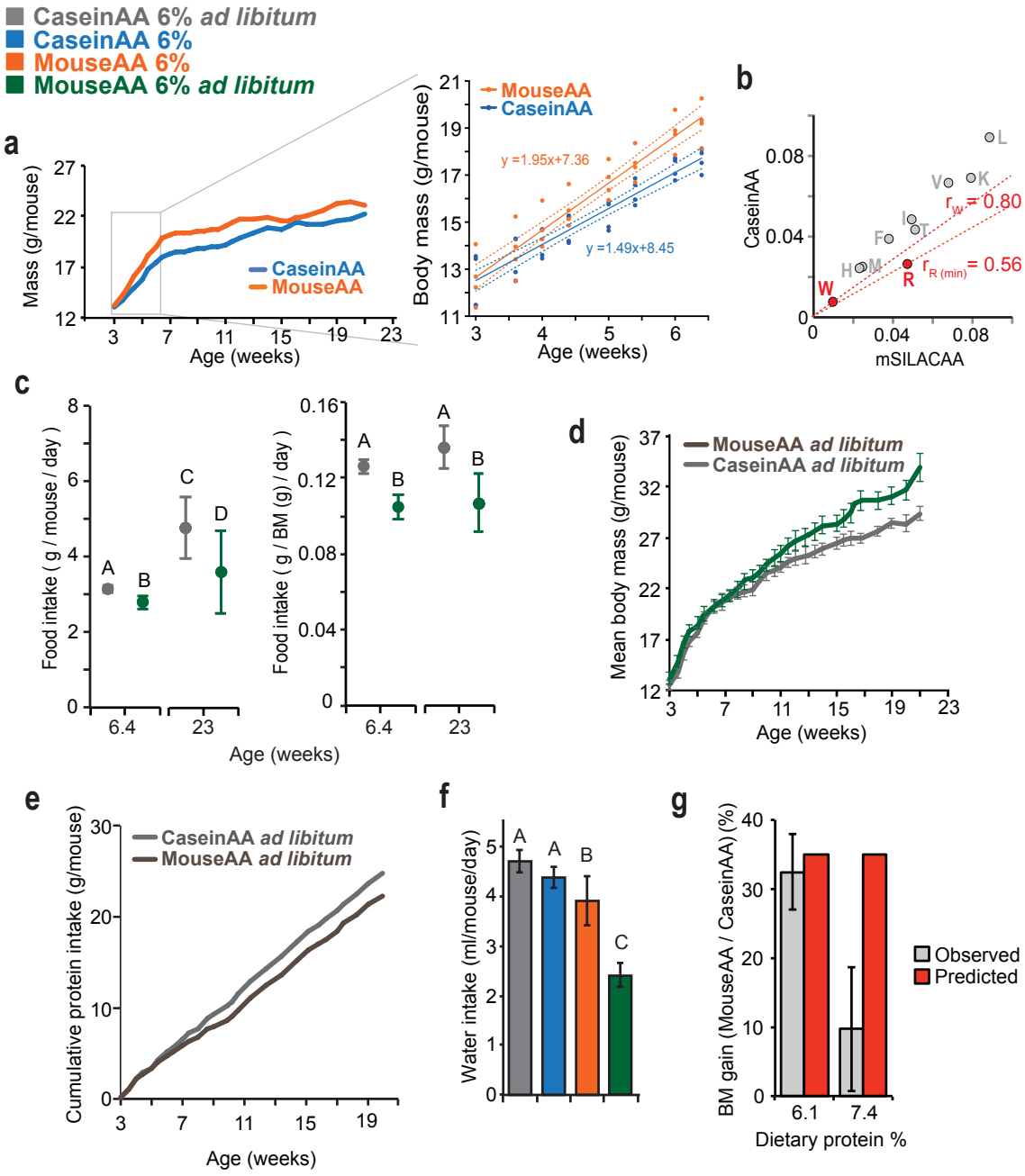


Figure 5.3

a. Growth rates for mice pair-fed CaseinAA or MouseAA. The linear part of the growth curves (3.0 to 6.4 weeks of age) indicates that CaseinAA suppresses growth compared to MouseAA, based on net mass gain for that period (linear regression analysis, P value = 0.0003). Growth curves are based on 20 female C3B6F1/J hybrids for each diet (N=20), housed 5 per cage (mean body mass per cage shown as single data point for each treatment). Curves represent the linear fit model for each treatment +/- 95% CI.

b. Comparison of AAs in CaseinAA to the mouse SILAC proteome AA usage (mSILACAA). Considering that the arginine-limitation in CaseinAA ($r_{R(min)}$ = 0.56) is conditionally essential, mSILACAA predicts that the next limiting EAA is tryptophan at $r_{W(min)}$ = 0.80, and therefore only a 20% limitation for CaseinAA.

c. Effect of dietary AA ratio on food intake. Mean *ad libitum* dry matter intake for mice fed the MouseAA and the CaseinAA ratio. Data are shown as food intake and normalized to body mass for two time points: at 6.4 weeks of age, representing the linear growth curve part, and at 23 weeks of age, representing the end-point. Mice fed on the CaseinAA diet consumed significantly more dry matter per day at both time points than mice fed the MouseAA diet, whether normalized to body mass or not ($p<0.05$, Student's t-test, N = 20).

d. Growth rates for mice *ad libitum*-fed CaseinAA or MouseAA. CaseinAA suppresses growth compared to MouseAA (polynomial regression analysis, P value < 0.05, N=20).

e. Protein intake for mice ad libitum-fed CaseinAA and MouseAA. Values represent the cumulative intake of 20 animals per diet for the first 20 weeks of age.

f. Water intake for mice *ad libitum*-fed and pair-fed on CaseinAA or MouseAA. Mice *ad libitum*-fed MouseAA consumed significantly less water compared to mice *ad libitum*-fed CaseinAA, but this difference was not significant between pair-fed animals (letters indicate significant change, $p<0.05$, Student's t-test, N = 4).

g. Relationship of dietary EAA limitation and dietary nitrogen levels on growth. The growth increase promoted by MouseAA over that promoted by CaseinAA is here expressed as a percentage ((mass gain in MouseAA / mass gain in CaseinAA)*100), for two dietary crude protein (CP) levels (N x 6.25). At lower CP levels (6.1%), the observed growth increase for MouseAA relative to that of CaseinAA is 32% and nearly matches the predicted increase of 35% (this value is a consequence of $r_{W(min)} = 0.65$ in CaseinAA, indicating a 35% limitation for Trp). However, at higher dietary CP levels (7.4%), the observed increase is only 10%, much shorter of the 35% predicted value, indicating that at high dietary AA levels the effect of the exome-defined imbalance diminishes. For the observed series, data are based on 20 female C3B6F1/J hybrids for each diet (N=20), while error bars represent SD.

5.3.2 Growth effect of exome-matching against an exome-mismatched AA supply

Similarly to the approach in flies, a MisMatched AA ratio for mice (MMAA) was designed that was predicted to be equally as mismatched as CaseinAA is to MouseAA, but with a different proportion of all 20 amino acids and with the identity, but not degree, of its most limiting EAA altered (Figure 5.4A). MouseAA supported a higher growth rate against MMAA (Figure 5.4B). We further assessed the exome-based identification of the most limiting AA in mMMAA, in an approach similar to what we did previously with Arginine in flies fed HuntAA. We generated two more AA ratios, a modified MMAA ratio (MMAA-T) that contained 30% less of the exome-predicted limiting AA (Thr) than mMMAA did, and a second modified MMAA ratio (MMAA-M) that contained 30% less of a predicted surplus AA (Met) than MMAA. According to our model, we anticipated that reducing the predicted most limiting AA (mMMAA-T) would further depress growth, while reducing a predicted surplus AA (MMAA-M) would have no impact on growth. This prediction was confirmed as mice fed the MMAA-Thr diet were growth-depressed compared to mice fed the original MMAA diet (Figure 5.4B), while in contrast mice fed the MMAA-Met ratio matched the growth rate of mice fed MMAA (Figure 5.4B). Mice fed CaseinAA ($r_{W\,(min)} = 0.65$), MMAA ($r_{T\,(min)} = 0.65$), or MMAA-M ($r_{T\,(min)} = 0.65$) also promoted food and protein efficiencies to a similar extent (Figure 5.4C-D). Combined these data strongly suggest that the exome defines AA requirements in mice, and consequently may determine the response of anabolic traits such as growth in mice feeding on diets of different AA profiles.

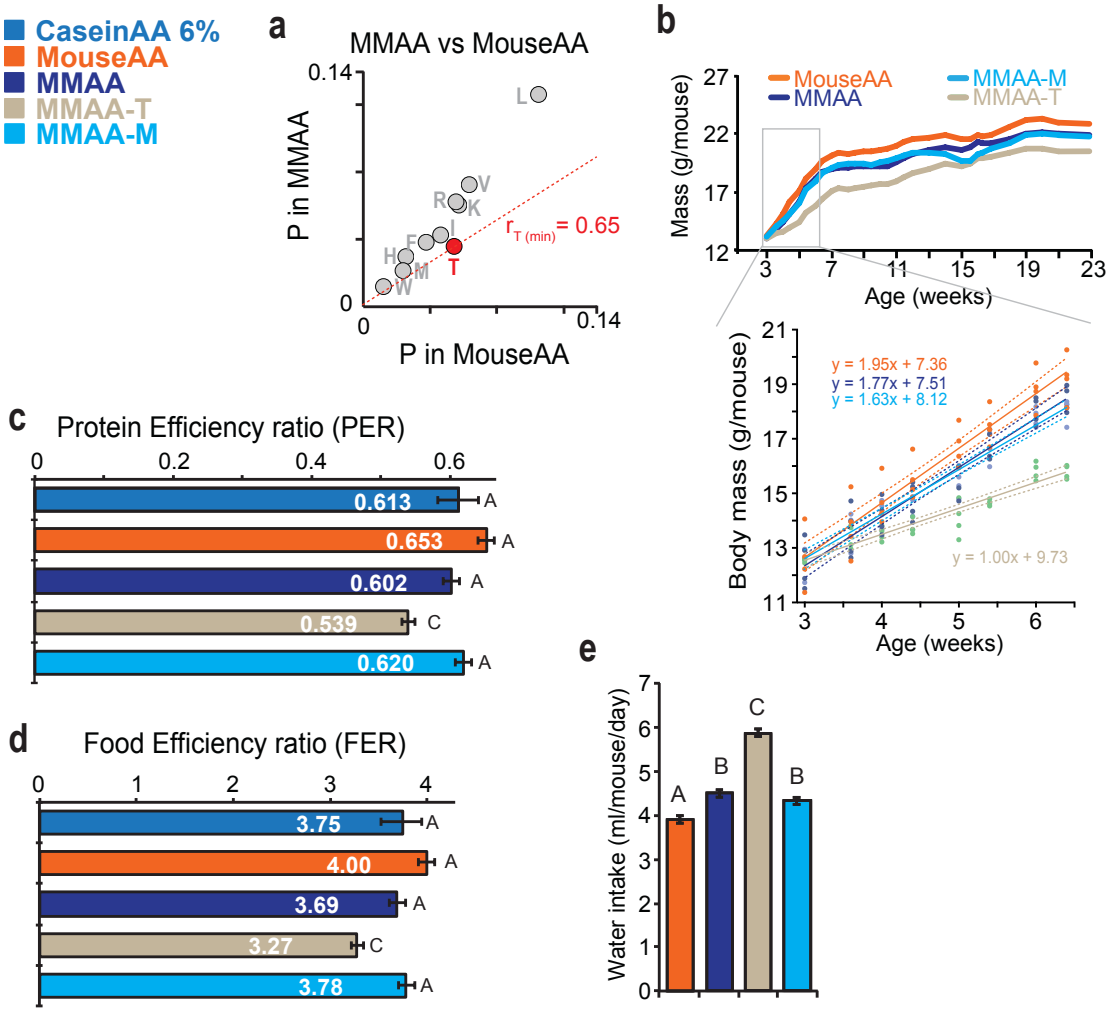


Figure 5.4

a. Comparison of mismatched (MMAA) and mouse exome (MouseAA) EAA ratios. Threonine (T) is the most limiting EAA in MMAA ($r_{T(min)}$ = 0.65). Therefore the degree of limitation for Thr in MMAA is the same to that for tryptophan in CaseinAA ($r_{W(min)}$ = 0.65).

b. Growth rates for females fed MouseAA, MMAA, MMAA-M, and MMAA-T. The linear part of the growth curves (3.0 to 6.4 weeks of age) indicates that MMAA and MMAA-M suppress growth by 14% and 20% respectively compared to MouseAA, based on net mass gain for that period (linear regression analysis, P values = 0.2265 and 0.0177). In contrast, MMAA-T suppressed growth by 48% (linear regression analysis, P value < 0.0001), nearly matching the 54% predicted suppression. Growth curves are based on 20 female C3B6F1/J hybrids for each diet (N=20), housed 5 per cage (mean body mass per cage shown as single data point for each treatment). Plots shown represent the linear fit model for each treatment, +/- 95% CI.

c/d. Effect of dietary AA ratio on protein (c) and food (d) efficiencies at 23 weeks of age. Both indices, the protein efficiency ratio (PER) and the food efficiency ratio (FER; see methods), shown after 20 weeks of dietary treatment (23 weeks of age). Although both indices were higher in MouseAA, and similarly reduced in CaseinAA, MMAA, and MMAA-M, most likely reflecting the identical degree of their limitation for Trp or Thr ($r_{EAA(min)}$ = 0.65), due to a large inter-cage variance and plateauing of the growth curves by this age, these changes were not significant. However, MMAA-T had the lowest indices, as a consequence of the degree of its limitation ($r_{T(min)}$=0.46). Bars shown represent the mean for each group (N = 20). Letters

indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).
e. Water intake for mice *ad libitum*-fed and pair-fed on MouseAA, MMAA, MMAA-T, and MMAA-M. Mice pair-fed MouseAA consumed significantly less water compared to all other pair-fed mice (letters indicate significant change, $p<0.05$, Student's t-test, $N = 4$).

5.3.3 Nitrogen excretion

The observed increase in body mass for mice fed MouseAA compared to CaseinAA suggested a better utilization of dietary AAs. To confirm whether utilization of ingested AAs was indeed higher for MouseAA, urea excretion normalized to water intake and body mass (227, 228) was first assessed. Mice fed CaseinAA (both *ad libitum* or pair-fed at 6% or 8% CP) a higher excretion of urine urea loss compared to mice fed MouseAA (6%) (Figure 5.6A). This result indicated that exome-matching reduces urea and nitrogen losses.

To further characterize nitrogen losses, a nitrogen balance analysis was carried out (186). Absolute nitrogen intake and losses were quantified in purpose-fit metabolic cages after 20 weeks of treatment, equivalent to 23 weeks of age (see Figure 5.4B for position on growth curve), with all mice allowed *ad libitum* access to food (see methods). Again, mice previously fed MouseAA consumed significantly less food and nitrogen than mice fed CaseinAA regardless of feeding mode (*ad libitum* or pair-fed; Figure 5.6B-C; Figure 5.key indicates treatment up to nitrogen balance assessment; for all results shown, all mice were allowed *ad libitum* access to food). There was also a strong correlation between dry matter intake and faecal output (Figure 5.6D). The amount of urine excreted was also higher in mice fed CaseinAA (Figure 5.s 3E-F), and it was also correlated to water intake across diets (Figure 5.6G). Again mice fed MouseAA secreted less urine than mice fed CaseinAA (Figure 5.6H). Moreover, the net protein utilization (NPU) is a measure of the efficiency of the ingested nitrogenous source, and is expressed as the ratio of utilized:ingested nitrogen. Although all animals had a positive nitrogen balance (more nitrogen utilized than excreted; data not shown), mice pair-fed CaseinAA had a significantly lower NPU value compared to mice fed MouseAA (Figure 5.6I). These results demonstrated that, as in flies, exome-matching increases the utilization of dietary nitrogen source in mice compared to common protein sources such as casein.

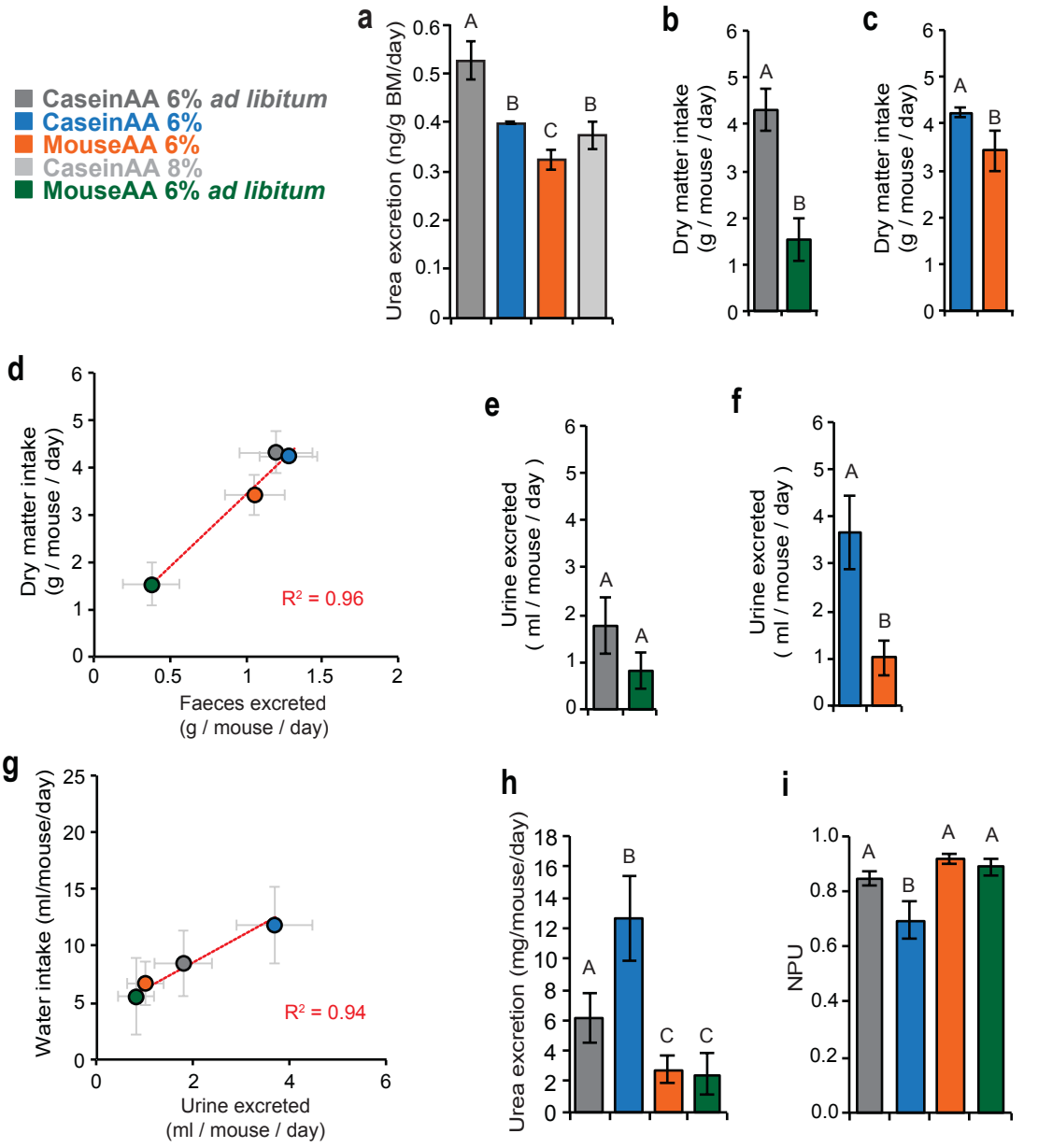


Figure 5.6

(Figure 5.key indicates treatment up to nitrogen balance assessment. For all experiments shown, all mice were allowed *ad libitum* access to food)

a. Daily urea excretion normalized to body mass, for mice after 20 weeks on the respective dietary regime. From the three regimes consuming equal amounts of nitrogen in the form of AAs (pair-fed CaseinAA 6%, MouseAA 6%, and CaseinAA 8%), MouseAA had the lowest levels of urea excretion. Bars represent the mean for each group (N = 5), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

b. Daily dry matter intake, for mice after 20 weeks on the respective *ad libitum* regime. Mice fed MouseAA had lower food consumption than mice fed CaseinAA. Bars represent the mean for each group (N = 5), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

c. Daily dry matter intake, for mice after 20 weeks on the respective pair-fed regime. Mice fed MouseAA had lower food consumption than mice fed CaseinAA. Bars represent the mean for each group (N = 5), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

d. Correlation of daily dry matter intake with daily fecal excretion. Data points represent the mean for each group (N = 5), and error bars represent SD. Regression analysis fit R^2 value = 0.96.

e. Daily urine volume excreted for mice after 20 weeks on the respective *ad libitum* regime. Mice fed MouseAA had lower urine excretions than mice fed CaseinAA. Bars represent the mean for each group (N = 5), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

f. Daily urine volume excreted for mice after 20 weeks on the respective pair-fed regime. Mice fed MouseAA had lower urine excretions than mice fed CaseinAA. Bars represent the mean for each group (N = 5), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

g. Correlation of daily water intake with daily urine excretion. Data points represent the mean for each group (N = 5), and error bars represent SD. Regression analysis fit R^2 value = 0.94.

h. Daily urea excretion (mg) for mice after 20 weeks on the respective dietary regime. Mice fed MouseAA had the lowest levels of urea excretion. Bars represent the mean for each group (N = 5), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

i. Net protein utilization (NPU) for mice after 20 weeks on the respective dietary regime. Mice fed MouseAA had the highest levels of NPU, with mice pair-fed (but not *ad libitum*-fed) CaseinAA having significantly lower NPU. Bars represent the mean for each group (N = 5), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

5.3.4 Lipid storage and body composition

To examine how body composition is affected by the different dietary AA ratios, fat and lean contents were determined both *in vivo* and histologically (Figure 5.7 data by George Soultoukis, Hanna Salmonowicz, Martin Purrio, Dr. Andrea Mesaros, Dr. Youssef Hassan, and Dr. Alain De Bruin). Although post-weaning mice started with identical fat contents, by week 23 mice fed MouseAA 6% had significantly elevated lean mass contents (Figure 5.7A), as well as increased body fat, matching the body fat profile of mice fed CaseinAA 8% (Figure 5.7B). To establish if the feeding mode (*ad libitum* vs. pair-fed) can alter the fat storage response to the ingested AAs, the body fat in animals fed MouseAA 6% and CaseinAA 6% *ad libitum* was also checked and it was observed that these differences persisted (Figure 5.7C). Differences in fat storage were also assessed histologically. Compared to CaseinAA 6%, abdominal white adipose tissue (aWAT) was greater in MouseAA 6%, and similar to CaseinAA 8%, while no change was seen between *ad libitum* and pair-fed CaseinAA 6% (Figure 5.7D). Moreover, the difference in fat mass between pair-fed or *ad libitum*-fed MouseAA 6% and CaseinAA 6% persisted after normalizing fat mass either to body size (nose-rump length; Student's t-test P value < 0.05) or after expressing it as a ratio to total lean mass (% lean mass x BM; Student's t-test P value < 0.05; data not shown). Abdominal fat lesions were not found to differ among groups (data not shown), but fat storage in skeletal muscle was significantly lower in CaseinAA 6% compared to MouseAA 6% or CaseinAA 8% (Figure 5.7E-F). Moreover mice fed CaseinAA 6% had reduced skin thickness (Figure 5.7G), which was primarily the consequence of reduced subcutaneous adiposity (SCAT) (Figure 5.7H) and a constant SCAT:skin thickness ratio (Figure 5.7I).

To establish how ingested AAs alter lipostasis in more detail, we investigated the molecular mechanisms behind these changes in adiposity. Mice fed CaseinAA 6% had no elevated levels of circulating ketones, higher levels of which are typically associated with increased lipolysis (Figure 5.7J). We also observed higher levels of the rate-limiting enzyme in *de novo* lipogenesis, fatty acid synthase (FASN; Figure 5.7K) in the livers of mice pair-fed MouseAA 6%. Moreover, although there were no changes in the hepatic levels of the rate-limiting enzyme in *de novo* triglyceride synthesis stearoyl-CoA desaturase 1 (SCD-1) (Figure 5.7L), mice fed MouseAA 6% had significantly increased levels of both liver triglycerides (Figure 5.7M) and plasma triglycerides, with the effect on circulating TAGs seen on both *ad-libitum* and pair-fed animals (Figure 5.7N).

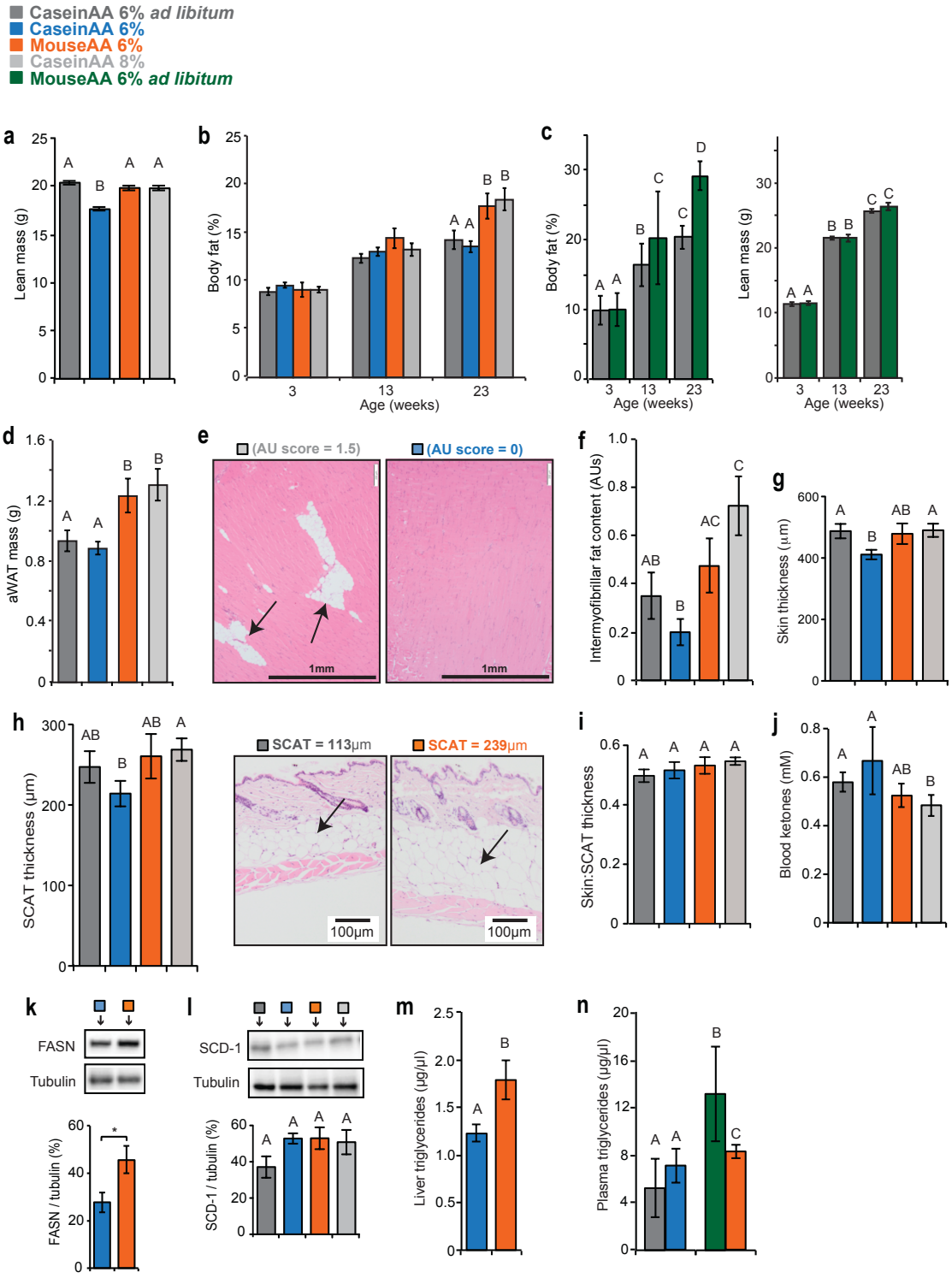


Figure 5.7

(Data by George Soultoukis, Hanna Salmonowicz, Martin Purrio, Dr. Andrea Mesaros, Dr. Youssef Hassan, and Dr. Alain De Bruin)

a. Effect of dietary AA ratio on lean mass after 20 weeks on the respective dietary regime. Lean mass was identical between *ad libitum*-fed CaseinAA (6%), and pair-fed MouseAA (6%) or CaseinAA (8%). In contrast, pair-fed CaseinAA (6%) had the lowest lean mass. Bars

represent the mean for each group (N = 20), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

b. Body composition (fat %) across dietary AA ratios. Post-weaning fat mass was identical between all groups, as was fat % after 10 weeks of treatment (age 14 weeks). However, fat % was significantly increased in mice pair-fed MouseAA (6%) or CaseinAA (8%) after 20 weeks of treatment (age 23 weeks). Bars represent the mean for each group (N = 20), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

c. Body composition (fat % and lean mass) across dietary AA ratios. Post-weaning fat mass was identical between the two groups, but fat % was significantly increased in mice *ad libitum*-fed MouseAA 6% compared to mice *ad libitum*-fed CaseinAA 6% after 10 and 20 weeks of treatment on the respective diet (age 13 and 23 weeks respectively). Lean mass was unchanged between the groups across the time points. Bars represent the mean for each group (N = 20 for weeks 3 and 13, N = 10 for week 23), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data by George Soultoukis, Martin Purrio and Dr. Andrea Mesaros.

d. Effect of dietary AA ratio on abdominal white adipose tissue (aWAT) wet mass. After 20 weeks of treatment on the respective diet (age 24 weeks), aWAT was significantly increased in mice pair-fed MouseAA (6%) or CaseinAA (8%). Bars represent the mean for each group (N = 20), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

e. Histological analysis of skeletal muscle intermyofibrillar fat. After 20 weeks of treatment on the respective diet (age 24 weeks), the right quadricep femoris muscle was assessed and given an arbitrary value reflecting the visible intermyofibrillar fat (as indicated by arrows). Two examples are shown here for mice pair-fed CaseinAA 6% (left panel) or CaseinAA 8% (right panel). Data Dr. Youssef Hassanand Dr. Alain De Bruin.

f. Quantified effect of dietary AA ratio on skeletal muscle intermyofibrillar fat, assessed as described above. Mice pair-fed CaseinAA 6% had the lowest score. Bars represent the mean arbitrary value for each group (N = 20), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data by George Soultoukis, Dr. Youssef Hassanand Dr. Alain De Bruin.

g. Skin thickness (epidermis to panniculus carnosus) thickness across dietary AA ratios. Mice pair-fed CaseinAA (6%) had the thinnest skin thickness. Bars represent mean values (N = 20), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data by George Soultoukis, Dr. Youssef Hassanand Dr. Alain De Bruin.

h. Subcutaneous adipose tissue (SCAT) thickness across dietary AA ratios. Mice pair-fed CaseinAA (6%) had the thinnest SCAT layers. Bars represent mean values (N = 20), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Panels on right show representative histological cross sections. Data by Dr. Youssef Hassanand Dr. Alain De Bruin.

i. The ratio of skin thickness (epidermis to panniculus carnosus) thickness to subcutaneous adipose tissue (SCAT) thickness across dietary AA ratios was constant across the dietary regimes. Bars represent mean values (N = 20), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data by George Soultoukis, Dr. Youssef Hassanand Dr. Alain De Bruin.

j. Blood ketones across dietary AA ratios. Mice pair-fed MouseAA (6%) or CaseinAA (8%) had the lowest levels of circulating ketones. Bars represent mean values (N = 20), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data by Martin Purrio and George Soultoukis.

k. Hepatic fatty acid synthase (FASN) across dietary AA ratios. Mice pair-fed MouseAA (6%) had significantly increased levels of FASN. Bars represent mean values (N = 6), and error bars represent SD. Stars indicate a statistically significant difference (P value < 0.05) in a pair-wise comparison (Student's t-test). Data by Hanna Salmonowicz and George Soultoukis.

l. Effect of dietary AA ratio on the hepatic level of the rate-limiting enzyme in *de novo* triglyceride synthesis stearoyl-CoA desaturase 1 (SCD-1), assessed by Western Blotting analysis. There were no detectable changes between the dietary AA profiles after 20 weeks of treatment (24 weeks of age) on the respective diet. Bars represent mean values of 4 trials (N = 4), each trial using 4-6 animals. Error bars represent SD and letters indicate no statistically significant difference (P value > 0.05) for pair-wise comparisons (Student's t-test).

m. Hepatic triacylglycerides (TAGs) across dietary AA ratios. Mice pair-fed MouseAA (6%) had significantly increased levels of liver TAGs. Bars represent mean values (N = 9-10), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data by Hanna Salmonowicz and George Soultoukis.

n. Plasma triacylglycerides (TAGs) across dietary AA ratios. Mice fed MouseAA (6%) had significantly increased levels of liver TAGs compared to mice fed CaseinAA (6%) independently of the feeding mode (*ad libitum* or pair-fed). Bars represent mean values (N = 9-10), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

5.3.5 Nutrient sensing

There was no significant change in the hepatic level or activation of the mammalian target of rapamycin (mTOR) (Figure 5.8A), of its downstream effector S6 kinase (S6K) (Figure 5.8B), or in the hepatic levels of the ribosomal subunit S6 (not shown). Moreover, there were no changes detected in the hepatic level or activation of the nutrient sensor AMP-activated protein kinase (AMPK), which senses low-energy states by detecting high AMP levels (Figure 5.8C), apart from a decrease in AMPK phosphorylation by *ad libitum* or higher (8%) CP regimes. No differences were detected in the levels of protein or activation of the nutrient responsive IIS pathway protein kinase B (Akt) (Figure 5.8D). Notably, these measurements were done on hepatic tissue extracted at 24 weeks of age, by which point the growth of animals had reached a plateau (Figure 5.2A). However, mice fed CaseinAA 6% had higher levels of p-GCN2 (Figure 5.8E).

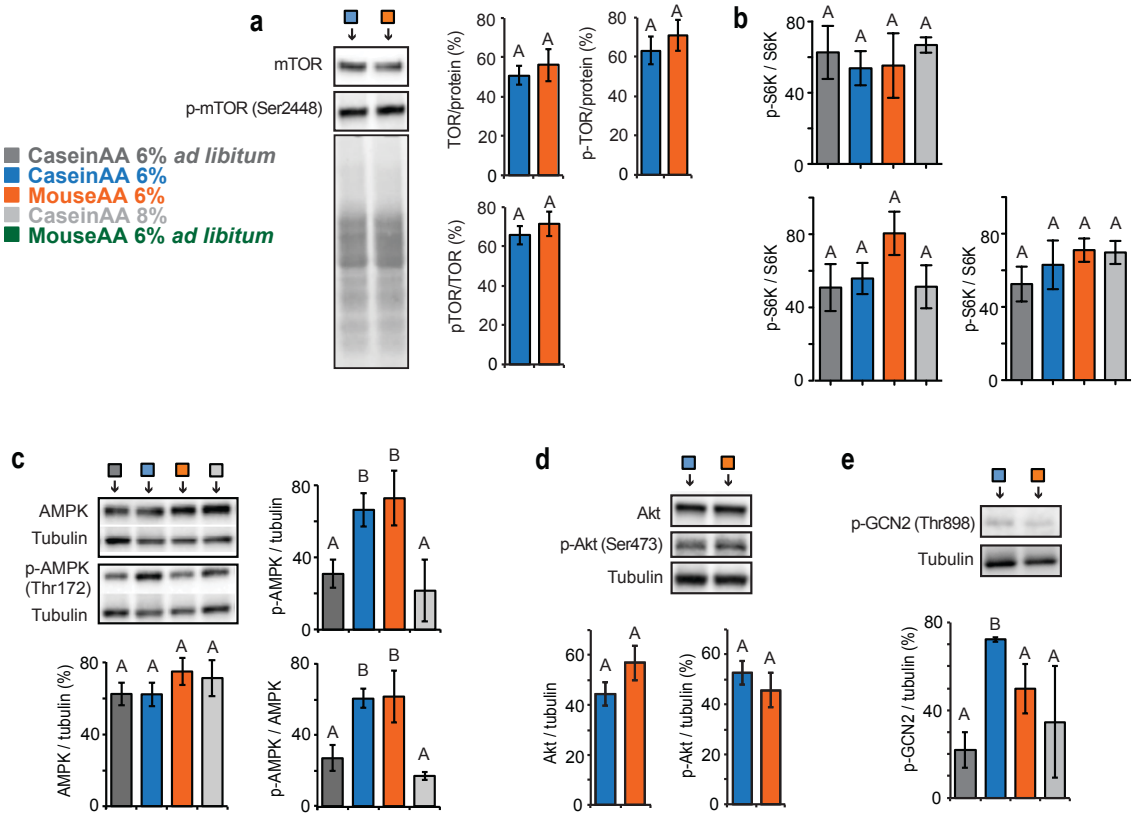


Figure 5.8

(data by George Soultoukis and Hanna Salmonowicz)

a. Effect of dietary AA ratio on the hepatic level and activation of a key mediator of AA sensing, the target or rapamycin (TOR), assessed by Western blotting. There were no detectable changes in the level or activation of TOR between the dietary AA profiles after 20 weeks of treatment (24 weeks of age) on the respective diet. Bars represent mean values of 4

blots (N = 4), each using 6-10 animals. Error bars represent SD and letters indicate no statistically significant difference (P value > 0.05) for pair-wise comparisons (Student's t-test).

b. Effect of dietary AA ratio on the hepatic level and activation of a key mediator of TOR signaling, S6 kinase (S6K), assessed by Western blotting. There were no detectable changes in the level or activation of S6K between the dietary AA profiles after 20 weeks of treatment (24 weeks of age) on the respective diet. Bars represent mean values of 4 blots (N = 4), each using 3-6 animals. Error bars represent SD and letters indicate no statistically significant difference (P value > 0.05) for pair-wise comparisons (Student's t-test). Data by Hanna Salmonowicz and George Soultoukis.

c. Effect of dietary AA ratio on the hepatic level and activation of the nutrient sensor AMP-activated protein kinase (AMPK), assessed by Western blotting. There were no detectable changes in the total AMPK levels between the dietary AA profiles after 20 weeks of treatment (24 weeks of age) on the respective diet. However, AMPK was activated less by mice *ad libitum*-fed CaseinAA (6%), or mice pair-fed CaseinAA (8%). Bars represent mean values of 6 blots (N = 6), each blot using 3-6 animals. Error bars represent SD and letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data by Hanna Salmonowicz and George Soultoukis.

d. Effect of dietary AA ratio on the hepatic level and activation of the key nutrient responsive IIS pathway protein kinase B (Akt), assessed by Western blotting. There were no detectable changes in the level or activation of Akt between the dietary AA profiles after 20 weeks of treatment (24 weeks of age) on the respective diet. Bars represent mean values of 4 blots (N = 4), each using 3-6 animals. Error bars represent SD and letters indicate no statistically significant difference (P value > 0.05) for pair-wise comparisons (Student's t-test).

e. Effect of dietary AA ratio on the hepatic level and activation of the key AA responsive protein GCN2, assessed by Western blotting. GCN2 was activated by pair-fed CaseinAA, as tested after 20 weeks of treatment (24 weeks of age) on the respective diet. Bars represent mean values of 4 blots (N = 4), each using 3-4 animals. Error bars represent SEM and letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data by Hanna Salmonowicz and George Soultoukis.

5.3.6 Respiration

It was next explored whether the nutritional efficiency differences observed between CaseinAA and MouseAA could affect energy homeostasis. To address this question, we analyzed oxygen consumption for mice chronically fed these diets in a pair-fed or *ad libitum* mode. Although no differences were seen between animals chronically fed these ratios *ad libitum*, animals pair-fed CaseinAA (6%) had increased oxygen consumption and carbon dioxide generation compared to mice pair-fed MouseAA (6%) (Figure 5.s 10A-B), so there were no changes in the respiratory exchange ratio (RER; Figure 5.10C). Energy expenditure was also decreased by pair-feeding MouseAA (6%) (Figure 5.10D), perhaps contributing to the non-significantly lower body temperature (Figure 5.10E). To further probe this phenotype, we investigated the abundance of the steady state levels of the five mitochondrial electron transport chain (ETC) complexes (I-V). The steady state levels of the mitochondrial CI were increased in mice fed CaseinAA (6%), compared to mice fed MouseAA (6%), as was the proportion of CI:CIV (Figure 5.10F).
The observed reduction in oxygen consumption for mice fed MouseAA (6%) was not due to lowered activity levels in these mice. When comparing mice pair-fed MouseAA (6%) or CaseinAA (6%), there were no significant changes in home cage horizontal or vertical activity levels (Figure 5.s 10G-J), or in the total distance traversed by these animals over a period of 24 hours (Figure 5.10K). However, animals *ad libitum*-fed MouseAA (6%) had lower levels of activity compared to animals pair-fed MouseAA, and there were no differences between the CaseinAA *ad libitum*-fed vs. pair-fed regimes (Figure 5.s 10G-K).

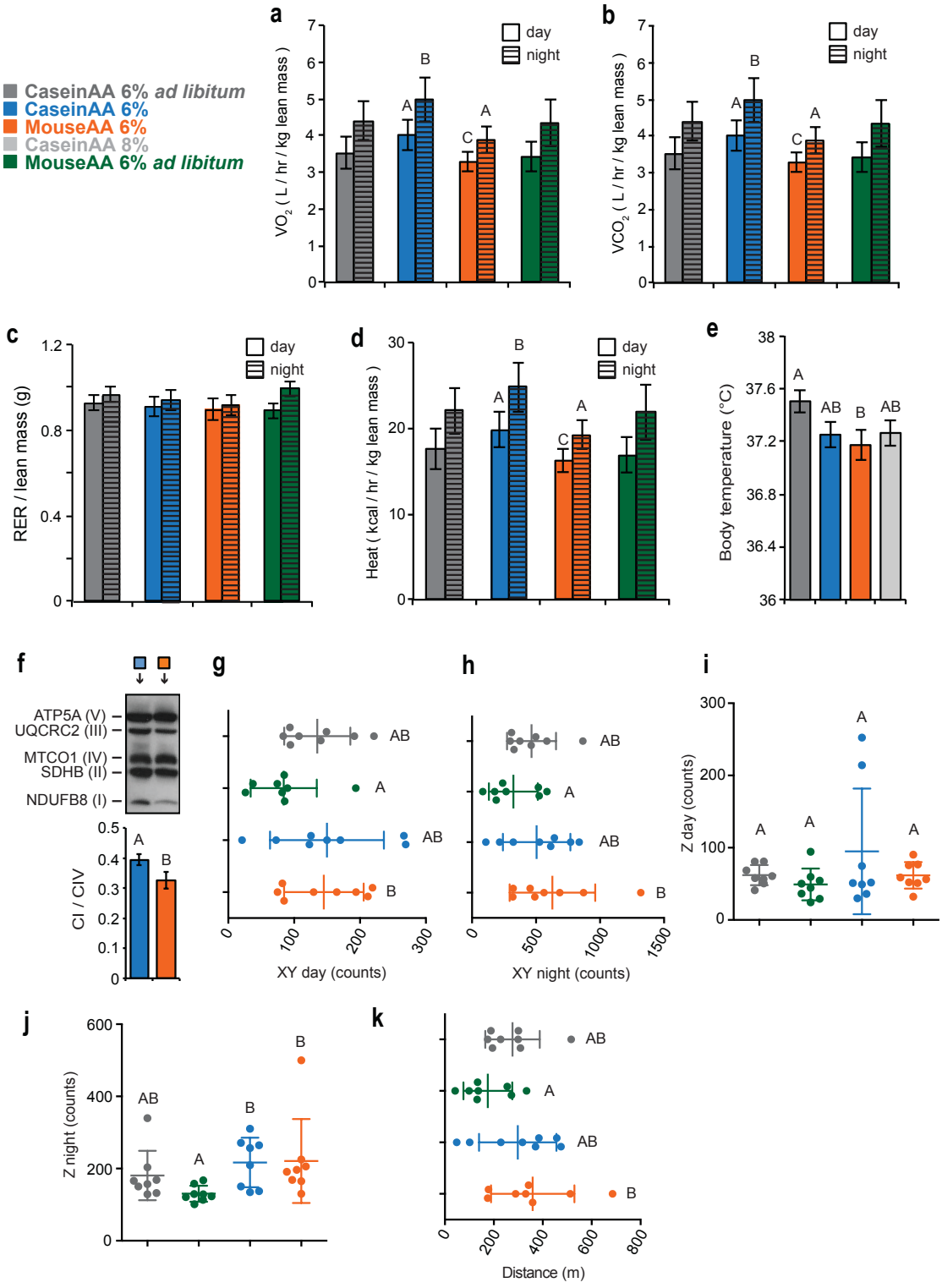


Figure 5.9

(Data by Martin Purrio, Dr. Andrea Mesaros, Stanka Matic, and George Soultoukis)

a. Oxygen consumption across dietary AA ratios. Mice pair-fed MouseAA (6%) had significantly decreased levels of oxygen consumption compared to mice pair-fed CaseinAA (6%). Bars represent mean values (N = 8), and error bars represent SD. Letters indicate

statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data by Martin Purrio and Dr. Andrea Mesaros.

b. Carbon dioxide generation across dietary AA ratios. Mice pair-fed MouseAA (6%) had significantly decreased levels of carbon dioxide generation compared to mice pair-fed CaseinAA (6%). Bars represent mean values (N = 8), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data by Martin Purrio and Dr. Andrea Mesaros.

c. Effect of dietary AA ratio on the respiratory exchange ratio (RER). The RER values remained unchanged across all treatments. Bars represent mean values (N = 8), and error bars represent SD. No statistically significant differences (P value < 0.05) were detected for pair-wise comparisons (Student's t-test). Data by Martin Purrio and Dr. Andrea Mesaros.

d. Heat production across dietary AA ratios. Mice pair-fed MouseAA (6%) had significantly decreased levels of heat generation compared to mice pair-fed CaseinAA (6%). Bars represent mean values (N = 8), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data by Martin Purrio and Dr. Andrea Mesaros.

e. Body temperature across dietary AA ratios. Mice pair-fed MouseAA (6%) had the lowest body temperature, but this decrease was not significant. Bars represent mean values (N = 20), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data by Martin Purrio and Dr. Andrea Mesaros.

f. Effect of dietary AA ratio on the level of the electron transport chain components, assessed by Western Blotting analysis of the relative steady state levels of the 5 OXPHOS complexes. Mice pair-fed MouseAA (6%) had significantly decreased levels of complex I. Bars represent mean values (N = 9-10), and error bars represent SD. Letters indicate a statistically significant difference (P value < 0.05) in a pair-wise comparison (Student's t-test). Data by Stanka Matic.

g-j. Effect of dietary AA ratio on horizontal and vertical activity levels. Bars represent mean values (N = 8), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data by Martin Purrio, Dr. Andrea Mesaros, and George Soultoukis.

k. Effect of dietary AA ratio on total distance traversed over a period of 24 hours. Bars represent mean values (N = 8), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data by Martin Purrio, Dr. Andrea Mesaros, and George Soultoukis.

5.3.7 Glucose homeostasis and glycogen synthesis

To examine the effects of exome-matching upon glucose homeostasis, we assessed blood glucose levels in both pair-fed and *ad-libitum* regimes. Although there were no changes in circulating glucose between the pair-fed CaseinAA and MouseAA regimes, mice *ad-libitum*-fed MouseAA had higher blood glucose (Figure 5.10A), and insulin (Figure 5.10B), but an increase in leptin was not significant (Figure 5.10C). Yet, as mentioned, there was no difference in the levels or activation of insulin-activated protein kinase Akt (Figure 5.8D). As higher circulating glucose and insulin levels are associated with decreased insulin sensitivity and Type 2 diabetes, it was decided to determine whether mice on MouseAA 6% had changes in insulin and glucose sensitivity. Interestingly, mice pair-fed MouseAA showed no change to mice pair-fed CaseinAA (Figure 5.10D-E), but when the two diets were consumed *ad-libitum* mice on MouseAA were both glucose tolerant and insulin resistant compared to CaseinAA (Figure 5.10F-G). To further probe the effects of exome-matching upon glucose homestasis, glycogen storage was also assessed histologically. There was a tendency for decreased liver glycogen storage in mice fed the exome ratio (Figure 5.10H), which corresponded to a higher activation of glycogen synthase kinase 3 (GSK-3) (Figure 5.10I), an inhibitor of glycogen synthase (GS), which converts glucose to glycogen.

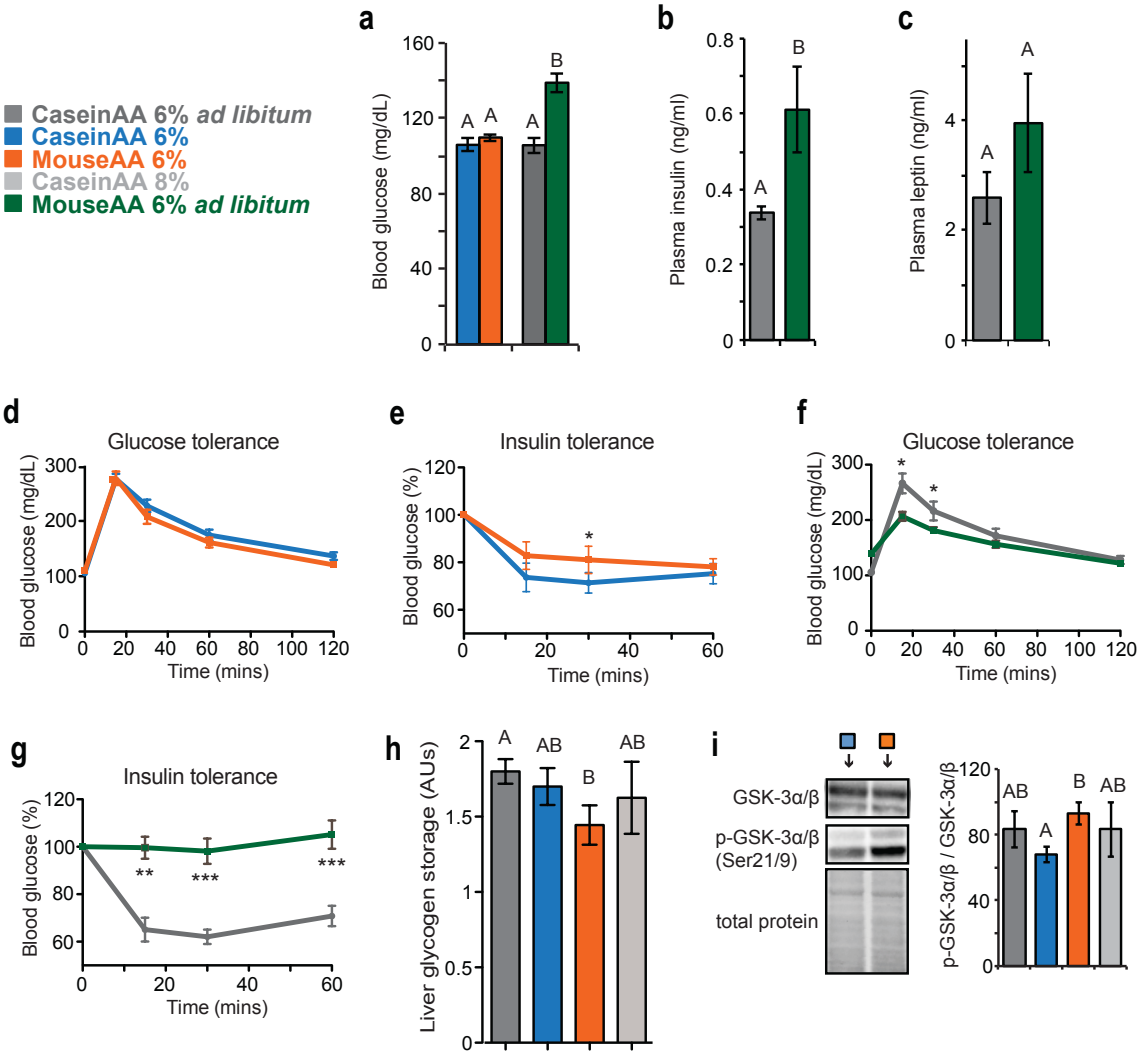


Figure 5.10

(Data by George Soultoukis, Martin Purrio, Dr. Andrea Mesaros, Dr. Youssef Hassan, Dr. Alain De Bruin, Joanna Goncalves, and Hanna Salmonowicz)

a. Blood glucose across dietary AA ratios. Mice *ad-libitum* MouseAA (6%) had significantly elevated levels of circulating glucose compared to all other treatments. Bars represent mean values (N = 10), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data by Martin Purrio, Dr. Andrea Mesaros, and George Soultoukis.

b. Plasma insulin across dietary AA ratios. Mice fed *ad-libitum* MouseAA (6%) had significantly elevated levels of circulating insulin compared to mice fed *ad-libitum* CaseinAA (6%). Bars represent mean values (N = 7-8), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data by Joanna Goncalves and George Soultoukis.

c. Plasma leptin across dietary AA ratios. Mice *ad-libitum* MouseAA (6%) had higher levels of circulating leptin compared to mice *ad-libitum* CaseinAA (6%), but this increase was not significant (Student's t-test, p=0.062). Bars represent mean values (N = 5-7), and error bars represent SD. Data by Joanna Goncalves and George Soultoukis.

d. Glucose tolerance test (GTT) across dietary AA ratios. The blood glucose levels of mice pair-fed MouseAA (6%) responded similarly to those of mice pair-fed CaseinAA (6%). Bars represent mean values (N = 10), and error bars represent SD. Curves were compared with 2-way ANOVA analysis and Bonferonni posthoc test (P value > 0.05). Data by George Soultoukis, Martin Purrio, and Dr. Andrea Mesaros.

e. Insulin tolerance test (ITT) across dietary AA ratios. The circulating insulin levels of mice pair-fed MouseAA (6%) responded similarly to those of mice pair-fed CaseinAA (6%). Bars represent mean values (N = 10), and error bars represent SD. Curves were compared with 2-way ANOVA analysis and Bonferonni posthoc test (P value > 0.05). Data by George Soultoukis, Martin Purrio, and Dr. Andrea Mesaros.

f. Glucose tolerance test (GTT) across dietary AA ratios. The blood glucose levels of mice *ad libitum*-fed MouseAA (6%) increased less than those of mice *ad libitum*-fed CaseinAA (6%). Bars represent mean values (N = 10), and error bars represent SD. Curves were compared with 2-way ANOVA analysis and Bonferonni posthoc test (P value < 0.05). Data by George Soultoukis, Martin Purrio, and Dr. Andrea Mesaros.

g. Insulin tolerance test (ITT) across dietary AA ratios. The circulating insulin levels of mice *ad libitum*-fed CaseinAA (6%) decreased significantly compared to those of mice *ad libitum*-fed MouseAA (6%). Bars represent mean values (N = 10), and error bars represent SD. Stars indicate a statistically significant difference (P value < 0.05) and curves were compared with 2-way ANOVA analysis and Bonferonni posthoc test. Data by George Soultoukis, Martin Purrio, and Dr. Andrea Mesaros.

h. Effect of dietary AA ratio on liver glycogen storage, assessed histologically. Mice pair-fed MouseAA (6%) had the lowest score. Bars represent the mean arbitrary value for each group (N = 5-10), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). (Data by George Soultoukis, Dr. Youssef Hassan, and Dr. Alain De Bruin)

i. Effect of dietary AA ratio on the level and activation of the glycogen synthase kinase 3 (GSK-3), the inhibitor of glycogen synthase, assessed by Western Blotting analysis. Mice pair-fed MouseAA (6%) had significantly increased levels of phospho-GSK-3, the activated form of GSK-3, compared to mice pair-fed CaseinAA (6%). Bars represent mean values (N = 6), and error bars represent SD. Letters indicate a statistically significant difference (P value < 0.05) in a pair-wise comparison (Student's t-test). (Data by George Soultoukis and Hanna Salmonowicz)

5.3.8 Proteostasis

As in flies, we tested the effects of exome-matching upon proteostasis in mice by assessing protein content, proteasome activity, poly-ubiquitination, and autophagy. No differences were detected in the polysome profiles between animals fed MouseAA and CaseinAA (Figure 5.11A) or in the monosome:polysome ratio (data not shown). Also unchanged were the amounts of protein content per wet mass in the brain, gut, muscle, or liver (Figure 5.11B), as well as the liver DNA:protein ratio (Figure 5.11C). However proteasome activity in the liver (Figure 5.11D) and muscle (Figure 5.11E) was reduced by MouseAA, contrasting the findings in flies. No differences were seen in the gut (Figure 5.11F) or brain (Figure 5.11G). Ubiquitination status in the liver was not significantly changed (Figure 5.11H). No changes in autophagy activation were induced in the liver by MouseAA (Figure 5.11I; data for Figure 5.11 by Hanna Salmonowicz).

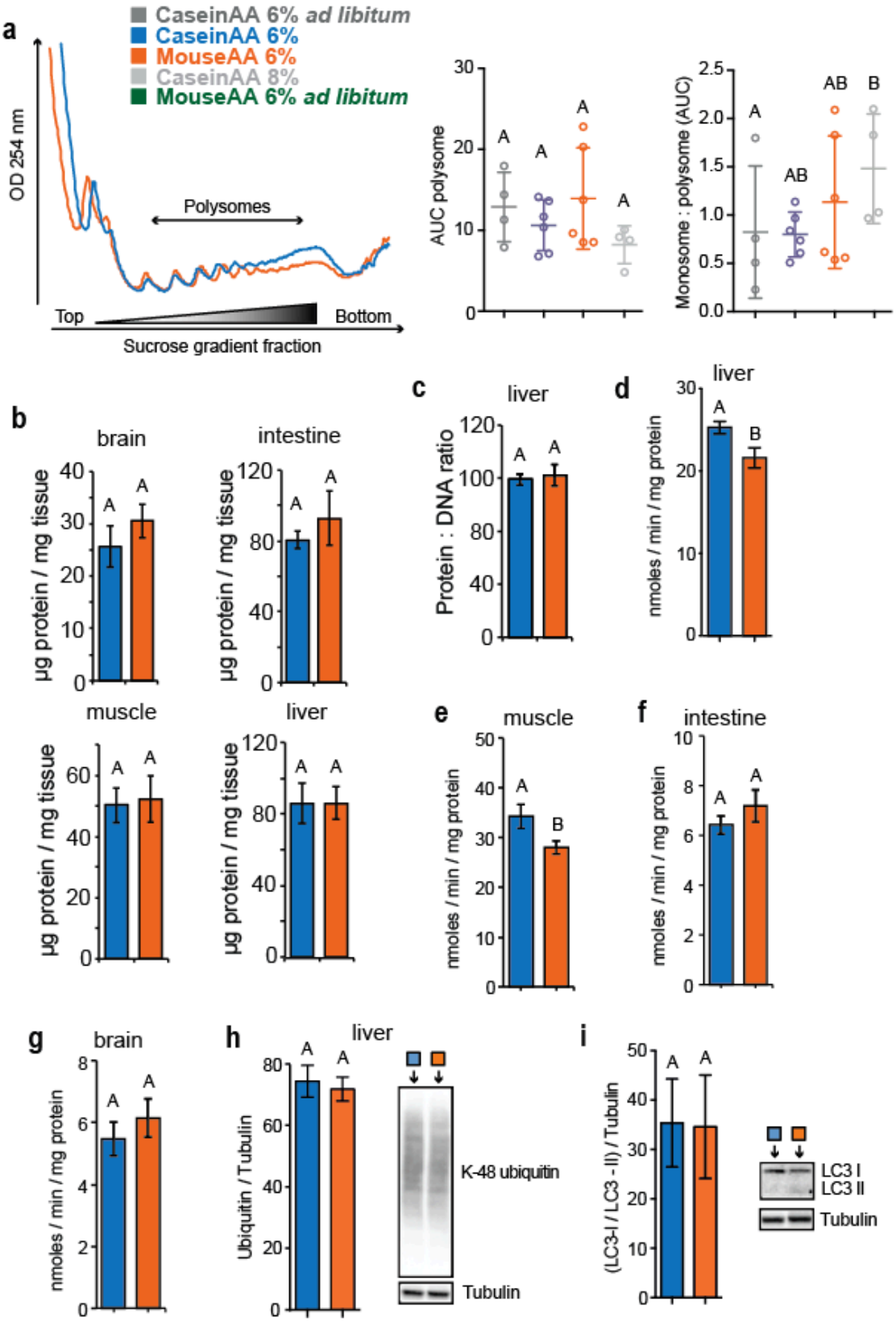


Figure 5.11

(data for Figure 5.11 by Hanna Salmonowicz)

a. Polysome profiling showing similar patterns across different dietary AA ratios. Curves represent the ribosomal/polysomal fractions across a sucrose gradient. Each curve shown represents a single representative biological replicate (N = 4-6) from animals treated for 20 weeks (23 weeks of age). Middle panel shows quantification of the AUC (area under curve) in these polysome profiles. Right panel shown monosome:polysome AUC ratio. No significant changes were detected between CaseinAA and MouseAA (N = 4-6, P value > 0.05 for pair-wise comparisons using Student's t-test; means are shown, while error bars represent SD).

b. Protein content per mass of tissue for brain, intestine, muscle, and liver showed no differences between CaseinAA and MouseAA. For each bar, N=8-10, while error bars represent SD and letters indicate no statistically significant difference (P value > 0.05) for pair-wise comparisons (Student's t-test).

c. Protein to DNA ratio in the liver of mice fed CaseinAA or MouseAA showed no differences between the two treatments. For each bar, N=10, while error bars represent SD and letters indicate no statistically significant difference (P value > 0.05) for pair-wise comparisons (Student's t-test).

d. Effect of dietary AA ratio on liver proteasome activity. The chart shows the activity based on the LLE-AMC substrate degradation as both substrates behaved similarly (data not shown). Each bar represents the mean proteasomal activity (N=8-10), and error bars represent SEM. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

e. Effect of dietary AA ratio on muscle proteasome activity. The chart shows the activity based on the LLE-AMC substrate degradation as both substrates behaved similarly (data not shown). Each bar represents the mean proteasomal activity (N=10), and error bars represent SEM. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

f. Effect of dietary AA ratio on gut proteasome activity. The chart shows the activity based on the LLE-AMC substrate degradation as both substrates behaved similarly (data not shown). Each bar represents the mean proteasomal activity (N=9), and error bars represent SEM. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

g. Effect of dietary AA ratio on brain proteasome activity. The chart shows the activity based on the LLE-AMC substrate degradation as both substrates behaved similarly (data not shown). Each bar represents the mean proteasomal activity (N=9), and error bars represent SEM. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

h. Quantification of Western blot analysis of hepatic ubiquitination levels across dietary AA ratios. Quantification performed on the basis on 5 biological replicates is expressed as arbitrary units and reproduced >3 times. Error bars represent SEM, letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

i. Quantification of Western blot analysis of microtubule-associated proteins 1A/1B light chain 3A (MAP1LC3A). A high ratio of LC3-I to LC3-II indicates defective autophagy induction. Quantification performed on the basis on 5 biological replicates is expressed as arbitrary units and reproduced >3 times. Error bars represent SEM, letters indicate statistically significant differences (P value < 0.05) for the pair-wise comparison (Student's t-test).

5.3.9 Organ growth and biosynthesis

To assess whether the growth suppression induced by CaseinAA has a general systemic growth-inhibitory effect or it affects tissues differentially, we performed a histological screen of a number of tissues. First, the wet mass of several organs with critical metabolic roles, including abdominal WAT, liver, kidney, heart, thymus, muscle, and brain was recorded (Figure 5.12A). The wet mass of abdominal WAT, kidney, liver, and brain was significantly lower in mice pair-fed CaseinAA (6%), compared to mice pair-fed MouseAA (6%) (Figure 5.12A). These differences diminished when data were normalized to the respective body or lean masses, but not when normalized to body size (nose-to-rump length) (Figure 5.12A).

As the fly SILAC proteome was also a good measure of defining anabolic traits (see Chapter 4), the mouse SILAC proteome was also considered. To further compare the predictive accuracy at the tissue level, the mass of several organs was measured and compared to the mSILACAA. However, neither MouseAA or mSILACAA were a reliable predictor for organ specific growth rates (Figure 5.12B). The tissue-specific mSILACAA (tsmSILACAA) usage was next quantified to establish if different tissues use AAs differently, and therefore if tsmSILACAA can better predict organ-specific growth. Nevertheless, tissue-specific AA usage was very similar for all tissues (Figure 5.12C), with glycine and glutamine elevated in the whole body possibly because in some mammals, including humans, glycine and glutamine are 10-50 times more concentrated in intracellular pools (4). Therefore, these results suggest that, as in flies, predictions based on dietary AA ingestion and on the principle of the minimum are not always accurate when assessing growth at the tissue-specific level. Moreover, upon assessing whole body lean mass growth, tsmSILACAA was a more accurate predictor for growth than MouseAA for a range of diets with different limiting AAs (Figure 5.12D).

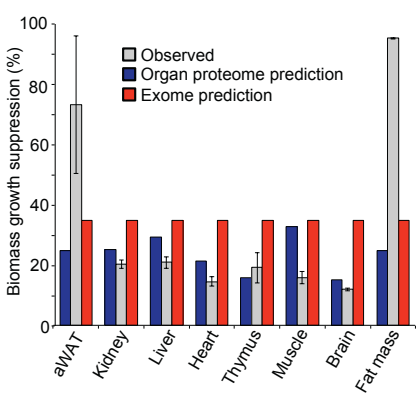
It was next asked if the abundance of circulating AAs can affect the accuracy of predictions for organ growth rates, by accounting for the bio-availability of ingested AAs into circulation. Ingested AAs are absorbed by the gut and into the portal vein, where they are available for first pass (splanchnic bed) metabolism. We therefore assessed portal vein AAs. No changes were detected in the portal vein plasma concentrations of amino acids between mice pair-fed CaseinAA or MouseAA (Figure 5.12E). Yet, when considering the relationship between diet and portal vein AA concentrations, a moderate correlation (R^2=0.494) was observed in the fold change between the diet amino acid concentration in CaseinAA compared to MouseAA, and the fold change between the mean portal vein plasma amino acid concentration in CaseinAA compared to that in MouseAA (Figure 5.12F). Although cysteine was not analysed, methionine (Figure 5.12E) or several sulphur-amino acid metabolites (Figure 5.12G) were unchanged. Of all 85 metabolites quantified (including nucleic acids, sugars, TCA cycle intermediates, and lipid metabolites), only three were found to be significantly changed. Nicotinamide-adenine dinucleotide (NAD), betaine, and phosphoethamolamine (PEA) were all significantly elevated in mice fed CaseinAA (Figure 5.12H).

CaseinAA 6% *ad libitum*
CaseinAA 6%
MouseAA 6%
CaseinAA 8%

a

Organ	Organ wet mass (g)					
					P value	/
White adipose tissue (abdominal)	0,9337	0,8837	1,2309	1,3035	0,0064	1,39
Kidney (r)	0,3220	0,2775	0,3052	0,3150	0,0003	1,10
Liver	1,2216	0,9168	1,0131	0,9958	0,0021	1,10
Heart	0,1438	0,1190	0,1246	0,1231	0,2005	1,05
Thymus	0,0747	0,0463	0,0505	0,0498	0,3276	1,09
Skeletal muscle (r) - quadricep femoris	0,2197	0,1935	0,2147	0,2084	0,1092	1,11
Brain	0,5102	0,5042	0,5155	0,5047	0,0500	1,02
	Normalised to whole body mass (g)					
White adipose tissue (abdominal)	0,0334	0,0368	0,0434	0,0456	0,0745	1,18
Kidney (r)	0,0116	0,0116	0,0110	0,0113	0,0703	0,95
Liver	0,0438	0,0383	0,0365	0,0359	0,1046	0,95
Heart	0,0052	0,0050	0,0045	0,0045	0,0303	0,91
Thymus	0,0026	0,0019	0,0018	0,0018	0,3736	0,93
Skeletal muscle (r) - quadricep femoris	0,0079	0,0081	0,0077	0,0075	0,4742	0,95
Brain	0,0183	0,0211	0,0187	0,0182	0,0001	0,89
	Normalised to body size (nose-rump cm)					
White adipose tissue (abdominal)	0,0921	0,0917	0,1260	0,1328	0,0092	1,37
Kidney (r)	0,0318	0,0288	0,0312	0,0322	0,0009	1,08
Liver	0,1206	0,0951	0,1035	0,1018	0,0051	1,09
Heart	0,0143	0,0123	0,0127	0,0126	0,3607	1,03
Thymus	0,0074	0,0048	0,0052	0,0051	0,4117	1,07
Skeletal muscle (r) - quadricep femoris	0,0217	0,0201	0,0219	0,0213	0,1717	1,09
Brain	0,0504	0,0523	0,0527	0,0516	0,5924	1,01
	Normalised to lean mass (g)					
White adipose tissue (abdominal)	0,0403	0,0441	0,0559	0,0595	0,0289	1,27
Kidney (r)	0,0139	0,0139	0,0140	0,0145	0,7567	1,01
Liver	0,0529	0,0458	0,0463	0,0460	0,6568	1,01
Heart	0,0063	0,0059	0,0057	0,0057	0,2591	0,96
Thymus	0,0032	0,0023	0,0023	0,0023	0,9413	0,99
Skeletal muscle (r) - quadricep femoris	0,0095	0,0097	0,0098	0,0096	0,8149	1,01
Brain	0,0221	0,0252	0,0236	0,0234	0,0002	0,94

b



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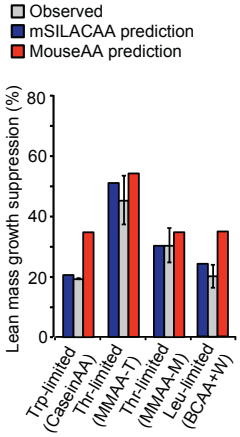

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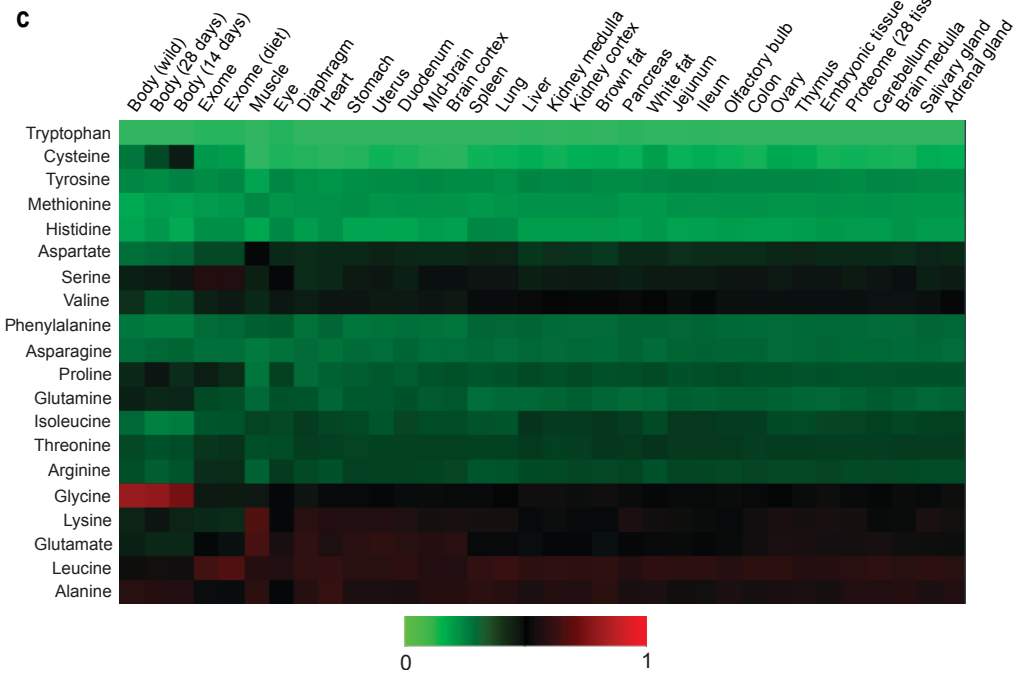


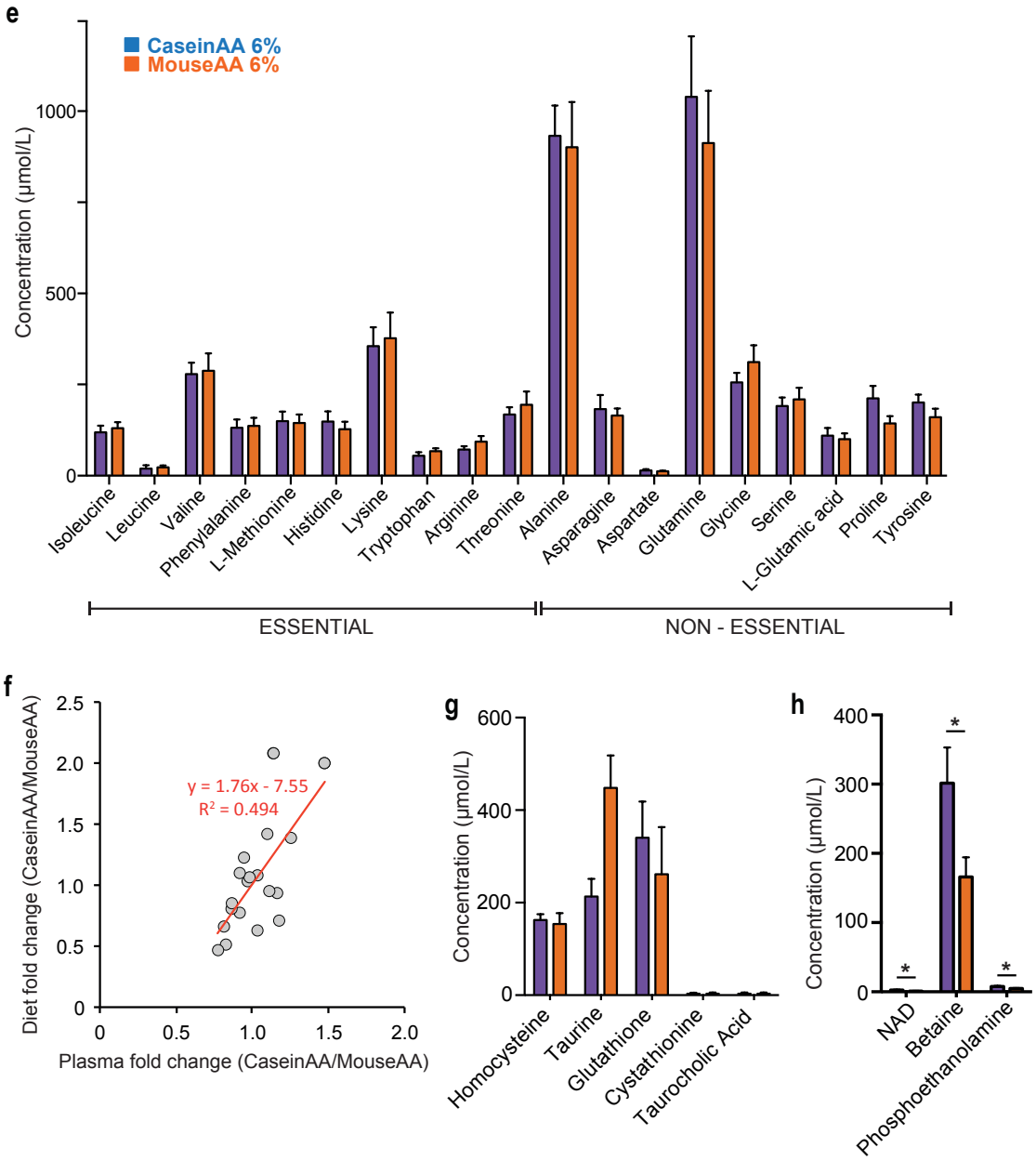


Figure 5.12

(Data by George Soultoukis, Martin Purrio, and Dr. Ilian Atanassov; metabolite analysis carried out at the FIMM (see methods))

a. The wet mass of abdominal WAT (aWAT), liver, kidney, heart, thymus, muscle, and brain. For each value shown, N = 20 per AA profile. The raw wet mass data shown are normalized to whole body mass, body size, or lean mass. P-value column shown represents the comparison of MouseAA 6% vs. CaseinAA 6%. Data by George Soultoukis and Martin Purrio.

b. Predictions for organ specific growth rates in mice fed CaseinAA. Predictions are based on the exome AA usage (MouseAA), or on the organ-specific weighted proteome usage (tsmSILACAA). Neither ratio is a good predictor for specific organ growth in mice fed CaseinAA. For organ masses, tissues were harvested and wet mass was recorded (N=10).

c. Tissue-specific AA content in the weighted proteome of different organs as determined by SILAC. Data by Dr. Ilian Atanassov.

d. The tissue-specific weighted proteome usage (tsmSILACAA) is a better predictor for lean mass growth than the exome AA usage (MouseAA), across four diets with 3 different limiting AAs (indicated in brackets).

e. Portal vein plasma amino acid concentrations for mice pair-fed CaseinAA (n=4) or MouseAA (n=6). No significant changes between the two groups were observed in any of the amino acids analysed (for each AA comparison, two-tailed Student's T-test, p-value > 0.05).

f. Relationship between diet and portal vein AA concentrations. The squared coefficient of correlation value (R^2=0.494) indicates a moderate but significant correlation in the fold change between the diet AA concentration in CaseinAA compared to MouseAA, and the fold change between the mean portal vein plasma AA concentration in CaseinAA compared to that in MouseAA (P value = 0.00055, two-tailed probability).

g. No significant changes were detected in the portal vein plasma sulphur-amino acid metabolite concentrations between mice pair-fed CaseinAA (n=4) or MouseAA (n=6) (for each metabolite comparison, two-tailed Student's T-test, p-value > 0.05). B. Portal vein plasma concentrations of metabolites that were significantly altered between mice pair-fed CaseinAA (n=4) and MouseAA (n=6) included betaine, NAD, and PEA (for each metabolite comparison, two-tailed Student's T-test, p-value < 0.05).

5.3.10 Histopathological effects of exome-matching

Histological analysis of livers showed that hepatocyte anisokaryosis, an indication of restricted cell size, occurred in a significantly higher occurrence in mice fed CaseinAA (Figure 5.13A). Animals pair-fed CaseinAA 6% also had the highest incidence of mild hepatic atrophy (Figure 5.13B). However, there were no differences in hepatocyte binucleation occurrence between the groups (Figure 5.13C). Another effect on livers was the higher incidence in vacuolar hepatopathy in animals fed CaseinAA 6% *ad-libitum* (Figure 5.13D). Moreover, staining analysis of the vacuoles to distinguish between three types of vacuolar contents (hydropic, lipid, and mixed contents) revealed differences in the vacuolar type. Mice pair-fed MouseAA 6% had a vacuolar profile which was distinct to that of mice CaseinAA 6% (*ad-libitum* or pair-fed) but similar to that of mice pair-fed CaseinAA 8% (Figure 5.13E). Although no differences in skeletal muscle lesions were seen, animals pair-fed CaseinAA 6% had the lowest quadricep muscle masses (Figure 5.12A), which was matched by their higher incidence of myofibrillar atrophy and necrosis (Figure 5.13F).

In rodents, protein deprivation reduces jejunal villus height and crypt depths, and supplementation of some AAs in the diet rescues these effects (229), so it was tested whether intestinal morphology was also defined by the ingested AA profile. However, no differences in cryptal length or villar length were seen in the small intestine (Figure 5.13G-H). Other parameters examined but not found to differ included kidney anisokaryosis, tubulonephrosis, glomerular lesions, sternal bone and cartilage lesions, bone marrow cellularity %, erythroid:myeloid ratio, and megakaryocyte content (data not shown).

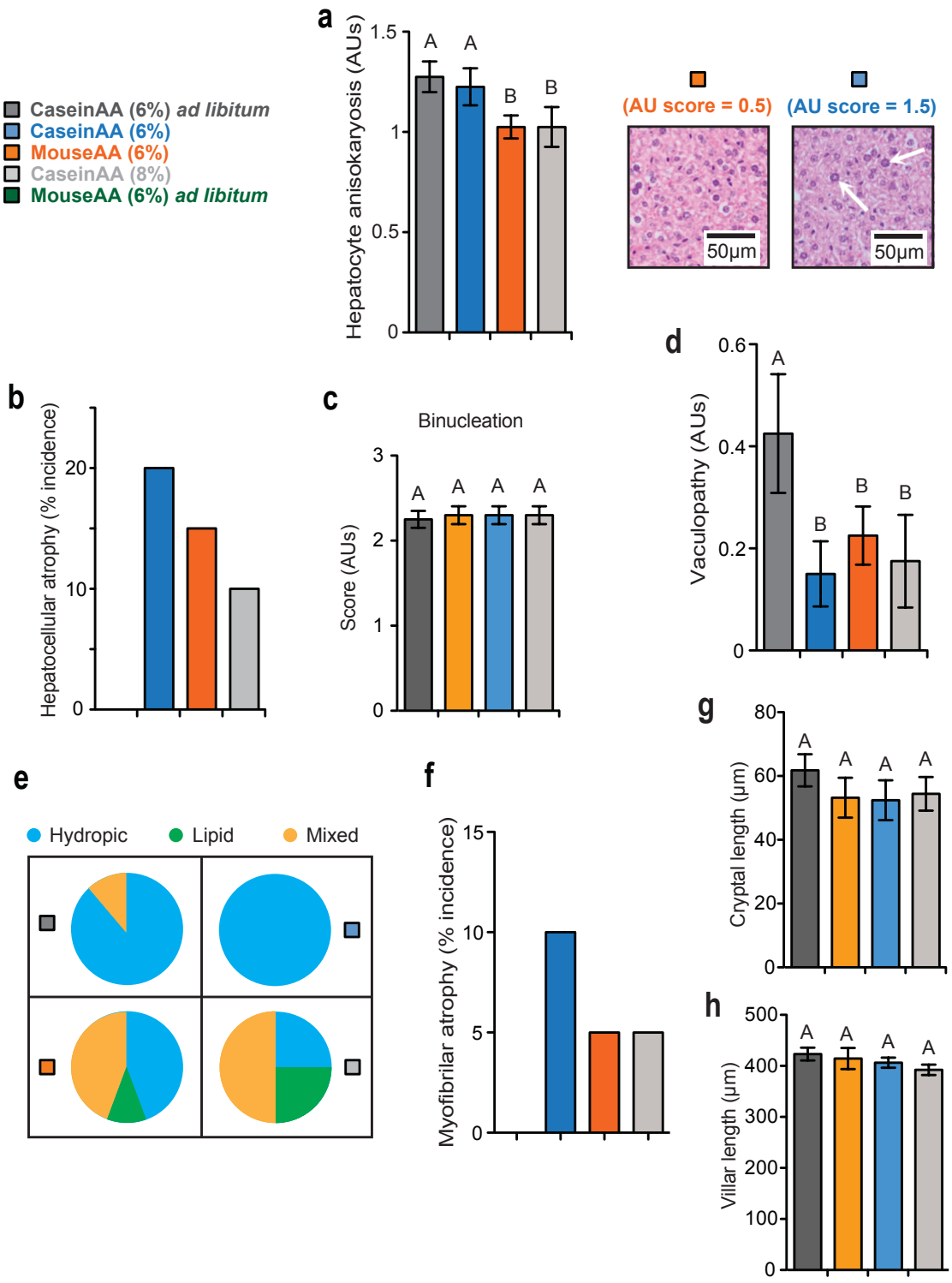


Figure 5.13

(Data by Dr. Youssef Hassan, Dr. Alain De Bruin and George Soultoukis)

a. Effect of dietary AA ratio on hepatocyte anisokaryosis, assessed histologically. Hepatocyte nuclei were assessed and given an arbitrary value reflecting the incidence of anisokaryosis. Mice pair-fed MouseAA (6%) or CaseinAA (8%) had the lowest score. Bars represent the

mean arbitrary value for each group (N = 20), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Cross-sections on the right show representative examples of scoring.

b. Effect of dietary AA ratio on hepatocellular atrophy, assessed histologically. Mice pair-fed CaseinAA (6%) had the highest incidence of hepatocellular atrophy. Hepatic atrophy incidence was as follows: pair-fed CaseinAA (6%) (4/20 mice), pair-fed MouseAA (6%) (3/20 mice), pair-fed CaseinAA (8%) (2/20 mice). Bars represent the % value for each group (N = 20).

c. Effect of dietary AA ratio on hepatocyte binucleation, assessed histologically. Liver tissue was assessed and given an arbitrary value reflecting the incidence of binucleation. No differences were observed between the tested dietary AA profiles. Bars represent the mean arbitrary value for each group (N = 20), and error bars represent SD. Letters indicate no statistically significant differences (P value > 0.05) for pair-wise comparisons (Student's t-test).

d. Effect of dietary AA ratio on hepatic vaculopathy, assessed histologically. Liver tissue was assessed and given an arbitrary value reflecting the incidence of vacuolar hepatopathy. Mice *ad libitum*-fed CaseinAA (6%) had the highest score. Bars represent the mean arbitrary value for each group (N = 20), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

e. Effect of dietary AA ratio on the type of hepatic vaculopathy. Liver tissue was stained for hydropic, lipid, or mixed vacuolar hepatopathy type (see methods) and given an arbitrary value reflecting the incidence of each type. The profile of mice *ad libitum*-fed CaseinAA (6%) was similar to that of mice pair-fed CaseinAA (6%), while the profile of mice pair-fed MouseAA (6%) resembled that of mice pair-fed CaseinAA (8%). Vacuolar hepatopathy types were assessed on the number of mice with a vaculopathy, which varied per group and was as follows: *ad libitum*-fed CaseinAA (6%) (9/20 mice), pair-fed CaseinAA (6%) (5/20 mice), pair-fed MouseAA (6%) (9/20 mice), pair-fed CaseinAA (8%) (4/20 mice).

f. Effect of dietary AA ratio on myofibrilar atrophy, assessed histologically. Mice pair-fed CaseinAA (6%) had the highest incidence of myofibrilar atrophy, whose incidence was as follows: pair-fed CaseinAA (6%) (2/20 mice), pair-fed MouseAA (6%) (1/20 mice), pair-fed CaseinAA (8%) (1/20 mice). Bars represent the % value for each group (N = 20).

g. Effect of dietary AA ratio on intestinal cryptal length, assessed histologically. Jejunum cross-sections were assessed for their cryptal length. No differences were observed between the tested dietary AA profiles. Bars represent the mean arbitrary value for each group (N = 10), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

h. Effect of dietary AA ratio on intestinal villar length, assessed histologically. Jejunum cross-sections were assessed for their villar length. No differences were observed between the tested dietary AA profiles. Bars represent the mean arbitrary value for each group (N = 10), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

5.3.11 Skeletal effects and bone quality

Apart from the differences in skeletal length evidenced by changes in nose-to-rump length between MouseAA and CaseinAA (Figure 5.2C), the quality of nutrition can also affect bone quality (230). Therefore several bone parameters were analysed by computer tomography (CT) scanning (see methods for details). For pair-fed mice, MouseAA resulted in increased cortical and trabecular structure thickness, and increased trabecular bone volume and bone mineral density (BMD) (Figure 5.14A-D). For *ad libitum* fed mice, in contrast, only cortical structure thickness was elevated in animals fed MouseAA compared to animals fed Casein AA (Figure 5.14E-H).

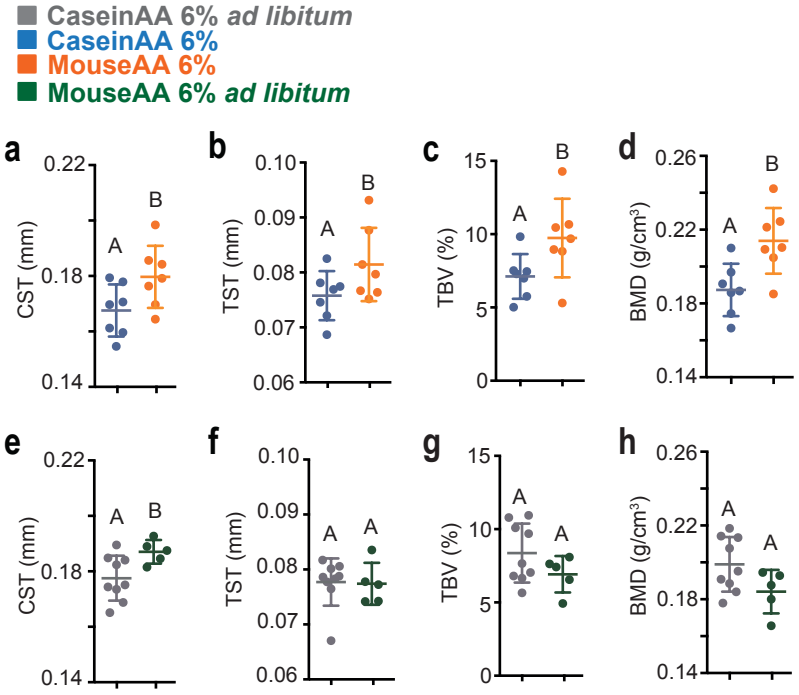


Figure 5.14

(Data by Dr. Andrea Mesaros, Martin Purrio, and George Soultoukis)

a. Effect of dietary AA ratio on cortical structure thickness (CST) as analysed by CT. Mice pair-fed MouseAA (6%) had a higher CST value. Bars represent the mean value for each group (N = 7), and error bars represent SD. Letters indicate a statistically significant difference (P value < 0.05) for the pair-wise comparison (Student's t-test).

b. Effect of dietary AA ratio on trabecular structure thickness (TST) as analysed by CT. Mice pair-fed MouseAA (6%) had a higher TST value. Bars represent the mean value for each group (N = 7), and error bars represent SD. Letters indicate a statistically significant difference (P value < 0.05) for the pair-wise comparison (Student's t-test).

c. Effect of dietary AA ratio on trabecular bone volume % (TBV %) as analysed by CT. Mice pair-fed MouseAA (6%) had higher TBV. Bars represent the mean value for each group (N = 7), and error bars represent SD. Letters indicate a statistically significant difference (P value < 0.05) for the pair-wise comparison (Student's t-test).

d. Effect of dietary AA ratio on bone mineral density (BMD) as analysed by CT. Mice pair-fed MouseAA (6%) had higher BMD. Bars represent the mean value for each group (N = 7), and error bars represent SD. Letters indicate a statistically significant difference (P value < 0.05) for the pair-wise comparison (Student's t-test).

e. Effect of dietary AA ratio on cortical structure thickness (CST) as analysed by CT. Mice *ad libitum*-fed MouseAA (6%) had higher CST. Bars represent the mean value for each group (N = 5-9), and error bars represent SD. Letters indicate a statistically significant difference (P value < 0.05) for the pair-wise comparison (Student's t-test).

f. Effect of dietary AA ratio on trabecular structure thickness (TST) as analysed by CT. Mice *ad libitum*-fed MouseAA (6%) had no change in their TST compared to mice *ad libitum*-fed CaseinAA (6%). Bars represent the mean value for each group (N = 5-9), and error bars represent SD. Letters indicate no statistically significant difference (P value > 0.05) for the pair-wise comparison (Student's t-test).

g. Effect of dietary AA ratio on trabecular bone volume % (TBV %) as analysed by CT. Mice *ad libitum*-fed MouseAA (6%) had no change in their TBV compared to mice *ad libitum*-fed CaseinAA (6%). Bars represent the mean value for each group (N = 5-9), and error bars represent SD. Letters indicate no statistically significant difference (P value > 0.05) for the pair-wise comparison (Student's t-test).

h. Effect of dietary AA ratio on bone mineral density (BMD) as analysed by CT. Mice *ad libitum*-fed MouseAA (6%) had no change in their BMD compared to mice *ad libitum*-fed CaseinAA (6%). Bars represent the mean value for each group (N = 5-9), and error bars represent SD. Letters indicate no statistically significant difference (P value > 0.05) for the pair-wise comparison (Student's t-test).

5.3.12 Exome-matching vs. proteome-matching and body-matching

The additive AA abundance in the proteome, in tissue free AAs, and in circulating AAs, is what ultimately shapes the body composition AA ratio. In mice this AA profile, which we refer to as BodyAA, has been reported and proposed as a measure of dietary AA requirements (153, 231). Therefore it was next tested whether BodyAA is different to the AA profile of the accrued mouse proteome (mSILACAA) or to the exome profile (MouseAA). With the exception of tryptophan ($r_{W(min)}$ = 0.65), BodyAA was very similar to ProteomeAA (Figure 5.15A), which is in accord with the observation that free AAs represent only a small fraction of the mammalian body's total AA content (4). However, comparison of BodyAA to the exome usage (MouseAA) predicted a more severe limitation in the tryptophan content of BodyAA ($r_{W(min)}$ = 0.53) (Figure 5.15B). Moreover, MouseAA was very similar to SILACAA ($r_{W(min)}$ = 0.82) (Figure 5.15C), with only an 18% limitation of tryptophan in SILACAA, so no significant growth effects were predicted and SILACAA was not tested experimentally. Therefore, as BodyAA showed a higher dissimilarity to MouseAA, it was decided to compare these two AA profiles for growth. Analysis of growth rates for mice pair-fed MouseAA or BodyAA showed a significant increase in growth for mice fed MouseAA (linear regression P value = 0.036; Figure 5.15D). Moreover addition of the exome-predicted limited EAA in BodyAA did not increase growth (linear regression P value = 0.0037; Figure 5.15D). These results demonstrate that the exome is a better measure for defining AA requirements than the whole body AA composition.

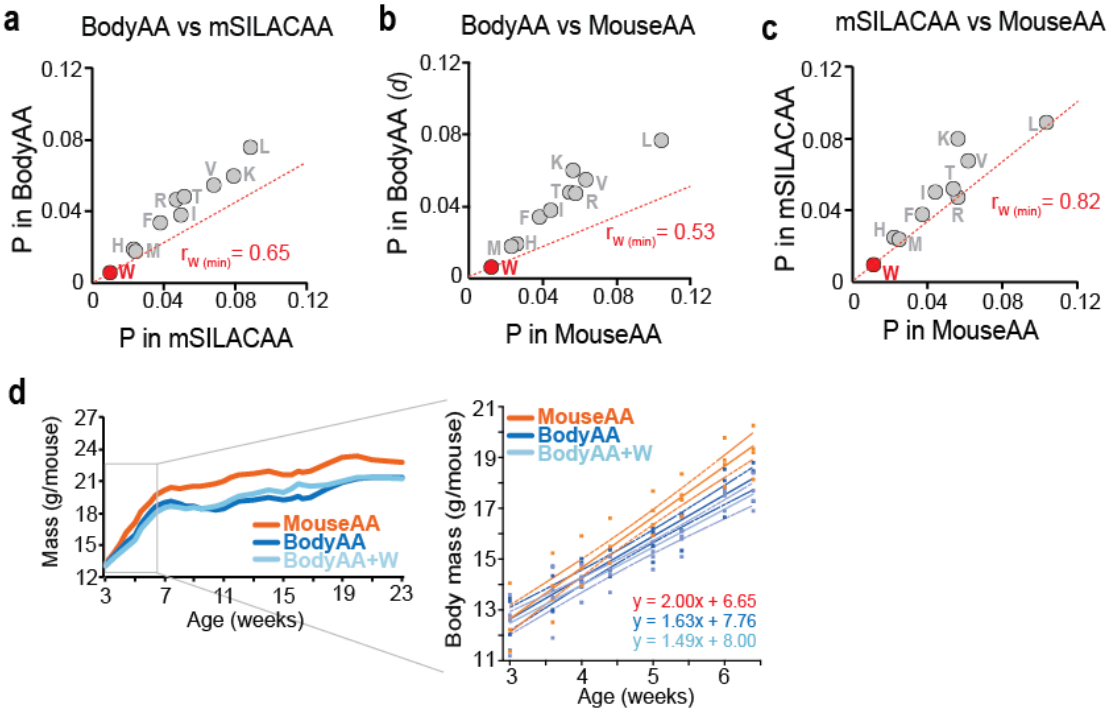


Figure 5.15

a. The mouse whole body AA ratio (BodyAA) vs. the mouse SILAC proteome (mSILACAA) ratio. The SILACAA-predicted most limiting EAA in the whole body AA ratio (BodyAA) is tryptophan ($r_{W(min)}$ = 0.65).

b. The mouse whole body AA ratio (BodyAA) vs. the mouse exome (MouseAA) ratio. The exome-predicted most limiting EAA in the whole body AA ratio (BodyAA) is tryptophan ($r_{W(min)}$ = 0.53).

c. The mouse SILAC proteome (mSILACAA) ratio vs. the mouse exome ratio (MouseAA). The exome-predicted most limiting EAA in mSILACAA is tryptophan ($r_{W(min)}$ =0.82). Therefore, the two ratios appear highly similar.

d. Growth rates for females fed BodyAA, MouseAA, and BodyAA + tryptophan (W). The linear part of the growth curves indicates that BodyAA suppresses growth by 20% compared to MouseAA (linear regression P value = 0.0306), based on net mass gain for that period (weeks 3 to 6.4 of age). Moreover addition of the exome-predicted limited EAA in BodyAA did not increase growth (MouseAA vs. BodyAA+W linear regression P value = 0.0037). Growth curves are based on 20 female C3B6F1/J hybrids for each diet (N=20).

5.3.13 Exome-matched against NRC-matched AA supply

As mentioned earlier, despite the many methods previously used in calculating dietary AA requirements, today there is consensus that the determination of dietary protein quality should take into account data from all existent methods (4, 25). Thus, in laboratory animal nutrition, recommendations are based on combining such experimental data along with empirical growth data. Yet, AA recommendations for well-defined model organisms can also vary considerably between such recommendations. In mice, independent sources, including the American Institute of Nutrition (AIN) (232) and the National Research Council (NRC) (28) recommend dietary AA profiles that are distinct to each other (Figure 5.16A), meaning that different expert panels arrive to different AA recommendations depending on the published information they use. Both ratios are also distinct to the mouse exome profile (Figure 5.16B-C). So it was next determined if the exome, as a less ambiguous measure for defining dietary AA requirements, can be used as an alternative and more reliable predictor of growth.

Based on the exome AA usage, it was predicted that the most limiting EAA in NRCAA would be tryptophan ($r_{W\,(min)} = 0.55$) (Figure 5.16C). However, NRCAA promoted growth more than MouseAA (Figure 5.s 16D-E). Moreover, when attempting to correct the NRCAA's limitations, as predicted by the exome, by increasing tryptophan by ~1.5 fold (to eliminate the W limitation; NRCAA+W) or by ~1.5-fold addition of tryptophan combined to a ~2-fold addition of arginine (NRCAA+W+R) as a control for arginine's dispensability, a significant growth suppression in both cases was observed (Figure 5.16E). Importantly, addition of arginine in NRCAA+W, resulting in the NRCAA+W+R diet, significantly suppressed growth by 14% in the linear part of the growth phase, a trend that persisted at least until 32 weeks of age (Figure 5.16E). Thus, although at limiting levels arginine did not promote growth (Figure 5.1E), surplus arginine in NRCAA+W significantly reduced growth. Mice fed NRCAA also had significantly increased lean and fat mass compared to mice fed MouseAA, NRCAA+W, and NRCAA+W+R, following both 10 or 20 weeks of treatment (Figure 5.16F), and the FER and PER indices were similarly affected, with NRCAA increasing the efficiency of both food and protein (Figure 5.16G). Mice fed NRCAA also had significantly reduced water consumption (Figure 5.16H).

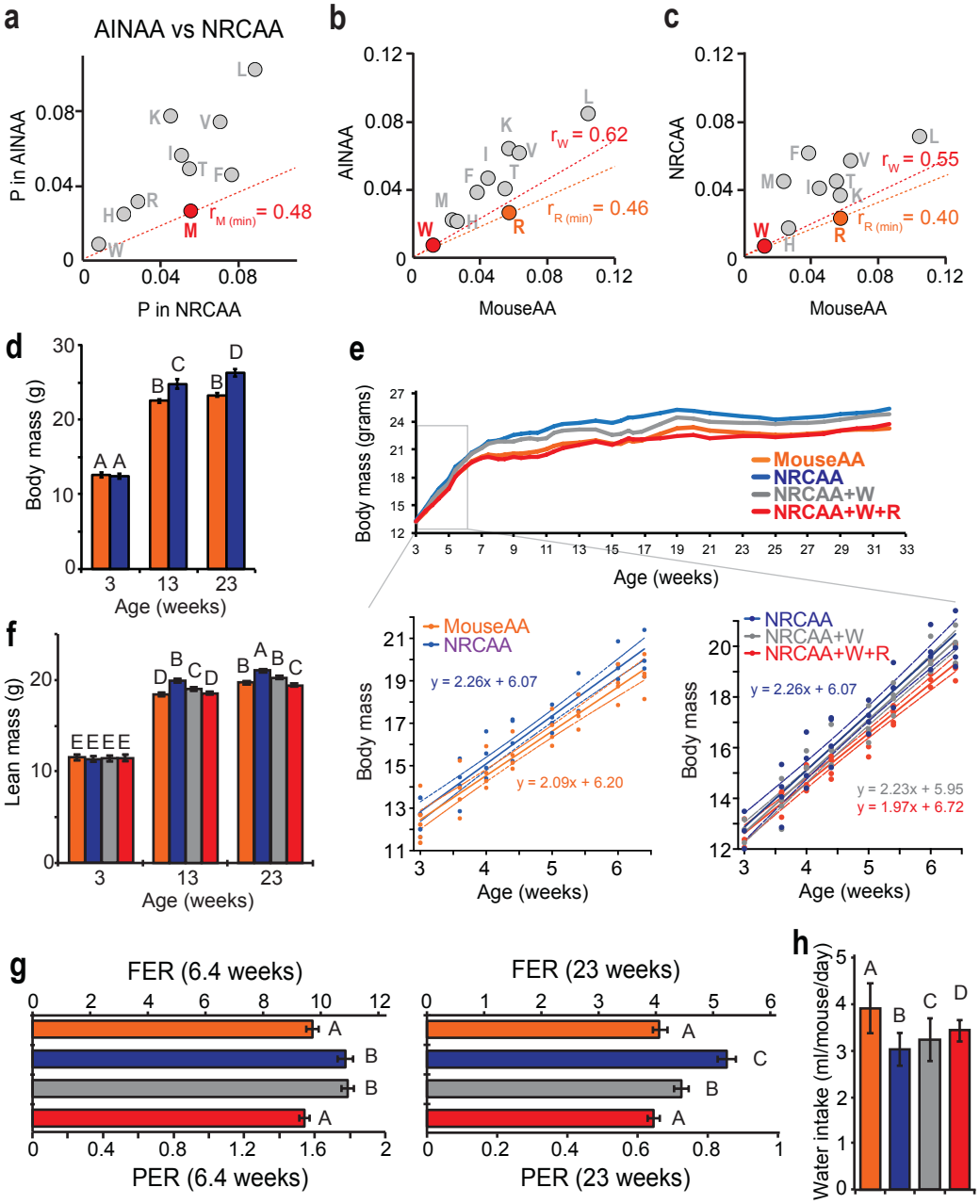


Figure 5.16

a. Comparison of the American Institute of Nutrition (AINAA) and the National Research Council (NRCAA) recommended dietary AA profiles. AINAA is limited for methionine compared to NRCAA.

b. Comparison of AINAA to the mouse exome AA usage (MouseAA) reveals that the AINAA is limited for Arginine and Tryptophan compared to MouseAA.

c. Comparison of NRCAA to the mouse exome AA usage (MouseAA) reveals that the NRCAA is limited for Arginine and Tryptophan compared to MouseAA.

d. Body mass across dietary AA ratios. Post-weaning body mass was similar between MouseAA and NRCAA, but was significantly increased in mice pair-fed NRCAA compared to

mice pair-fed MouseAA 6% after 10 and 20 weeks of treatment on the respective diet (age 13 and 23 weeks respectively). Bars represent the mean for each group (N = 20 for weeks 3 and 14, N = 10 for week 24), and error bars represent SEM. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

e. Body mass growth comparison of mice fed MouseAA and NRCAA. Mice fed NRCAA had an increased growth rate, and this improvement was ~11% at week 6.4 (polynomial regression analysis P value = 0.03), and ~30% at week 9 (polynomial regression analysis slope P value < 0.05, linear mixed effects analysis P value = 0.12). Moreover, correcting NRCAA by matching its tryptophan (NRCAA+W) or arginine and tryptophan (NRCAA+W+R) contents to the exome requirement decreased growth against MouseAA (for both MouseAA vs. NRCAA+W or vs. NRCAA+W+R, polynomial regression analysis slope P value < 0.05)

f. Lean mass across dietary AA ratios. Post-weaning lean mass was similar between the groups, but was significantly increased in mice pair-fed NRCAA compared to mice pair-fed MouseAA 6% after 10 and 20 weeks of treatment on the respective diet (age 13 and 23 weeks respectively). Bars represent the mean for each group (N = 20 for weeks 3 and 14, N = 10 for week 24), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test). Data by George Soultoukis, Martin Purrio and Dr. Andrea Mesaros.

g. Food efficiency and protein efficiency were improved by NRCAA compared to MouseAA, both at 6.4 weeks of age, and at 18 weeks of age. Bars represent the mean for each group (N = 20 for weeks 3 and 14, N = 10 for week 24), and error bars represent SD. Letters indicate statistically significant differences (P value < 0.05) for pair-wise comparisons (Student's t-test).

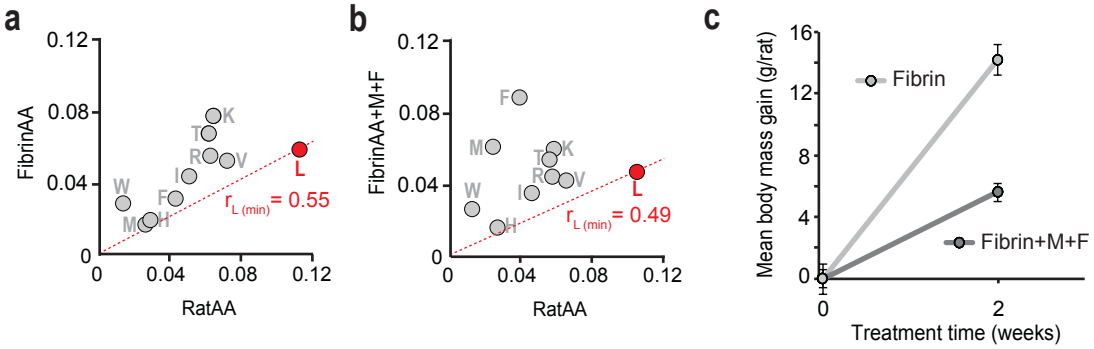
h. Water intake for mice *ad libitum*-fed and pair-fed on MouseAA, MMAA, MMAA-T, and MMAA-M. Mice pair-fed MouseAA consumed significantly less water compared to all other pair-fed mice (letters indicate significant change, $p<0.05$, Student's t-test, N = 4).

The observation that moderate additions of tryptophan or arginine in NRCAA suppress growth suggest that complex interactions between specific AAs, such as substrate antagonisms or other less well-understood effects (26), may have significant effects upon growth. It is notable that NRCAA has twice the content in methionine and phenylalanine compared to MouseAA. Autoclaving and irradiation, both common sterilization steps in laboratory rodent food preparation, can diminish the levels of certain AAs including methionine and cysteine (177) and may result in a lower methionine content in the MouseAA food than that required by the exome AA usage.

Moreover in the liver, methionine enters many transulfation, transmethylation, and folate metabolism reactions (56), and an estimated 35%-100% of the ingested methionine, phenylalanine, and branched-chain AAs, is used by the splanchnic bed (first pass metabolism), never reaching systemic circulation (48, 56). Therefore, it is possible that the high content of NRCAA in methionine and phenylalanine allows a sufficient amount of these two AAs to reach circulation, to meet AA usage demands, and consequently to promote growth more efficiently than MouseAA. This could explain why mice fed NRCAA grow faster than mice fed MouseAA. However, this explanation does not account for the growth-suppressing effect induced by increasing tryptophan by ~1.5 fold (to match the W content in MouseAA; NRCAA+W) or by ~1.5-fold addition of tryptophan combined to a ~2-fold addition of arginine (NRCAA+W+R). Yet, previous growth studies have observed that some tryptophan additions become toxic and suppress growth (26), and this could be an explanation for these effects. Alternatively, complex interactions between AAs could contribute to these effects. However, this is a poorly understood topic: for example, antagonisms between lysine and arginine can suppress growth upon addition of lysine or arginine only in the diet (26). Excess leucine or methionine depress rat growth independently of food intake, with excess leucine increasing the growth requirement for tryptophan (26). Yet, there is a lack of comprehensive studies due to the many dimensions of potential interactions, and the effects of AA imbalance-induced decreases in growth, and upon health and lifespan, are poorly characterized. In a previously published study, moderately increasing methionine and phenylalanine to proportions similar to those in the NRCAA proportions did not increase rodent growth (180, 233), and actually decreased it significantly. A 6% fibrin diet (Figure 5.17A) with supplementations of 0.4% w/w methionine and 0.6% w/w phenylalanine (Figure 5.17B) decreased growth in rats by ~30% compared to the unsupplemented 6% fibrin diet (Figure 5.17C). Therefore it is unclear if the additions of tryptophan or tryptophan+methionine are toxic, or if the excess methionine and phenylalanine simply cause interactions with surplus tryptophan to suppress growth.

Nonetheless, NRCAA, an empirical-based recommended AA profile promoted anabolism more than exome-matching did. Nevertheless, exome-matching is a valid measure of defining requirements, and is based on a method that considers quantifiable genomic information, and is not simply empirical as NRCAA is. This also means exome-matching is immediately more applicable to new model organisms with sequenced genomes, compared to other methods that are simply empirical, arduous, and consider data accumulated

over decades to derive some recommendations; such methods are arguably less efficient for immediate use. Therefore exome-matching is a quick, accessible, and quantifiable approach for defining the AA requirements in mice.



a. Comparison of the AA profile in fibrin (FibrinAA) and the rat exome AA usage (RatAA) profile. FibrinAA is limited for Leucine compared to RatAA.

b. Comparison of the AA profile in fibrin supplemented with Methionine and Phenylalnine (FibrinAA+M+F) and the rat exome AA usage (RatAA) profile. FibrinAA+M+F is limited for Leucine compared to RatAA.

c. Rat body mass gain following 2 weeks of treatment on FibrinAA and on FibrinAA+M+F. Data replotted from those publiched in (233).

5.4 Discussion

5.4.1 Mouse growth

As reported in Chapter 4, the degree of limitation in the most limiting essential AA (EAA) as defined by the exome can be used as an accurate predictor of anabolism in flies. Using the same model in mice, it is demonstrated in this Chapter that an imbalanced ratio also suppresses anabolic traits and growth in mice. Limiting dietary EAAs were predicted by the mouse exome, and dietary AA ratios were qualitatively defined by the degree of their disproportion to the exome AA usage more accurately than when compared to other approaches. Mouse exome-matching (MouseAA) promoted growth more so than casein, the common protein source in commercial mouse feeds. *Ad libitum*–fed mice also consumed less MouseAA food than CaseinAA food, suggesting exome-matching defines murine diets to be more satiating. MouseAA was also more efficient for anabolic traits than the whole body AA ratio (BodyAA), a previously used measure of AA requirements (153). Importantly, across species, AA intake can itself alter the body's AA composition (189, 234), which introduces a critical confounding variable in this approach. Therefore, exome-matching is an effective measure of determining AA requirements across species compared to previously reported measures. Mice fed at higher CP levels (CaseinAA 8%) had growth rates matched by an exome-matched diet fed at lower CP levels (MouseAA 6%). Morevover the fly findings supported a conditionally-essential role of arginine for egg-laying, and it was therefore set out to confirm the nature of the essential role of arginine for anabolic traits in mice. Previous studies in mice have shown that arginine is essential (28) but replenishable for rodent growth (235), with limited effects on growth when restricted in the diet. Yet, excess dietary arginine can also suppress growth (26). Indeed our results provide further support to these findings, as it was observed that arginine addition in an arginine-limited diet did not improve growth, but even small arginine additions in a diet containing adequate amounts of arginine (NRCAA) resulted in significant growth suppression. It is likely that these growth-suppressing effects of surplus arginine occur because arginine can exhibit substrate antagonism against certain AAs for specific transporters (46). Indeed, it has been observed that antagonisms between lysine and arginine can suppress growth upon addition of lysine or arginine only in the diet (26). Thus, a conditionally essential AA whose restriction has no effect on growth (arginine) can in fact suppress growth when consumed in excess. Recent findings on the unique mechanisms involved in AA sensing by TOR, and in specific, on the unique mechanism involved in arginine sensing, also provide intrigue into the unique role of arginine in AA sensing and growth signaling (236).

5.4.2 Mouse metabolic rate

In contrast to flies, exome-matching in mice resulted in a decreased oxygen consumption, both during the day and night cycles. In mice compared to higher protein diets, low protein diets increase thermogenesis, body surface

temperature (237), energy expenditure, mitochondrial activity, and oxygen consumption (3, 238), all of which were observed in mice fed the exome-mismatched CaseinAA diet. CaseinAA therefore caused a decreased utilization of nutrients (lower food and protein efficiencies) and mimicked a lower protein diet consumption by elevating all these aspects of metabolic rate. However, the RER was not changed by exome-matching, suggesting no shift to fat as an energy fuel. Finally, as mice were kept at temperatures (22-23°C) below their thermoneutral zone (30°C) it is worth mentioning that SCAT thickness also affects heat loss. Indeed, exome-matching increased SCAT, and decreased energy expenditure and body temperature (although the body temperature was not decreased significantly).
Oxygen consumption depends on mitochondrial function, and mitochondrial activity is decreased in type 2 diabetes (218). Moreover, diet induced obesity is associated with impaired mitochondrial function and mitochondrial dynamic processes, and impacts on mitochondrial bioenergetics and on the metabolic response to nutrients (239, 240). In agreement to these observations, exome-matching resulted in lower oxygen consumption, higher insulin-resistance, and higher levels of obesity compared to mice fed CaseinAA. As a decrease in hepatic proteasomal activity was also observed, it is also possible that compromised hepatic proteasome function contributed to the decreased mitochondrial function in a feedback loop process between the two systems (241). However, exome-matching reduced the abundance of complex I in the mouse liver, which could confer metabolic advantages. Hepatic mitochondrial complex I contents can be reduced by dietary protein restriction (242), and a low abundance of complex I protein levels can promote mitochondrial efficiency and longevity in mice (243). Indeed fatter, more insulin-resistant mice can be longer lived due to the C:P ratio consumed (3). Moreover, high protein diets can reduce fat deposition, unlike what was seen in MouseAA-fed mice here, and this can shorten lifespan in mice (244). Therefore, more work is needed to further appreciate the role of mitochondrial function across a range of dietary protein levels and AA profiles. For example, more steady state single subunit levels could be assessed, as well as complex, supercomplex, and activity levels (including complex I activity).
Finally, in rodents, DR increases spontaneous activity levels (245), including voluntary wheel activity (246). In our set-up, *ad libitum* fed mice had reduced levels of spontaneous activity compared to pair-fed mice, and this effect was significant in *ad libitum*-fed exome-matched mice.

5.4.3 Mouse body composition

In *ad libitum*-fed mice, low dietary protein increases adiposity as a consequence of a higher food intake, but these animals are as healthy as DR ones (3, 247). However, here we show that under a fixed AA intake, the profile of ingested AAs determines fat storage. In wild type mice, GCN2 represses liver fatty acid synthesis and promotes fat mobilization when animals are fed a leucine-deprived diet (248). Our results show that a tryptophan-limited CaseinAA diet also reduces adiposity in mice. Therefore, more usable AA sources (MouseAA) enable fat storage, an effect that is apparent particularly at low dietary protein conditions. However, a decrease in

fat storage and an improved insulin sensitivity is seen upon an increase in the total AA intake both in mice and humans (3) (24), so it remains possible that a balanced AA profile will reduce adiposity at higher levels of dietary protein compared to an imbalanced one, as was observed in flies. In humans, protein sources with a high nutritional value, such as whey protein, promote a reduction of adipose tissue during diet restriction-induced weight loss (142), which decreases both fat and lean mass, thereby exacerbating an unfavourable body composition known as sarcobesity (249). Therefore, the assessment of fat storage across various levels of dietary AA intake would better elucidate how the profile of ingested AAs interacts with the total AA intake to determine fat storage. Moreover, the ability of animals to maintain their adiposity despite DR is critical for gaining the health benefits of DR (92), and a lifespan analysis in exome-matched and exome-mismatched dietary AA profiles would establish the interaction between dietary AA profiles, total AA intake, adiposity, and health. Therefore, as the role of fat deposition in mediating the health benefits of protein restriction, single AA restriction, and AA imbalance requires further investigation.

In mice and humans, DR reduces triglycerides and increases some ketones (including β-hydroxybutarate) (250), and our results indicated similar effects in mice fed the exome-imbalanced CaseinAA. Additionaly, imbalanced AA intakes can inhibit lipogenesis, and methionine or tryptophan restrictions reduce fat storage in rodents, while methionine restriction also reduces hepatic triglycerides (13, 14). In leucine or methionine-restricted diets, fibroblast growth factor 21 (FGF21) is a common downstream effector of GCN2 activation that represses liver fatty acid synthesis and increases fatty acid mobilization (13) (79, 251). The results in this Chapter are in accord with these observations, as exome-mismatching reduced liver and plasma triglycerides, and increased oxygen consumption. An increased oxidative capacity and reduced lipogenic function is a critical component of the hepatic response to diets with limiting dietary AAs including methionine-restricted diets (13). Interestingly, mice on the exome-matched diet also had a significantly increased subcutaneous fat and skin thickness, which presumably contributes to a better insulation and decreased heat loss, thereby resulting in less need for thermogenesis. Therefore although mice pair-fed MouseAA had a significantly lower energy expenditure, their surface body temperature was not significantly reduced, which is in agreement with previous studies showing that subcutaneous fat differences as a function of dietary protein do not necessarily explain differences in thermogenesis and body surface temperature (237).

Another tissue-specific effect of protein restriction in rodents is the positive correlation seen between intestinal villus length and growth rate (229, 252). This effect is presumably linked to the abundance and usability of intraluminal AAs (229, 253). However, although in our exome-matched mice we saw a higher growth rate, no changes in villus or cryptal length were detected at 24 weeks of age, although it is possible that an effect may occur at younger ages. Brain mass, which can be spared during growth-depressing nutritional stress, was also significantly increased by exome-matching but the difference disappeared upon normalization to body mass. Lean and muscle mass was

also increased by exome-matching, a higher quality AA source, which is in accord with the findings that a balanced AA source such as whey protein, or essential AA supplementation can promote an increase in muscle protein synthesis during diet restriction-induced weight loss in elderly obese individuals (142), or during muscle atrophy induced by casting in mice (254). Interestingly, mice fed CaseinAA had a higher incidence of hepatic and muscle atrophy, and its been previously shown that a higher induction of IGF-1, PI3K, and Akt suppresses muscle atrophy (255). The insulin levels of mice fed CaseinAA were decreased compared to mice fed MouseAA, indicating a possible suppression of the IIS pathway in mice fed CaseinAA, consistent with their higher muscle atrophy incidence. Moreover, although hepatic levels or activation of Akt was not decreased at 24 weeks, when growth curves approach a plateau phase, differences in Akt levels or activation could have occurred during the more critical linear growth phase, thereby contributing to their higher muscle atrophy incidence.

5.4.4 Mouse glucose homeostasis

Accumulation of fat and lipids in tissues and circulation can interfere with insulin signaling and lead to insulin resistance (256), while in mice and humans DR reduces circulating glucose (250). Mice fed MouseAA utilize a significantly higher proportion of the ingested AAs and display insulin resistance, suggesting that the higher utilization of dietary AAs in MouseAA results in an effect similar to that observed upon increasing the total amount of ingested AAs. As more dietary AAs are used by mice fed MouseAA (both *ad libitum* and pair-fed), more AA catabolism occurs, generating carbon skeleton products, including glucose. However, glucose levels were elevated in *ad libitum*-fed MouseAA mice only, indicating that pair-fed mice in a low protein diet enable homeostatic mechanisms to prevent an increase in basal glucose levels as a function of the ingested AA profile. Although glycogen accumulation is one of the protective mechanisms of DR in yeast and worms, elevated glycogen storage is not a hallmark of the DR-induced benefits in mammals (257), and in humans DR actually reduces glycogen stores (250). Yet, exome-matching increased adiposity and decreased glycogen storage, assessed histologically, under pair-fed conditions suggesting that an AA profile with a higher nutritional value can shift the metabolism of glucose away from glycogen synthesis and towards fat synthesis. In skeletal or heart muscle, the inhibitor of glycogen synthase GSK-3 is induced by insulin-like growth factor-1 (IGF1) (255) or insulin (258). Mice fed MouseAA diet had higher levels of circulating insulin, a greater body size, and increased levels and activation of GSK-3, resulting in decreased liver glycogen storage. High protein diets are known to reduce hepatic glycogen storage in mice, and insulin resistance can lead to a reduction in glycogen synthesis (259). Given the decreased respiration in mice fed MouseAA, the most probable end product for glucose and its by-products may be fat precursors. Interestingly, mice *ad libitum*-fed MouseAA had a similar glucose homeostasis profile to long-lived insulin receptor substrate 1 null mice ($Irs1^{-/-}$), which also show glucose tolerance, insulin resistance, and elevated basal levels of insulin (260). Insulin and leptin are satiety hormones, and high circulating levels of

these hormones reduce food intake (111, 261), in accord with what was observed for mice fed the exome-matched diet. Such hormonal modulations induced by dietary AA profiles can reflect long-term metabolic adaptations to a diet, as opposed to short-term responses including GCN2 mediated responses (111). Combined, our results support these findings and further show that a dietary AA profile that is highly utilizable can phenocopy a higher protein diet, and result in obesity, higher insulin resistance, increased glucose tolerance, reduced food intake, and decreased glycogen storage.

5.4.5 Mouse proteostasis

Insulin resistance is associated with proteasome dysfunction, exhibited as increased levels of proteasome subunit transcripts and decreased proteasome activity (262). The decreased proteasomal activities of mouse liver and muscle tissues upon exome-matching might, therefore, be secondary to the increased fat accumulation and increased insulin resistance observed in these animals. As mentioned, in the liver a decrease in proteasomal activity caused by impairment of proteasome function can drive the malfunction of mitochondrial function in a feedback loop process between the two systems (241). However, more work would be required to establish the cause of the observed reduction in hepatic proteasome activity. In addition, although no changes were seen in the modulation of TOR protein levels or activation as a function of the ingested AA profile, recent studies suggest that proteostatic mechanisms can function independently of TOR modulation (263). Interestingly, in both mice and flies (Chapter 4) proteasomes proved to be more sensitive to dietary modulations than the autophagic machinery, regardless of whether the observed effects are primary or secondary. Moderate, rather than severe, modulations in the AA proportions were sufficient to exert responses to dietary AAs. This may provide a valuable hint about the function of degradation machineries, and about which one of them responds more upon chronic dietary AA interventions.

5.4.6 Mouse nitrogen balance

It has long been recognized that diets containing an imbalanced AA ratio can reduce nitrogen retention compared to a balanced AA ratio (26), while the observation that imbalanced AA sources reduce nitrogen retention is also widely accepted in humans (25). An imbalanced dietary AA supply can induce excessive water intake and urine loss. The urine output in methionine-restricted mice, for example, is higher than for controls presumably due to the osmotic demands of excreting nutrients that cannot be metabolized into protein due to the limiting levels of sulphur AAs (203). Our results are in agreement with these observations, as mice ingesting an exome-mismatched AA profile had a lower nitrogen retention, owing to poor AA utilization, which is a consequence of this AA imbalance. Another important aspect of such feeding regimes, however, involves feeding frequency. *Ad libitum*-fed mice snack throughout the day much more often, whereas DR mice eat all food at

one go as soon as its offered to them (less feeding episodes) (245). Water consumption per mass of food ingested is same for DR animals, but again through less drinking episodes. It is likely that such differences in feeding patterns can alter certain phenotypes including energy homeostasis, insulin signaling, and nitrogen utilization.

5.4.7 Mouse bone quality

Apart from the increase in skeletal length observed in pair-fed animals on the MouseAA regime, the AA profiles also affected several bone quality parameters. Bone health is affected by many nutrients, with the type and quality of dietary protein playing a predominant role (264). High quality protein sources that promote growth hormones are associated with increased BMD and lower porosity (264). For pair-fed mice, exome-matching resulted in increased cortical and trabecular structure thickness, increased trabecular bone volume and bone mineral density (BMD) compared to CaseinAA-fed mice. These observations are in accord with the hypothesis that MouseAA is a higher quality nutritional source than CaseinAA. In mice, several nutrient-responsive pathways modulate skeletal development, and bone mineralization and ossification processes, including Akt-Foxo, mTOR, and GSK-3 mediated pathways (265). The observation that mice pair-fed CaseinAA had decreased levels of activated GSK-3 may explain their lower levels of bone volume and mineralization (265). Moreover, reduction in IGF-I, typically associated with reduced growth such as that observed in animals pair-fed CaseinAA, is also linked to decreased bone quality and strength with age (266). Therefore, it would be interesting for future studies to explore the effects of dietary AA-mediated effects on bone health with ageing.
For *ad libitum* fed mice, in contrast, only cortical structure thickness was elevated in animals fed MouseAA compared to animals fed Casein AA. The fact that several parameters were unchanged in the *ad libitum*-fed (more obese) animals is in accord with the finding that bone quality in rodents is more sensitive to leaner than it is to more obese animals (267). Moreover, DR in mice increases BMD (268) but DR mice are typically leaner and BMD can also partially reflect body mass (269). Accordingly, mice pair-fed MouseAA were heavier and also had elevated BMD compared to mice pair-fed CaseinAA. In humans too, decreased trabecular and cortical strength and lower BMD, consistent with increased risk of fracture, are associated with dietary restriction (230, 270). Therefore, the findings described here suggest that exome-matching can have a significant impact upon bone quality, primarily by increasing BMD. Moreover, it is possible that as in mice, exome-matching in humans could define diets for the promotion of long-term bone health. In this respect, it would be interesting to investigate the long-term effects of exome-matching in murine bone health with age.

5.5 Conclusions

Malnutrition is a leading cause of growth attenuation in children (271). In this Chapter it is observed that an ingested AA profile matched to the mouse

exome requirements can prevent mouse growth attenuation. Moreover, the results demonstrate that small dietary AA modulations can dictate many physiological processes that ultimately impact on long-term health in mice. Critical health aspects, such as obesity and insulin resistance, were determined by the ingested AA profile. It is thus important to understand how modulations in dietary AAs exert their biochemical and physiolometabolic effects. However, the role of fat metabolism in promoting health in DR is still not clear, and the role of fat deposition in mediating the health benefits of protein or single AA restriction also requires further investigation.

The weighted proteome's AA usage was a better predictor for mouse lean mass growth than the exome requirement. However, no correlation was seen between the tissue-specific weighted proteome (tsmSILACAA) and the growth rate for the specific organs in question. In flies, proteasome peptide biosynthesis could not be predicted or modulated simply through dietary AA manipulation on the basis of AA requirement by the proteasome's proteome. As in flies, these results suggest that ingested AA profiles do not directly modulate the biosynthesis of single organs on the basis of the principle of minimum. The biosynthesis of specific organs or of sets of proteins (e.g. proteasome complex) is not merely a function of what AAs are ingested, but are rather modulated by other regulatory processes possibly including transcriptional, translational, and metabolic parameters.

Chapter 6: General discussion

6.1 Summary

The results presented in this thesis show that an exome-matched supply of dietary AAs promoted production traits in both flies and mice better than other commonly used dietary AA sources, including AAs provided in the proportion found in yeast or in casein respectively. In both animals, exome-defined limiting EAAs decreased dietary protein efficiency for anabolic processes. These results show that dietary AA balance is critical for the modulation of key life history traits, including growth, reproduction, and for key health aspects including fat metabolism in both flies and mice. Because this model is driven by the genome sequence of the organism in question, the principle can be applied to any organism whose genome sequence is known. Therefore our findings carry possible implications for interventional applications in human nutrition.

The data in this thesis also suggest a quantitative relationship between the levels of dietary nitrogen (in the form of AAs), the dietary AA profile, and key life history traits such as development, reproduction, and health. Failing to account for the interaction between the limiting EAA and the total nitrogen intake can lead to fallacies in growth data interpretation (180, 272, 273). The principle of the minimum poses that the most limiting nutrient in a diet can prevent successful utilization of other, non-limiting nutrients that are, as a result, acquired in excess (31). Accordingly, we find that exome-defined limiting EAAs prevent the anabolic usage of non-limiting EAAs. In addition, the law of diminishing returns holds that each succeeding increment of the limiting essential nutrient will produce a smaller increment of growth than the preceding increment (31, 190). In agreement, we observed a decrease in the effect of the limiting EAA with incremental increases in its concentration, or in the concentration of dietary nitrogen, upon fly development, growth, and fecundity, as well as mouse growth. At high AA supply, the physiological needs for development were equally met by diverse dietary AA ratios, but at low dietary nitrogen they were adequately met only by the exome-matched diet. Interestingly, certain AA intakes, such as that in casein, have been shown in the past to delay fly growth and development time (145) but to be adequate for lifespan (274). The resonation of the effects of a dietary AA imbalance upon anabolic traits at low dietary AA supply is not a new observation in rodents (180).

However, although the degree of limitation of a limiting EAA in the diet is important, the findings described in this thesis also support a key role of the identity of the limiting EAA in mediating the physiological responses to a dietary EAA imbalance. This is in accord with previous studies showing that the identity of the limiting EAA is critical for the effects of protein and single EAA utilization for fly fecundity (212), fly development (199), and for rat growth (273). Other studies in rodents also support a key role for the proportion of the limiting EAA to other, non-limiting EAAs (233). Moreover, even minor disproportions of EAAs can be detrimental for health (26). Thus,

although a measure to aid the identification of limiting EAAs is of great importance (that measure here defined as the exome), further work is required to resolve the precise biological reasons for such indiscrepancies between different identities of the most limiting EAA.

Finally, the weighted proteome's AA usage was a better predictor for mouse lean mass growth than the exome requirement. However, no correlation was seen between the tissue-specific weighted proteome (tsmSILACAA) and the growth rate for the specific organs in question. In flies, proteasome peptide biosynthesis could not be predicted or modulated simply through dietary AA manipulation on the basis of AA requirement by the proteasome's proteome. These results suggest that ingested AA profiles do not directly modulate the biosynthesis of single organs or of specific gene ontologies on the basis of the principle of minimum. Thus the biosynthesis of specific organs or of sets of proteins (e.g. proteasome complex) is not merely a function of what AAs are ingested, but are rather modulated by other regulatory processes possibly including transcriptional, translational, and metabolic parameters.

A complication in the evaluation of the effects of ingested AAs may stem from the fact that dietary-AA dependent processes are not necessarily regulated in an identical way across species. In many model organisms, as well as humans, DR results in leanness, limiting obesity to confer multiple metabolic advantages that favour longevity (5). However, DR or PR flies increase their fat storage (275, 276). In mice DR reduces fat storage (6) but PR increases it, yet both DR and PR promote healthspan and lifespan (8, 13). Moreover, mice subjected to DR have an extended lifespan particularly when they can maintain their adiposity (6, 92). The physiological effects of single AA modulations may also be inconsistent across species. While long-lived methionine-restricted (MR), leucine-restricted (LR), or tryptophan-restricted (TR) mice have depleted fat storage (13, 14, 79), increasing evidence suggests that flies fed diets limited in single AAs have higher levels of body lipids. Mice subjected to DR or MR also have a higher energetic efficiency than ad libitum-fed mice aiding thermoregulation (13), in contrast to flies which are poikilothermic. Thus life-extending interventions through dietary AAs appear to cause disparate effects in lipostasis and energy homeostasis between flies and mice. Indeed, some findings were contrasting between flies and mice. Exome-matching in flies resulted in lower lipid content, but in mice it increased fat storage. Nonetheless, many common features between the two organisms were observed, including a general induction in anabolic processes.

6.2 Conclusions

The genetic composition of a species may affect the response to ingested AAs and contribute to the diversity of protein sources consumed across species. In humans, recent nutrigenomics methods employ the use of genetic information on inborn errors of metabolism to direct the use of dietary AAs in clinical research (106). Yet, the advance of multiple –omics technologies has opened the possibility of evaluating dietary AA requirements across species

through novel approaches. Here, a genome-based technique for designing an enhanced quality protein is presented, and it is shown that the exome is a powerful measure for defining AA requirements across species.

Malnutrition is a leading cause of growth attenuation in children (271). In both flies and mice, an ingested AA profile matched to the organism's exome requirements prevented growth attenuation at low dietary protein levels. However, such response mechanisms appear to be species-dependent. Stark contrasting physiometabolic responses to dietary AA modulations were observed between mice and flies. The role of fat metabolism in promoting health in DR is still not clear, and the role of fat deposition in mediating the health benefits of protein or single AA restriction also requires further investigation.

However, here it is shown for the first time how the interaction between nitrogen levels and limiting AAs impacts on multiple physiometabolic aspects. Following ingestion, the net amount of usable AAs depends both on the total nitrogen intake and on the AA profile ingested, and determines both production traits and health. In *Drosophila*, an exome-balanced AA source is more usable and maximizes anabolism, suggesting that optimized anabolic traits increase growth signaling. In contrast, exome-imbalanced dietary AA ratios are less usable and maximize anabolism only at higher dietary nitrogen levels. Nonetheless, traits such as growth, development time, and reproductive output, however, require high nitrogen supply to approach their maximum levels, regardless of the AA profile. Importantly, exome-matching also defines anabolic traits in mice as exome-mismatched AA ratios supported a slower growth rate, which can be a predictor of murine lifespan (36). Specifically, the growth rate at ~20 weeks of age, which is also report here, has been proposed as an accurate predictor of lifespan in mice (36). Therefore, it is possible that the observed phenotypes linking dietary AAs to anabolic traits and health also determine lifespan. This is an interesting prospect that warrants further investigation, as it would directly link an exome-matched profile of ingested AAs to longevity, with a wide range of implications for both human and animal nutrition.

Despite the complexity of the multitude of factors that can alter AA usage, nutrigenomics may be the most promising approach for defining AA requirements. The exome-based approach can be used in designing enhanced protein quality in diets, thereby improving growth and reproduction, and predicting the effects of dietary nitrogen upon key life history traits. An optimally balanced nitrogen source can define nitrogen requirements for anabolic traits and health when the total intake of dietary amino acids is relatively low. In humans, a higher satiety effect is linked to higher protein diets used in clinical weight management (23). The results in this thesis may have broad implications to the application of dietary regimes aimed at weight loss as it is shown that apart from the protein content of a diet, the AA profile also determines satiety and food selection in mammals. Enhancing dietary protein quality is also a keen interest of the food industry, with economic advantages in increasing the efficiency of biomass production and environmental advantages in minimising pollution from nitrogenous waste

production. Moreover, protein efficiency optimization can also find application in improved animal health and growth for sustainable food production (277), enhanced recovery for humans from intense exercise (278) and surgery, and in maximising the nutritional value of food to aid recovery from malnutrition.

References

1. Keenan KP, Wallig MA, Haschek WM. 2013. *Toxicol Pathol*. 41(2):190–209
2. Simpson SJ, Raubenheimer D. 2010. *Calorie Restriction, Aging and Longevity*. Dordrecht: Springer Netherlands. 12 p.
3. Solon-Biet SM, McMahon AC, Ballard JWO, Ruohonen K, Wu LE, et al. 2014. *Cell Metabolism*. 19(3):418–30
4. Lupton JR, Brooks GA, Butte NF, Caballero B, Flatt JP, et al. 2001. *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids*. Washington D.C.: The National Academies Press. 1 p.
5. Fontana L, Partridge L. 2015. *Cell*. 161(1):106–18
6. Weindruch R, Walford RL. 1988. *The Retardation of Aging and Disease by Dietary Restriction*. Springfield, IL: Charles C. Thomas Pub Ltd. 1 p.
7. Mair W, Piper MDW, Partridge L. 2005. *Plos Biol*. 3(7):e223
8. Grandison RC, Piper MDW, Partridge L. 2009. *Nature*. 462(7276):1061–64
9. Horáková M, Deyl Z, Hausmann J, Macek K. 1988. *Mechanisms of Ageing and Development*. 45(1):1–7
10. Nakagawa S, Lagisz M, Hector KL, Spencer HG. 2012. *Aging Cell*. 11(3):401–9
11. Mirzaei H, Suarez JA, Longo VD. 2014. *Trends Endocrinol. Metab.* 25(11):558–66
12. Douris N, Melman T, Pecherer JM, Pissios P, Flier JS, et al. 2015. *Biochim. Biophys. Acta*. 11(1852):2056–65
13. Orgeron ML, Stone KP, Wanders D, Cortez CC, Van NT, Gettys TW. 2014. *Progress in molecular biology and translational science*. 121:351–76
14. Zimmerman JA, Malloy V, Krajcik R, Orentreich N. 2003. *Experimental Gerontology*. 38:47–52
15. Shin AC, Fasshauer M, Filatova N, Grundell LA, Zielinski E, et al. 2014. *Cell Metabolism*. 20(5):898–909
16. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. 2013. *Cell*. 153(6):1194–1217
17. Rizza W, Veronese N, Fontana L. 2014. *Ageing Research Reviews*. 13:38–45
18. Brandhorst S, Choi IY, Wei M, Cheng C-W, Sedrakyan S, et al. 2015. *Cell Metabolism*. 22(1):86–99
19. Kabil H, Kabil O, Banerjee R, Harshman LG, Pletcher SD. 2011. *Proceedings of the National Academy of Sciences*. 108(40):16831–36
20. Hine C, Harputlugil E, Zhang Y, Ruckenstuhl C, Lee BC, et al. 2015. *Cell*. 160(1-2):132–44
21. Li P, Yin Y-L, Li D, Woo Kim S, Wu G. 2007. *BJN*. 98(02):237
22. Parrella E, Maxim T, Maialetti F, Zhang L, Wan J, et al. 2013. *Aging Cell*. 12(2):257–68
23. Westerterp-Plantenga MS, Nieuwenhuizen A, Tome D, Soenen S, Westerterp KR. 2009. *Annu. Rev. Nutr.* 29:21–41
24. Tremblay F, Lavigne C, Jacques H, Marette A. 2007. *Annu. Rev. Nutr.*

27:293–310
25. Consultation ROAJWFUE. 2007. *Protein and amino acid requirements in human nutrition*. Geneva: World Health Organization. 1 p. WHO Technical Report Series ed.
26. Harper AE, Benevenga NJ, Wohlhueter RM. 1970. *Physiol Rev*. 50(3):428–558
27. Park B-C. 2006. *Asian-Aust. J. Anim. Sci.* 19(9):1361–68
28. Benevenga NJ, Calvert C, Eckhert CD, Fahey GC, Greger JL, et al. 1995. *Nutrient Requirements of Laboratory Animals*. Washington, D.C.: National Academy Press. 1 p. Fourth Revised Edition ed.
29. Piper MDW, Blanc E, Leitão-Goncalves R, Yang M, He X, et al. 2014. *Nat Meth*. 11(1):100–105
30. Tucker LA, Erickson A, LeCheminant JD, Bailey BW. 2015. *J Diabetes Res*. 2015:206959
31. Brody S. 1945. *Reinhold Publishing Corp.*, p. 1023
32. Almquist HJ. 1953. *Arch. Biochem. Biophys.* 44(1):245–47
33. McCay CM, Crowell MF, Maynard LA. 1935. *The Journal of Nutrition*. 10:63–79
34. Junger MA, Rintelen F, Stocker H, Wasserman JD, Vegh M, et al. 2003. *J. Biol.* 2(3):20
35. Enesco HE, Samborsky J. 1986. *Arch Gerontol Geriatr*. 5(3):221–33
36. Miller RA, Harper JM, Galecki A, Burke DT. 2002. *Aging Cell*. 1(1):22–29
37. Selman C, Nussey DH, Monaghan P. 2013. *Current Biology*. 23(10):R451–53
38. Rollo CD. 2002. *Evol Dev*. 4(1):55–61
39. Bartke A. 2012. *Gerontology*
40. Partridge L, Gems D, Withers D. 2005. *Cell*. 120:461–72
41. O'Brien DM, Kyung-Jin Min, Larsen T, Tatar M. 2008. *Current Biology*. 18(4):R156
42. Hoffman JM, Creevy KE, Promislow DEL. 2013. *PLoS ONE*. 8(4):e61082
43. Tabatabaie V, Atzmon G, Rajpathak SN, Freeman R, Barzilai N, Crandall J. 2011. *Aging*. 3(12):1–4
44. Jahan-Mihan A, Luhovyy BL, Khoury El D, Anderson GH. 2011. *Nutrients*. 3(5):574–603
45. Rutherfurd SM, Moughan PJ. 2012. *BJN*. 108 Suppl 2:S298–305
46. Bröer S. 2008. *Physiol Rev*. 88(1):249–86
47. Zhang Z, Adelman AS, Rai D, Boettcher J, Lőnnerdal B. 2013. *Nutrients*. 5(12):4800–4821
48. Davila A-M, Blachier F, Gotteland M, Andriamihaja M, Benetti P-H, et al. 2013. *Pharmacol. Res.* 68(1):95–107
49. Metges CC, Petzke KJ. 2007. *BJN*. 94(05):621–22
50. Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, et al. 2009. *Proceedings of the National Academy of Sciences*. 106(10):3698–3703
51. McDonald P, Edwards RA, Greenhalgh JFD, Morgan CA, Sinclair LA. 2010. *Animal Nutrition*. London: Pearson. 1 p. 7 ed.
52. Zhang C, Li S, Yang L, Huang P, Li W, et al. 2013. *Nat Commun*. 4:2163

53. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y, et al. 2011. *Science*. 334(6052):105–8
54. Erkosar B, Storelli G, Defaye A, Leulier F. 2013. *Cell Host Microbe*. 13(1):8–14
55. Neuman H, Debelius JW, Knight R, Koren O. 2015. *FEMS Microbiol Rev*. 39(4):509–21
56. Stoll B, Burrin DG. 2006. *Journal of Animal Science*. 84 Suppl:E60–E72
57. Hinton T, Noyes DT, Ellis J. 1951. *Physiological Zoology*. 24(4):335–53
58. Ball RO, Courtney-Martin G, Pencharz PB. 2006. *The Journal of Nutrition*, pp. 1682S–1693S
59. Sarup P, Pedersen SMM, Nielsen NC, Malmendal A, Loeschcke V. 2012. *PLoS ONE*. 7(10):e47461
60. Tomás-Loba A, Bernardes de Jesus B, Mato JM, Blasco MA. 2012. *Aging Cell*. 12(1):93–101
61. Selman C, Kerrison ND, Cooray A, Piper MDW, Lingard SJ, et al. 2006. *Physiological Genomics*. 27(3):187–200
62. Richards SE, Wang Y, Claus SP, Lawler D, Kochhar S, et al. 2013. *J. Proteome Res.* 12(7):3117–27
63. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, et al. 2011. *Nat. Med.* 17(4):448–53
64. Wijeyesekera A, Selman C, Barton RH, Holmes E, Nicholson JK, Withers DJ. 2012. *J. Proteome Res.* 11(4):2224–35
65. Kilberg MS, Pan YX, Chen H, Leung-Pineda V. 2005. *Annu. Rev. Nutr.* 25(1):59–85
66. Wauson EM, Lorente-Rodríguez A, Cobb MH. 2013. *Molecular Endocrinology*. 27(8):1188–97
67. Pochini L, Scalise M, Galluccio M, Indiveri C. 2014. *Front Chem*. 2:61
68. Nicklin P, Bergman P, Zhang B, Triantafellow E, Wang H, et al. 2009. *Cell*. 136(3):521–34
69. Berg JM, Tymoczko JL, Stryer L. 2006. *Biochemistry*. New York: W. H. Freeman. 1 p. 6 ed.
70. Lemaitre B, Miguel-Aliaga I. 2013. *Annu. Rev. Genet.* 47(1):377–404
71. Libert S, Pletcher SD. 2007. *Cell*. 131(7):1231–34
72. Bjordal M, Arquier N, Kniazeff J, Pin JP, Leopold P. 2014. *Cell*. 156(3):510–21
73. Gietzen DW, Hao S, Anthony TG. 2007. *Annu. Rev. Nutr.* 27(1):63–78
74. Chaveroux C, Lambert-Langlais S, Cherasse Y, Averous J, Parry L, et al. 2010. *Biochimie*. 92(7):736–45
75. Chantranupong L, Wolfson RL, Sabatini DM. 2015. *Cell*. 161(1):67–83
76. Alves VS, Motta FL, Roffé M, Delamano A, Pesquero JB, Castilho BA. 2009. *Biochemical and Biophysical Research Communications*. 378(1):41–44
77. Efeyan A, Comb WC, Sabatini DM. 2015. *Nature*. 517(7534):302–10
78. Dang Do AN, Kimball SR, Cavener DR, Jefferson LS. 2009. *Physiological Genomics*. 38(3):328–41
79. De Sousa-Coelho AL, Relat J, Hondares E, Pérez-Martí A, Ribas F, et al. 2013. *J. Lipid Res.* 54(7):1786–97
80. Leib DE, Knight ZA. 2015. *Cell Rep*. 13(6):1081–89

81. Karnani MM, Venner A, Jensen LT, Fugger L, Burdakov D. 2011. *J. Physiol. (Lond.)*. 589(Pt 3):639–51
82. Steculorum SM, Paeger L, Bremser S, Evers N, Hinze Y, et al. 2015. *Cell*. 162(6):1404–17
83. Deval C, Chaveroux C, Maurin A-C, Cherasse Y, Parry L, et al. 2009. *FEBS J.* 276(3):707–18
84. Suryawan A, O'Connor PMJ, Bush JA, Nguyen HV, Davis TA. 2009. *Amino Acids*. 37(1):97–104
85. Stenesen D, Suh JM, Seo J, Yu K, Lee K-S, et al. 2013. *Cell Metabolism*. 17(1):101–12
86. Chotechuang N, Azzout-Marniche D, Bos C, Chaumontet C, Gausserès N, et al. 2009. *Am. J. Physiol. Endocrinol. Metab.* 297(6):E1313–23
87. Tews JK, Kim YW, Harper AE. 1980. *The Journal of Nutrition*. 110(3):394–408
88. Davis AJ, Austic RE. 1994. *The Journal of Nutrition*. 124(9):1667–77
89. Grant TD, Snell EH, Luft JR, Quartley E, Corretore S, et al. 2012. *Nucleic Acids Res.* 40(8):3723–31
90. Palii SS, Kays CE, Deval C, Bruhat A, Fafournoux P, Kilberg MS. 2009. *Amino Acids*. 37(1):79–88
91. Takenaka A, Oki N, Takahashi SI, Noguchi T. 2000. *The Journal of Nutrition*. 130(12):2910–14
92. Liao C-Y, Rikke BA, Johnson TE, Gelfond JAL, Diaz V, Nelson JF. 2011. *Aging Cell*. 10(4):629–39
93. Linford NJ, Beyer RP, Gollahon K, Krajcik RA, Malloy VL, et al. 2007. *Aging Cell*. 6(5):673–88
94. Demetriades C, Doumpas N, Teleman AA. 2014. *Cell*. 156(4):786–99
95. Kim J, Guan K-L. 2011. *Annu. Rev. Biochem.* 80(1):1001–32
96. Chantranupong L, Wolfson RL, Orozco JM, Saxton RA, Scaria SM, et al. 2014. *Cell Rep*. 9(1):1–8
97. Wolfson RL, Chantranupong L, Saxton RA, Shen K, Scaria SM, et al. 2015. *Science*
98. Wang S, Tsun Z-Y, Wolfson RL, Shen K, Wyant GA, et al. 2015. *Science*, pp. 188–94
99. Tsun Z-Y, Bar-Peled L, Chantranupong L, Zoncu R, Wang T, et al. 2013. *Molecular Cell*, pp. 495–505
100. Li L, Kim E, Yuan H, Inoki K, Goraksha-Hicks P, et al. 2010. *J. Biol. Chem.* 285(26):19705–9
101. Jewell JL, Kim YC, Russell RC, Yu F-X, Park HW, et al. 2015. *Science*. 347(6218):194–98
102. Lamming DW, Ye L, Katajisto P, Goncalves MD, Saitoh M, et al. 2012. *Science*. 335(6076):1638–43
103. Saha AK, Xu XJ, Balon TW, Brandon A, Kraegen EW, Ruderman NB. 2011. *Cell Cycle*. 10(20):3447–51
104. Lindgren O, Pacini G, Tura A, Holst JJ, Deacon CF, Ahrén B. 2015. *J. Clin. Endocrinol. Metab.* 100(3):1172–76
105. Saxton RA, Knockenhauer KE, Wolfson RL, Chantranupong L, Pacold ME, et al. 2015. *Science*
106. Norheim F, Gjelstad IMF, Hjorth M, Vinknes KJ, Langleite TM, et al. 2012. *Nutrients*. 4(12):1898–1944

107. Journel M, Chaumontet C, Darcel N, Fromentin G, Tomé D. 2012. *Advances in nutrition*, pp. 322–29
108. Kohl S, Behrens M, Dunkel A, Hofmann T, Meyerhof W. 2013. *J. Agric. Food Chem.* 61(1):53–60
109. Alfa RW, Park S, Skelly K-R, Poffenberger G, Jain N, et al. 2015. *Cell Metabolism*. 21(2):323–33
110. Jiang Y, Rose AJ, Sijmonsma TP, Bröer A, Pfenninger A, et al. 2015. *Mol Metab*. 4(5):406–17
111. Crespo CS, Cachero AP, Jiménez LP, Barrios V, Ferreiro EA. 2014. *Front Endocrinol (Lausanne)*. 5:58–58
112. Jordi J, Herzog B, Camargo SMR, Boyle CN, Lutz TA, Verrey F. 2013. *J. Physiol. (Lond.)*. 591(Pt 22):5611–21
113. McCormick MA, Tsai SY, Kennedy BK. 2010. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 366(1561):17–27
114. Edwards C, Canfield J, Copes N, Brito A, Rehan M, et al. 2015. *BMC Genet.* 16(1):8
115. Santos J, Leao C, Sousa MJ. 2012. *Oxid Med Cell Longev*. 2012:10
116. Sikalidis AK, Mazor KM, Kang M, Liu H, Stipanuk MH. 2013. *J Amino Acids*. 2013:864757
117. Cai Z, Zhou Y, Xiao M, Yan L-J, He W. 2015. *NDT*, pp. 1015–30
118. Kimball SR, Jefferson LS. 2006. *Am. J. Clin. Nutr.* 83(2):500S–507S
119. Bunpo P, Dudley A, Cundiff JK, Cavener DR, Wek RC, Anthony TG. 2009. *J. Biol. Chem.* 284(47):32742–49
120. Rousakis A, Vlassis A, Vlanti A, Patera S, Thireos G, Syntichaki P. 2013. *Aging Cell*, pp. 742–2751
121. Pal S, Ellis V. 2010. *BJN*, pp. 1241–48
122. Hoffman JR, Falvo MJ. 2004. *Journal of Sports Science and Medicine*. 3:118–30
123. van Milgen J. 2002. *The Journal of Nutrition*. 132(10):3195–3202
124. Chin RM, Fu X, Pai MY, Vergnes L, Hwang H, et al. 2014. *Nature*. 510(7505):397–401
125. D'Antona G, Ragni M, Cardile A, Tedesco L, Dossena M, et al. 2010. *Cell Metabolism*. 12(4):362–72
126. Poff AM, Ari C, Arnold P, Seyfried TN, D'Agostino DP. 2014. *Int. J. Cancer*. 135(7):1711–20
127. Gibson AA, Seimon RV, Lee CMY, Ayre J, Franklin J, et al. 2014. *Obesity Research & Clinical Practice*. 8(8):36
128. Pedroso JAB, Zampieri TT, Donato J. 2015. *Nutrients*. 7(5):3914–37
129. Komar B, Schwingshackl L, Hoffmann G. 2015. *J Nutr Health Aging*. 19(4):437–46
130. Jakubowicz D, Froy O. 2013. *Journal of Nutritional Biochemistry*. 24:1–5
131. McCarty MF, Barroso-Aranda J, Contreras F. 2009. *Medical Hypotheses*. 72(2):125–28
132. Melnik BC, John SM, Schmitz G. 2013. *Nutr J*. 12:103
133. Newgard CB. 2012. *Cell Metabolism*. 15(5):606–14
134. Poulain M, Herm A, Pes G. 2013. *Vienna Yearbook of Population Research - JSTOR*. 11:87–108
135. Trichopoulou A, Vasilopoulou E. 2000. *Br. J. Nutr.* 84 Suppl 2:S205–9

136. Buckland G, Agudo A, Travier N, Huerta JM, Cirera L, et al. 2011. *Br. J. Nutr.* 106(10):1581–91
137. Crowe FL, Key TJ, Allen NE, Appleby PN, Roddam A, et al. 2009. *Cancer Epidemiol Biomarkers Prev*. 18(5):1333–40
138. Kalu DN, Masoro EJ, Yu BP, Hardin RR, Hollis BW. 1988. *Endocrinology*. 122(5):1847–54
139. Zhang X, Beynen AC. 1993. *Br. J. Nutr.* 70(1):139–46
140. Bounous G, Gervais F, Amer V, Batist G, Gold P. 1989. *Clin Invest Med*. 12(6):343–49
141. Shertzer HG, Woods SE, Krishan M, Genter MB, Pearson KJ. 2011. *The Journal of Nutrition*. 141(4):582–87
142. Coker RH, Miller S, Schutzler S, Deutz N, Wolfe RR. 2012. *Nutr J*. 11:105
143. Noguchi T. 2000. *Br. J. Nutr.* 84 Suppl 2:S241–44
144. O'Neill B. 2010. *Bioscience Horizons*. 3(2):197–212
145. Geer BW. 1963. *J. Exp. Zool.* 154(3):353–64
146. Metaxakis A, Partridge L. 2013. *PLoS ONE*. 8(9):e74681
147. Liao C-Y, Rikke BA, Johnson TE, Diaz V, Nelson JF. 2010. *Aging Cell*. 9(1):92–95
148. Ram R, Mehta M, Balmer L, Gatti DM, Morahan G. 2014. *Genetics*. 198(1):75–86
149. Miller DS, Payne PR. 1968. *Experimental Gerontology*. 3:231–34
150. Gilani GS, Sepehr E. 2003. *The Journal of Nutrition*. 133(1):220–25
151. Sun L, Sadighi Akha AA, Miller RA, Harper JM. 2009. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*. 64A(7):711–22
152. Ozanne SE, Hales CN. 2004. *Nature*. 427(6973):411–12
153. John AM, Bell JM. 1976. *The Journal of Nutrition*. 106(9):1361–67
154. Grönke S, Clarke D-F, Broughton S, Andrews TD, Partridge L. 2010. *PLoS Genet*. 6(2):e1000857
155. Bass TM, Grandison RC, Wong R, Martinez P, Partridge L, Piper MDW. 2007. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*. 62(10):1071–81
156. Piper MD, Blanc E, Leitão-Goncalves R, Yang M, He X, et al. 2013. *Nat Meth*
157. FAO. 2013. *FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS*, p. 79
158. Van Etten CH, Hubba JE, Mallan JM, Smith AK, Blessin CW. 1959. *J. Agric. Food Chem.* 7(2):129–31
159. Zezulka AY, Galloway DH. 1976. *J. Nutr.* 106:212–21
160. Nielsen K, Kondrup J, Elsner P, Juul A, Jensen ES. 1994. *Br. J. Nutr.* 72(1):69–81
161. Acheson KJ, Blondel-Lubrano A, Oguey-Araymon S, Beaumont M, Emady-Azar S, et al. 2011. *Am. J. Clin. Nutr.* 93(3):525–34
162. Burnet B, Sang JH. 1968. *Genetics*. 59(2):211–35
163. Sury MD, Chen J-X, Selbach M. 2010. *Mol Cell Proteomics*. 9(10):2173–83
164. Wiśniewski JR, Rakus D. 2014. *J Proteomics*. 109:322–31
165. Wiśniewski JR, Ostasiewicz P, Duś K, Zielińska DF, Gnad F, Mann M. 2012. *Molecular Systems Biology*. 8:611

166. Geiger T, Velic A, Macek B, Lundberg E, Kampf C, et al. 2013. *Molecular & Cellular Proteomics*. 12(6):1709–22
167. Cox J, Mann M. 2008. *Nat. Biotechnol.* 26(12):1367–72
168. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. 2011. *J. Proteome Res.* 10(4):1794–1805
169. Wong R, Piper MDW, Wertheim B, Partridge L. 2009. *PLoS ONE*. 4(6):e6063
170. Cooper DM. 1960. *Evolution*. 14(1):41–55
171. Vargas MA, Luo N, Yamaguchi A, Kapahi P. 2010. *Curr. Biol.* 20(11):1006–11
172. Gomes AV, Waddell DS, Siu R, Stein M, Dewey S, et al. 2012. *FASEB J.* 26(7):2986–99
173. Spindler SR. 2012. *AGE*. 34(1):111–20
174. Warner HR, Ingram D, Miller RA, Nadon NL, Richardson AG. 2000. Program for testing biological interventions to promote healthy aging.
175. Haseman JK, Bourbina J, Eustis SL. 1994. *Fundam Appl Toxicol*. 23(1):44–52
176. Claassen V. 1994. *Neglected factors in pharmacology and neuroscience research*, Vol. Volume 12. Amsterdam and New York: Elsevier. 2 p.
177. Hedrich HJ, Bullock GR. 2004. *The Laboratory Mouse*. London: Elsevier. 1 p.
178. Aletor V, Hamid I, Nieß E, Pfeffer E. 2000. *Journal of the Science of Food and Agriculture*. 80:547–54
179. Orentreich N, Mataas JR, Defelice A, Zimmerman JA. 1993. *The Journal of Nutrition*, pp. 269–74
180. Harper AE, Kumta US. 1959. *Fed. Proc.*, pp. 1136–42
181. Percy DH, Barthold SW. 2007. *Pathology of Laboratory Rodents and Rabbits*. Iowa: Blackwell Publishing. 1 p. Third ed.
182. Keenan KP, Ballam GC, Soper KA, Laroque P, Coleman JB, Rakesh D. 1999. *Toxicological Sciences*. 52:24–34
183. Oka K, Sakuarae A, Fujise T, Yoshimatsu H, Sakata T, Nakata M. 2003. *J. Dent. Res.* 82(6):491–94
184. Beamer WG, Shultz KL, Donahue LR, Churchill GA, Sen S, et al. 2001. *Quantitative trait loci for femoral and lumbar vertebral bone mineral density in C57BL/6J and C3h/HeJ inbred strains of mice*, Vol. 20. Journal of Bone and Mineral Research. 120000 p.
185. Halloran BP, Ferguson VL, Simske SJ, Burghardt A, Venton LL, Majumdar S. 2002. *J Bone Miner Res*. 17(6):1044–50
186. Millward DJ. 2012. *BJN*. 108 Suppl 2:S3–S21
187. Young VR, el-Khoury AE. 1995. *Proc. Natl. Acad. Sci.* 92:300–304
188. Sofola-Adesakin O, Castillo-Quan J, Rallis C, Tain LS, Bjedov I, et al. 2014. *Frontiers in Aging Neuroscience*. 6(190):1–10
189. Pellett PL, Kaba H. 1972. *The Journal of Nutrition*. 102(1):61–68
190. Fuller MF, McWilliam R, Wang TC, Giles LR. 2007. *BJN*. 62(02):255–67
191. Dewey KG, G B, C F, B L, P R. 1996. *Eur J Clin Nutr.* 50(1):S119–47, S147–50
192. Reeds PJ. 2000. *The Journal of Nutrition*. 130(7):1835S–40S
193. Dabrowski K, Zhang Y, Arslan M, Terjesen BF. 2009. *Rev Col Cienc*

Pec. 20(4):508–11
194. Fuller MF, Garlick PJ. 1994. *Annu. Rev. Nutr.* 14:217–41
195. Pencharz PB, Ball RO. 2003. *Annu. Rev. Nutr.* 23:101–16
196. Fürst P, Stehle P. 2004. *The Journal of Nutrition*. 134:1558s–1565s
197. Rippon WP. 1959. *Br. J. Nutr.* 13:243–60
198. Takruri HR, Humeid MA, Umari MAH. 1990. *Journal of the Science of Food and Agriculture*. 50(3):319–27
199. Hunt V. 1970. *Drosophila Information Services*, p. 179
200. Koehnle TJ, Russell MC, Gietzen DW
201. Hao S, Sharp JW, Ross-Inta CM, McDaniel BJ, Anthony TG, et al. 2005. *Science*. 307(5716):1776–78
202. Rogers QR, Harper AE. 1965. *The Journal of Nutrition*. 87(3):267–73
203. Miller RA, Buehner G, Chang Y, Harper JM, Sigler R, Smith-Wheelock M. 2005. *Aging Cell*. 4(3):119–25
204. Yu BP. 1994. *Proc Soc Exp Biol Med*. 205:97–105
205. Wootton JC. 1994. *Current Opinion in Structural Biology*. 4(3):413–21
206. MacLeod EL, Ney DM. 2010. *Ann Nestlé [Engl]*. 68(2):58–69
207. Chintapalli VR, Wang J, Dow JAT. 2007. *Nat Genet*. 39(6):715–20
208. Breslin T, Edén P, Krogh M. 2004. *BMC Bioinformatics*. 5:193
209. Nikoh N, Hosokawa T, Moriyama M, Oshima K, Hattori M, Fukatsu T. 2014. *Proceedings of the National Academy of Sciences*. 111(28):10257–62
210. Su Q, Xie W, Wang S, Wu Q, Liu B, et al. 2014. *PLoS ONE*. 9(2):e89002
211. Chwalibog A, Sawosz E, Niemiec T, Grodzik M, Thorbek G. 2008. *Archives of Animal Nutrition*. 62(1):82–85
212. Sang JH, King RC. 1961. *J. Exp. Biol.* 38:793–809
213. Chou H-Y, Lin Y-H, Shiu G-L, Tang H-Y, Cheng M-L, et al. 2014. *J. Biomed. Sci.* 21(1):64
214. Ingolia NT, Lareau LF, Weissman JS. 2011. *Cell*. 147(4):789–802
215. Rodrigues MA, Martins NE, Balancé LF, Broom LN, Dias AJS, et al. 2015. *Journal of Insect Physiology*. 81:69–80
216. Taormina G, Mirisola MG. 2014. *Biomed Res Int*. 2014:308690
217. Lanet E, Maurange C. 2014. *Front Physiol*. 5:117
218. Vernochet C, Kahn CR. 2012. *Aging*. 4(12):859–60
219. Elia M. 1992. In *Protein-Energy Interactions*, eds NS Scrimshaw, B Schurch. Lausanne Switzerland: International Dietary Energy Consultancy Group (I/D/E/C/G). Proceedings of an I/D/E/C/G Workshop held in Waterville Valley, NH, USA ed.
220. Owen OE, Smalley KJ, D'Alessio DA, Mozzoli MA, Dawson EK. 1998. *Am. J. Clin. Nutr.* 68(1):12–34
221. Vigne P, Frelin C. 2007. *Mechanisms of Ageing and Development*. 128(5-6):401–6
222. Pant R. 1988. *Proc. Indian Acad. Sci.* 97(5):379–415
223. Magwere T, Goodall S, Skepper J, Mair W, Brand MD, Partridge L. 2006. *The Journal of Gerontology*. 61A(1):36–47
224. Zhang Y, Nicholatos J, Dreier JR, Ricoult SJH, Widenmaier SB, et al. 2014. *Nature*. 513(7518):440–43
225. Zahn JM, Poosala S, Owen AB, Ingram DK, Lustig A, et al. 2007. *PLoS Genet*. 3(11):e201

226. Walther DM, Kasturi P, Zheng M, Pinkert S, Vecchi G, et al. 2015. *Cell*. 161(4):919–32
227. Green EL. 1967. ***Biology of the Laboratory Mouse***. New York, : Blakiston Division, McGraw-Hill. 1 p. 2nd ed.
228. Drickamer LC. 1995. *J. Chem. Ecol.* 21(10):1481–93
229. Belmonte L, Coëffier M, Le Pessot F, Miralles-Barrachina O, Hiron M, et al. 2007. *World J. Gastroenterol.* 13(20):2833–40
230. Kueper J, Beyth S, Liebergall M, Kaplan L, Schroeder JE. 2015. *Int J Endocrinol*. 2015:628740
231. Kremen NA, Calvert CC, Larsen JA, Baldwin RA, Hahn TP, Fascetti AJ. 2013. *Journal of Animal Science*. 91(3):1270–76
232. Reeves PG, Nielsen FH, Fahey GC. 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet.
233. Deshpande PD, Harper AE, Elvehjem CA. 1958. *Journal of Biological Chemistry*. 230(1):327–33
234. Sklan D, Noy Y. 2005. *Poult. Sci.* 84(3):412–18
235. Rose WC. 1937. *Science*. 86(2231):298–300
236. Wang S, Tsun Z-Y, Wolfson RL, Shen K, Wyant GA, et al. 2015. *Science*. 347(6218):188–94
237. Huang X, Hancock DP, Gosby AK, McMahon AC, Solon SMC, et al. 2013. *Obesity*. 21(1):85–92
238. Leto S, Kokkonen GC, Barrows CH. 1976. *J Gerontol*. 31(2):149–54
239. Shamoto Nagai M, Maruyama W, Kato Y, Isobe KI, Tanaka M, et al. 2003. *Journal of Neuroscience Research*. 74(4):589–97
240. Rosalba Putti RSVMLL. 2015. *Front Physiol*. 6:
241. Ross J, Olson L, Coppotelli G. 2015. *International Journal of Molecular Sciences 2015, Vol. 16, Pages 22830-22855*. 16(8):19458–76
242. Ayala V, Naudí A, Sanz A, Caro P, Portero-Otín M, et al. 2007. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*. 62(4):352–60
243. Miwa S, Jow H, Baty K, Johnson A, Czapiewski R, et al. 2014. *Nat Commun*. 5:3837
244. Simpson SJ, Raubenheimer D. 2012. *Princeton University Press*
245. Hart RW, Neumann DA, Robertson RT. 1995. *Dietary restriction: implications for the design and interpretation of toxicity and carcinogenicity studies.* International Life Sciences Institute
246. Olewine DA, Barrows CH, Shock NW. 1964. *J Gerontol*. 19:230–33
247. Sørensen A, Mayntz D, Raubenheimer D, Simpson SJ. 2008. *Obesity*. 16(3):566–71
248. Guo F, Cavener DR. 2007. *Cell Metabolism*. 5(2):103–14
249. Rotella CM, Dicembrini I. 2015. *World J Methodol*. 5(1):1–9
250. Mahoney LB, Denny CA, Seyfried TN. 2006. *Lipids Health Dis*. 5:13
251. Lees EK, Król E, Grant L, Shearer K, Wyse C, et al. 2014. *Aging Cell*. 13(5):817–27
252. Hill RBJ, Prosper J, Hurschfield JS, Kern F Jr. 1968. *Experimental and molecular pathology*, pp. 66–74
253. Hirschfield JS, Kern F Jr. 1969. *J. Clin. Invest.* 48(7):1224–29
254. Magne H, Savary-Auzeloux I, Migné C, Peyron M-A, Combaret L, et al. 2013. *PLoS ONE*. 8(8):e70130

255. Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyva Y, et al. 2004. *Molecular Cell*. 14:395–403
256. Mehlem A, Hagberg CE, Muhl L, Eriksson U, Falkevall A. 2013. *Nat Protoc*. 8(6):1149–54
257. Fontana L, Partridge L, Longo VD. 2010. *Science*. 328(5976):321–26
258. Ni YG, Wang N, Cao DJ, Sachan N, Morris DJ, et al. 2007. *Proceedings of the National Academy of Sciences*. 104(51):20517–22
259. Ulusoy E, Eren B. 2006. *Histol. Histopathol.* 21(9):925–30
260. Selman C, Lingard S, Choudhury AI, Batterham RL, Claret M, et al. 2008. *FASEB J.* 22(3):807–18
261. Vajiheh Izadi SS-BLA. 2014. *ARYA Atherosclerosis*. 10(5):266
262. Otoda T, Takamura T, Misu H, Ota T, Murata S, et al. 2013. *Diabetes*. 62(3):811–24
263. Drake JC, Bruns DR, Peelor FF, Biela LM, Miller RA, et al. 2015. *Aging Cell*
264. Heaney RP, Layman DK. 2008. *Am. J. Clin. Nutr.* 87(5):1567S–1570S
265. Rokutanda S, Fujita T, Kanatani N, Yoshida CA, Komori H, et al. 2009. *Dev. Biol.* 328(1):78–93
266. Courtland H-W, Kennedy OD, Wu Y, Gao Y, Sun H, et al. 2013. *AGE*. 35(5):1691–1703
267. Hawkins J, Cifuentes M, Pleshko NL, Ambia-Sobhan H, Shapses SA. 2010. *The Journal of Nutrition*. 140(1):31–37
268. Brochmann Murray EJ, Beamer WG, Duarte ME, Behnam K, Grisanti MS, Murray SS. 2001. *Metabolism*. 50(4):436–42
269. Brochmann EJ, Duarte ME, Zaidi HA, Murray SS. 2003. *Metabolism*. 52(10):1265–73
270. Villareal DT, Fontana L, Weiss EP, Racette SB, Steger-May K, et al. 2006. *Arch. Intern. Med.* 166(22):2502–10
271. Gat-Yablonski G, Phillip M. 2015. *Nutrients*. 7(1):517–51
272. Almquist HJ. 1954. *Arch. Biochem. Biophys.* 50(2):503
273. Taylor S, Coburn SP, Townsend DW. 1996. *Mathematical Modeling in Experimental Nutrition: Vitamins, Proteins, Methods*, Vol. 40. Academic Press. 1 p. 1st ed.
274. Min K-J, Tatar M. 2006. *Mechanisms of Ageing and Development*. 127(7):643–46
275. Katewa SD, Demontis F, Kolipinski M, Hubbard A, Gill MS, et al. 2012. *Cell Metabolism*. 16(1):97–103
276. Kristensen TN, Overgaard J, Loeschcke V, Mayntz D. 2011. *Biology Letters*. 7(2):269–72
277. Pimentel D, Pimentel M. 2003. *Am. J. Clin. Nutr.* 78(3 Suppl):660S–663S
278. Wolfe RR. 2000. *American Journal of Clinical Nutrition*. 72(2):551s–557s

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22.02.16

Curriculum Vitae

PERSONAL INFORMATION

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PUBLICATIONS

ACCEPTED

1. Matthew D W Piper, Eric Blanc, Ricardo Leitão-Gonçalves, Mingyao Yang, Xiaoli He, Nancy J Linford, Matthew P Hoddinott, Corinna Hopfen, **George A Soultoukis**, Christine Niemeyer, Fiona Kerr, Scott D Pletcher, Carlos Ribeiro, and Linda Partridge. A holidic medium for Drosophila melanogaster. ***Nature Methods*** **11**, 100–105 (2014).

2. **George A. Soultoukis** and Linda Partridge. Protein, Metabolism and Ageing. ***Annual Reviews of Biochemistry*** **85**, xxx-xxx, (2016), (accepted).

IN PREPARATION

1. **George A Soultoukis***, Matthew D Piper*, Eric Blanc, Ilian Athanassov, Paula Juricic, Hanna Salmonowicz, Matthew Hoddinott, Carlos Ribeiro, Andrea Mesaros, Stephen J Simpson, and Linda Partridge. Exome matching of essential amino acids in the diet increases anabolic efficiency without limiting lifespan. ***eLife***, (submitted)

2. **George A. Soultoukis**, Andrea Mesaros, Hanna Salmonowicz, Stanka Matic, Chirag Jain, Joana Goncalves, Youssef Hassan, Alain de Bruin, and Linda Partridge. Disparate metabolic effects of promoting anabolism through dietary exome matching in vertebrates and invertebrates. (in preparation)

*authors contributed equally

RESEARCH EXPERIENCE

Max Planck Institute for Biology of Ageing – (01/02/2012 – till present)

- Graduate studies: Doctoral student placement at Prof. Linda Partridge Department Biological Mechanisms of Ageing.

- Project Title: Elucidating mechanisms of mouse and fly lifespan regulation through nutrients in the diet.
- Special techniques: Mouse and fly dissections, Insulin (ITT) and glucose tolerance (GTT) assays, histopathology, statistical and bioinformatics analyses (including data analysis and visualisation on R), nitrogen balance assays, body composition analysis by TD-NMR, blood and hemolymph extraction, defined media preparation, *Drosophila melanogaster in vivo* assays for nutrient intake (PEB assay, EDS blue dye assay), fluorescent microscopy, foraging behaviour assays (DPI), uric acid and urea assay, enzyme-linked immunosorbent assay (ELISA), triacylglyceride (TAG) assay.

Max Planck Institute for Biology of Ageing – (13/06/2011 – 31/01/2012)

- Scientific research assistant: Pre-doctoral student placement in the Department Biological Mechanisms of Ageing.
- Project Title: Foxo dependence of lifespan extension in a dominant negative insulin receptor ($dInR^{DN}$) mutant fly.
- Special techniques: rtPCR genotyping, semi-defined media preparation, genetic crossing strategies, microscopy, *Drosophila melanogaster in vivo* assays for xenobiotic stress resistance, starvation resistance, fecundity, development, and lifespan.

University College London - (04/10/2010 – 25/03/2011)

- 3rd Year Undergraduate Research Project at the Institute of Neurology, National Hospital of Neurology and Neurosurgery.
- Project Title: 'Biochemical consequences of methylmalonic acid on human neuroblastoma SH-SY5Y cells: inhibition of mitochondrial electron transport chain complex I and other effects on mitochondrial metabolism'.
- Special techniques: cell culture and microplating (human neuroblastoma SH-SY5Y cells), cell (LDH) and mitochondrial (GSH) toxicity assays, fluorimetry, spectrophotometry, WB, light microscopy.

Sanofi-Aventis, Alnwick Research Centre - (03/08/2009 – 17/09/2010)

- Extramural Industrial Placement at the Metabolism Group of the Global Metabolism and Pharmacokinetics Department, Alnwick Research Centre.
- Main Project Title: 'A metabonomics preclinical study on rats with CNS toxicity caused by 5-ethoxymethyl pyridine-2-amine, an impurity in a drug substance'.
- Special techniques: metabolomics, sample preparation (oxidation, drying, reconstitution, etc.), RP/HPLC-MS, MS data analysis (PCA, PLS-DA, MVDA, UVDA), R programming, data mining, radiolabelled sample scintillation analysis (LSC, MSC).

University College London - (01/06/2009 – 31/07/2009)

- BBSRC Vacation Scholarship at the Institute of Structural and Molecular Biology, UCL.
- Project Title: 'Structural investigation of the synthesis of alpha-synuclein (αSyn) on the ribosome by NMR Spectroscopy'.

- Special techniques: plasmid purification, gene isolation (endonuclease cleavage and gel electrophoresis) and amplification (PCR), DNA quantification, gene insertion in vector, cell transformation and culture (*E. coli* DH5a cells), NMR spectroscopy.

EDUCATION

Doctoral studies: University of Cologne, Graduate School for Biological Sciences

- PhD in Genetics (Start date: Feb 2012, Award date: Jul 2016)
- Department Biological Mechanisms of Ageing, Max Planck Institute for Biology of Ageing, Cologne, Germany.
- Supervisor: Prof. Linda Partridge, PhD Thesis Title: Elucidating mechanisms of mouse and fly lifespan regulation through nutrients in the diet

Bachelor studies: University College London

- BSc, Molecular Biology (Start date: Sep 2007, Award date: Aug 2011)
- Recommended Degree Class: Upper Second Class Hons.

College education: Kingston University

- Science Degree Foundation Course (Sep 2006 - June 2007)
- Kingston College, Kingston University, London, UK.
- *Result: 79% (Passed with distinction)*, Modules & Marks: Biology 78%, Chemistry 81%, Mathematics 78%, Physics 93%, Information & Communication Technology 86%.

FUNDING & AWARDS

- **2010** - Winner of the Sanofi-Aventis Alnwick Research Center Student 'Poster of the Year' Award. Poster title: 'A metabonomics preclinical study on rats with CNS toxicity caused by 5-ethoxymethyl pyridine-2-amine'.

- **2009** - €3,000 - BBSRC Vacation Scholarship at the Institute of Structural and Molecular Biology, University College London. Project Title: 'Structural investigation of the synthesis of alpha-synuclein (αSyn) on the ribosome by NMR Spectroscopy'.

- **2007** - Graduated with Distinction from the Kingston University Science Degree Foundation Course.

STUDENTS SUPERVISED

- **2014 - 2015** - Hanna Salmonowicz (26/01-13/07/2015 – Master Thesis*)
  (21/04-30/06/2014 & 01/10-23/01/2015 – Undergraduate*)

- **2014 - 2015** - Nina Grisard (01/07/2014-31/03/2015 – Undergraduate*)

*University of Cologne

ONLINE CONTRIBUTIONS

Interview for news release: 'A standardized chemical diet for flies allows to reduce conflicting results across labs and might thus speed up science' http://www.age.mpg.de/news-events/news/singleview-news/?no_cache=1&tx_ttnews%5Btt_news%5D=132&cHash=c8dd85530ce2f62d79f150a434563b27

RELEVANT QUALIFICATIONS & TRAINING

- Felasa B Course (Theoretical and practical mouse handling course with qualification awarded) - 06/2012 (5 days) - Berlin, Germany
- FRAME: Experimental design and statistical analysis of biomedical experiments (Workshop on minimizing animal use in basic research experiments) - 07/12 (3 days) - University of Nijmegen, Netherlands
- Mouse pathology workshop (Theoretical and practical training) - 05/2013 (1 day) - Utrecht University, Dutch Molecular Pathology Center, Netherlands
- National and European research funding for young researchers (MPI Age – CECAD Information Event) – 10/2013 (1 day) - MPI Age, Robert Koch Strasse, Cologne Germany
- Data visualization workshop (Science Craft training seminar) - 11/2014 (3 days) - University of Cologne, Germany
- Statistical literacy workshop (Science Craft theoretical and practical workshop) - 03/2015 (3 days) - University of Cologne, Germany
- Effective writing for biomedical professionals (Oxford University scientific writing workshop) – 11/2015 (2 days) – Oxford, UK

PRESENTATIONS & POSTERS

- **George Soultoukis**, Matthew Piper, and Linda Partridge (2012) 4th International Symposium Crossroads in Biology, Cologne, Germany, 9-10/02/2012. Title: 'Elucidating dietary effects on *Drosophila* physiology using a holidic medium'. Poster.
- **George Soultoukis**, Matthew Piper, and Linda Partridge (2012) Eurosymposium on Healthy Ageing, Brussels, Belgium, 12-14/12/2012. Title: 'Elucidating the effects of dietary amino acids on *Drosophila* life history traits using a holidic medium'. Poster.

REFEREES

Name: Prof. Linda Partridge
Connection: PhD supervisor

Address: Department Biological Mechanisms of Ageing, Max Planck Institute for Biology of Ageing, Joseph-Stelzmann-Str. 9b, D-50931, Cologne, Germany
Contact No.: +49 221 37970 600
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Name: Prof. Aleksandra Trifunovic,
Connection: PhD thesis committee member
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Name: Dr Lisa Cabrita,
Connection: Lecturer, Tutor, and Vacation Scholarship Supervisor
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