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The Impact of GCN2 on Lifespan and Proteomic Changes in *Drosophila* Under Phenylalanine Deprivation

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Abstract

Diet plays a crucial role in aging and longevity, influencing both health and lifespan through evolutionary conserved biological mechanisms. Moderate dietary restriction (DR) without malnutrition has long been recognized for its potential to extend lifespan and delay the onset of age-related diseases. Recent research has highlighted that amino acid (AA) availability is now known to be central in mediating the effects of DR, with specific AA sensing pathways mediating their effects on metabolism, stress resistance, and cellular maintenance. One key player in this process is the General Control Nonderepressible 2 (GCN2) protein, which is sensitive to AA deprivation and stress. Especially when essential AAs are scarce, GCN2 phosphorylates the translation initiation factor eIF2α, a crucial step that reduces global protein synthesis but selectively enhances the production of stress response proteins that protect against AA insufficiency. The aim of this project was to elucidate the role of GCN2 and eIF2α in maintaining lifespan of *Drosophila melanogaster* under phenylalanine (Phe) restriction. Lifespan experiments revealed that flies lacking GCN2 or eIF2α exhibit significantly shorter lifespans than wild type controls under severe Phe restriction, highlighting the critical roles of both proteins in maintaining lifespan in this stress conditions. Because of their central role in regulating protein synthesis, proteomic data were analysed, focusing on differential protein expression between wild-type and *GCN2*-knockout flies under Phe deprivation. This revealed significant enrichment in processes related to AA metabolism, cellular stress response, and protein folding, reflecting adaptive changes to nutrient scarcity. Specifically, we identified 33 upregulated and 56 downregulated proteins that required GCN2 for differential expression in response to Phe deprivation, pointing to a coordinated response aimed at conserving energy and enhancing stress resilience. Notably, the upregulation of Larval Serum Protein 2 (Lsp2) in wild-type flies, but not *GCN2* nulls, suggests it has role as an essential reservoir for Phe, crucial for sustaining functions during adult Phe deprivation. This research supports a model where GCN2 mediates adaptive strategies to preserve lifespan by activating autophagy and utilizing stored AAs, thus elucidating new mechanisms for GCN2 and eIF2α in managing nutrient stress.

1 Introduction

The necessity of obtaining adequate nutritional resources both in terms of quantity and quality is an intrinsic condition of every living organism, yet what is an "optimal diet" that maximizes health and aging remains an open question. As humans, we are living in a paradoxical situation where excessive food consumption, insufficient intake of food, and overall imbalanced diets all coexist in the population, contributing to the insurgence of diseases that limit optimal health and aging (Townsend et al., 2023). Simultaneously, we are gaining more knowledge about the role of food as a crucial determinant of health and longevity (Rattan & Kaur, 2022). Research has shown the significant impact that essential micronutrients and macronutrients exert on biological processes, spanning from cellular functions to the performance of entire organisms (Warne, 2014). We have observed how specific foods, nutrients, and dietary patterns can significantly impact disease risk and mortality in humans, as well as influence the lifespan of model organisms (Ekmekcioglu, 2020).

Accordingly, it has been argued that adjusting the amount, variety, and timing of food intake represents the most effective, practical, and safest method to prolong lifespan (Longo & Anderson, 2022). In addition, it is also an effective approach to extend the period of maintained health and functional abilities (i.e., healthspan), an effect that is observable across a range of different species (Longo & Anderson, 2022). Nevertheless, there is still considerable debate surrounding the specific types, amounts, and combinations of nutrients that promote optimal healthy aging and increase lifespan.

With the primary goal of understanding the fundamental biological mechanisms underlying nutrients availability and aging, a vast branch of research is focusing on dietary macronutrient (i.e., carbohydrates, proteins, and fats) modifications and their effects on health and lifespan of model organisms (Heymsfield & Shapses, 2024; Solon-Biet et al., 2015). Interestingly, recent studies have shown that the availability and quality of these macronutrients is crucial in mediating aging-related processes (Liu et al., 2021). Protein availability in particular has been established as a major determinant of an organism's health and aging. Proteins are essential biomolecules for all living organisms, serving roles as enzymes, transporters, sensors, and structural components of cells and they constitute the main dietary source of amino acids (AAs). The relative proportion of protein to carbohydrates in the diet has shown

strong effects on lifespan and fecundity across various species. Research indicates that the majority of these effects can be attributed to dietary proteins and the constituent AAs. For instance, studies on flies have shown that lifespan is maximized on low-protein, high-carbohydrate diets, while fecundity requires higher protein intake (Carey et al., 2022; Ng'oma et al., 2019).

The synthesis of new proteins from AAs is a complex and energetically demanding process, thus accurate sensing of AA levels is crucial for cells to maintain protein synthesis, catabolism, and energy balance (Lushchak et al., 2019). Studying AA signalling, especially driven by the availability of specific AAs, can unveil some of the fundamental biological mechanisms which underly the connection between diet and aging. Therefore, the focus of this thesis is on specific AA availability and its effect on fundamental molecular pathways that are believed to impact lifespan and healthspan of living organisms.

1.1 Why do we age and how does it happen?

Most aging theories agree that aging is the result of an accumulation of molecular damage at the cellular level, which leads to impaired tissue regeneration, chronic age-related diseases, and overall organismal disfunction (Aunan et al., 2016; Di Micco et al., 2021). However, understanding why aging occurs and why different aging rates are observed in different species is still an unresolved question.

Classical evolutionary theories suggest that extrinsic mortality factors, such as predation, disease, and environmental challenges, are key drivers of how quickly an organism ages (Johnson et al., 2019). In the wild, where most animals do not survive to old age due to these harsh conditions, there is minimal evolutionary pressure to develop genetic traits that ultimately slow aging or extend lifespan beyond the point at which extrinsic mortality curtails life. Consequently, evolutionary pressures would favour genes that enhance relatively early survival, growth, and reproduction (Johnson et al., 2019; Kirkwood & Shanley, 2005). Moreover, due to the decreased strength of natural selection for self-maintenance later in life, a "selection shadow" towards ageing-related diseases would be present (Figure 1A), allowing physiological decline to manifest at older age without being selected against (Fabian & Flatt, 2011).

This concept is particularly central to Medawar's "mutation accumulation" theory (Medawar, 1952), which suggests that life-threatening harmful mutations manifesting later in life (beyond the peak reproductive period) can accumulate with little resistance. This accumulation occurs because natural selection prioritizes early-life reproductive success over late-life maintenance, leading to a trade-off where resources are directed towards reproduction at the expense of repair mechanisms, thereby allowing deleterious mutations to arise and persist (Figure 1B). Building on this, Williams' "antagonistic pleiotropy" theory (Williams, 1957) posits that alleles that cause aging can be selected for if they are both beneficial for early life, but also detrimental late in life (Figure 1C). In this sense, antagonistic pleiotropy describes a situation in which alleles control multiple phenotypic traits, some of which are beneficial for fitness and reproduction but detrimental later on. Therefore, the decline in the force of natural selection with age allows these detrimental effects to persist, and even be selected for, because the beneficial effects early in life outweigh the negative effects (aging) later on (Williams, 1957).

The Disposable Soma Theory of aging proposed by Kirkwood (1977) adds a mechanism to these theories which involve resource allocation. It argues that aging occurs because of the presence of trade-offs between reproduction and survival, and selection works to maximise fitness by tuning resource reallocation between traits. One of the classic trade-offs is between reproduction and somatic maintenance, which in turn affects lifespan. Therefore organisms would have evolved the ability to differentially prioritize the allocation of resources to reproduction or maintenance and repair, which can lead to an accumulation of damage and a decline in function with age (Figure 1D) (Kirkwood, 1977). This theory offers a comprehensive framework for understanding how the aging process can be explained as an inevitable consequence of the evolved anatomy and physiology of the body under the constraint of limited environmental resources.

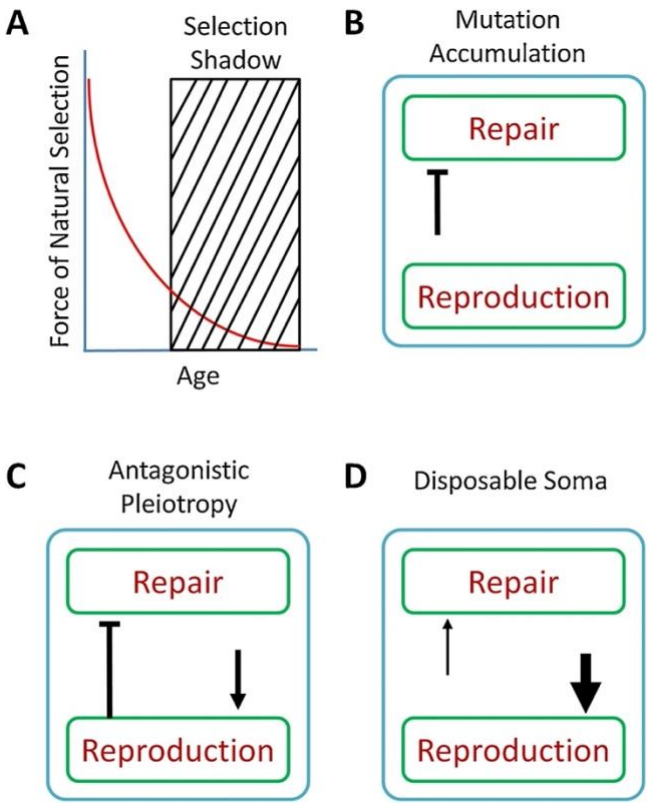

Figure 1. Classical theories on the evolution of aging. A) Medawar's concept of the "selection shadow," which illustrates how harmful mutations face reduced selective pressure after at older age. B) The mutation accumulation theory, which highlights the absence of selection against mutations that cause damage later in life. C) The antagonistic pleiotropy theory, which proposes that mutations that enhance reproduction but impair repair are favoured, leading to a direct trade-off. D) The disposable soma theory, which proposes the trade-off between repair and reproduction, driven by competition for limited resources. Source: Johnson et al. (2019).

Both the Antagonistic pleiotropy and Disposable Soma Theory predict an obligatory trade-off between aging and reproduction (Flatt & Partridge, 2018), and all three theories suggest that higher extrinsic mortality should drive the evolution of shorter lifespans, because it is not evolutionary priority to invest in body maintenance for more than the typical survival period of the organism. However, we know that is not always the case. For instance, some species that live with high extrinsic mortality, such as certain guppies and nematodes, have evolved longer lifespans (Johnson et al., 2019). Additionally, theoretically immortal animals like planarians and hydra challenge these classical theories (Elliott & Alvarado, 2018). This suggests that evolutionary response to extrinsic mortality is influenced by additional factors, which may include food availability, population density, reproductive costs, and the specific sources of mortality (Johnson et al., 2019).

Recently, Lemaître et al. (2024) have combine all the classical models to proposed a new hierarchical model linking genes to “vital rates”, i.e. growth and death rates or processes that promote health. This new framework emphasizes the role of genes in determining vital rates, suggesting that genetic pathways and higher-level homeostatic mechanisms are crucial determinants of longevity. While recognising that the allocation of resources between growth, reproduction, and maintenance is a key factor in determining lifespan, the new model recognises that the degree to which they do so is a product of the specific genetic and environmental interactions that shape these processes, thus allowing for apparent exceptions to the rules invoked by the Disposable Soma Theory (Fedarko, 2018; Lemaître et al., 2024). By doing this, it also provides a conceptual foundation to link through those studying aging from a biomedical perspective, which focuses on twelve biological hallmarks of aging proposed by López-Otín et al. (2023). These hallmarks include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, disabled macroautophagy, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis(López-Otín et al., 2023). Each hallmark fulfils three criteria: it manifests during aging, its experimental accentuation accelerates aging, and therapeutic interventions to block their manifestation can decelerate, halt, or reverse aging(López-Otín et al., 2023). Table 1 summarizes the twelve hallmarks and includes a brief description of how each are thought to contribute to aging (Aunan et al., 2016; Guerville et al., 2020; López-Otín et al., 2013; López-Otín et al., 2023). The integration of these hallmarks provides a more biomedical and mechanistic understanding of the aging process, which is useful in the context of studying aging-related phenotypes. Interestingly, many of the underlying biological mechanisms determining an organism’s lifespan appear to be evolutionary conserved across species, thus understanding the mechanisms that cause these dysfunctions can guide research into interventions aimed at extending human healthspan and lifespan by targeting the genetic and molecular pathways involved in aging (Lemaître et al., 2024).

Table 1. List of twelve hallmarks of aging identified by López-Otín et al. (2023) with a brief description. Source: López-Otín et al. (2023).

Hallmark of Aging	Description
Genomic Instability	Accumulation of DNA damage over time, leading to mutations and chromosomal aberrations.
Telomere Attrition	Progressive shortening of telomeres, the protective caps at the ends of chromosomes, triggering cellular senescence or apoptosis.
Epigenetic Alterations	Changes in DNA methylation, histone modification, and chromatin remodelling that affect gene expression without altering the DNA sequence.
Loss of Proteostasis	Decline in the cellular machinery responsible for protein folding, maintenance, and degradation, leading to the accumulation of misfolded or damaged proteins.
Disabled Macroautophagy	Impairment in the process of degrading and recycling cellular components, leading to the accumulation of cellular debris.
Deregulated Nutrient-Sensing	Disruption in pathways like insulin/IGF-1 signalling, mTOR, AMPK, and sirtuins, which modulate metabolism and cellular growth, playing pivotal roles in aging.
Mitochondrial Dysfunction	Impairment in mitochondrial biogenesis and function, resulting in reduced energy production and increased oxidative stress.
Cellular Senescence	Irreversible arrest of cell division, accompanied by the secretion of pro-inflammatory cytokines, growth factors, and proteases, known as the senescence-associated secretory phenotype (SASP).
Stem Cell Exhaustion	Diminished regenerative capacity of stem cells, leading to tissue degeneration and impaired repair mechanisms.
Altered Intercellular Communication	Disruption in endocrine, neuroendocrine, and neuronal signaling, affecting tissue homeostasis and function.
Chronic Inflammation ("Inflammaging")	Persistent, low-grade inflammation that exacerbates tissue damage and dysfunction.
Dysbiosis	Imbalance in the composition and function of the gut microbiota, linked to age-related diseases and systemic inflammation.

1.2 The connection between diet and aging

As already mentioned, there is strong evidence from evolutionary theory that aging results from a nutrient based trade-off between somatic maintenance and reproduction, suggesting that organisms may prioritize reproductive success over the maintenance of somatic cells, which leads to the accumulation of cellular damage over time (Kirkwood, 1977; Malavolta & Mocchegiani, 2016). According to this theory, as nutritional resources were limited throughout evolution, living organisms have fine-tuned allocation of these resources to strategically distribute them to different biochemical processes, modifying phenotypes to optimize fitness (Figure 2)(Kirkwood & Shanley, 2005; Piper et al., 2023).

As a proposed consequence, when resources are available in relatively high amounts, organisms tend to allocate them to reproduction, increasing the number and viability of their offspring. When resources are scarce, they are allocated towards somatic maintenance at the expense of reproduction (Malavolta & Mocchegiani, 2016). This strategy of resource reallocation ensures that the body is maintained in the most optimal manner based on the individual's circumstances, ensuring optimal timing and investment in reproduction(Maklakov & Chapman, 2019; Rodrigues & Flatt, 2016).

As a consequence, it becomes crucial to explore what exactly constitutes somatic maintenance. Current literature refers to it as a series of cellular processes involved in energy metabolism, gene expression, protein turnover, immune function, and oxidative stress responses, all of which are pivotal to preserve the body(Boye & Grallert, 2020; Longo & Anderson, 2022; Masoro, 2003). One key solution to solving the paradox of supplying somatic maintenance with resources at a time when nutrients are scarce is the acceleration of recycling processes that target old and/or damaged cellular components for degradation. This process is thought to explain how restricting nutrient intake slows aging by both reducing damage as well as fuelling the organism’s capacity for repair (López-Otín et al., 2013). Autophagy is a highly conserved process where cells degrade and recycle their own intracellular components through lysosomes, to liberate raw materials for anabolism(Gómez-Virgilio et al., 2022). This process helps remove damaged organelles, misfolded proteins, and other cellular debris, thus preventing the accumulation of cellular damage that contributes to aging(Wong et al., 2020). Interestingly, enhanced autophagy has been shown to delay aging

and extend lifespan in various organisms by improving cellular function and stress resistance(Li et al., 2024).

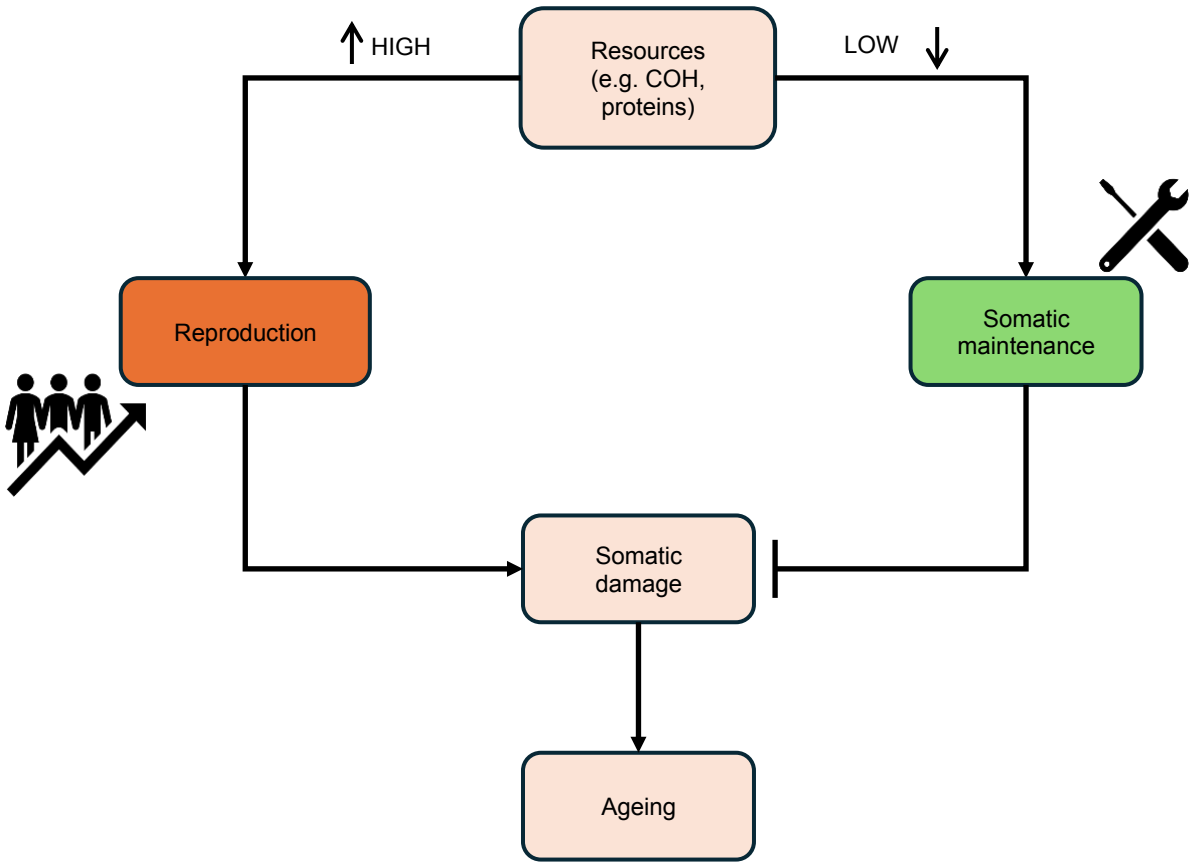

Figure 2. Schematic representation of resource reallocation according to the Disposable Soma Theory. When nutritional resources are low, their allocation towards somatic maintenance is prioritised, at the expense of reproduction. This results in an acceleration of recycling and repair processes that reduce cellular damage and slow aging. When resources are high, they are directed towards reproduction, to increase the number and viability of offspring.

1.3 Dietary restriction and longevity

Dietary restriction (DR) is a moderate restriction of food intake without malnutrition that extends healthy lifespan. The beneficial effects of DR were initially observed in 1917 by Osborne(Osborne et al., 1917) and is recognised as one of the most effective non-genetic interventions for maximizing both lifespan and healthspan in many different organisms, spanning from yeasts to flies, rodents and humans(Boye & Grallert, 2020; Fontana et al., 2010; Green et al., 2022; Masoro, 2003).

Today, it is generally recognized that a 30-40% reduction in total food intake, measured in caloric value, compared to control groups with unrestricted access to food, can extend lifespan (Balasubramanian et al., 2017; Mattison et al., 2012). However, the mechanisms through which DR enhances health and longevity were almost entirely obscure until the 1980s, when the discovery of the first single-gene mutations that prolong lifespan in nematode worms were found to target essential nutrient-sensing pathways (Green et al., 2022; Klass, 1977). Interestingly, one critical pathway influenced by DR is autophagy, which is upregulated under DR, thereby helping to maintain cellular health and function by removing dysfunctional mitochondria and other damaged organelles. This autophagic activity not only clears harmful cellular debris but also ensures the recycling of nutrients required for anabolism, which is especially beneficial during periods of low nutrient availability (Madeo et al., 2015).

DR has also been shown to enhance mitochondrial function by promoting mitochondrial biogenesis and improving the efficiency of existing mitochondria, thus reducing oxidative stress. This is crucial because excessive reactive oxygen species (ROS) can damage cellular components, leading to aging and age-related diseases (Giorgi et al., 2018). Furthermore, DR reduces inflammation, a major contributor to aging and many chronic diseases. Chronic low-grade inflammation is linked to the decline in immune function and the progression of diseases such as cancer, cardiovascular disease, and neurodegeneration. DR has been shown to downregulate pro-inflammatory cytokines and signalling pathways which play a pivotal role in the inflammatory response (Fontana et al., 2018).

Another critical benefit of DR is the enhancement of DNA repair mechanisms. As cells age, DNA damage accumulates due to factors such as oxidative stress and replication errors. DR has been linked to the activation of DNA repair pathways, including those involved in the repair of double-stranded breaks and oxidative DNA damage. By improving the efficiency of these repair mechanisms, DR helps maintain genomic stability, thereby reducing the incidence of mutations that could lead to cancer and other age-related diseases (Gredilla & Barja, 2005).

Collectively, these processes—enhanced autophagy, improved mitochondrial function, reduced inflammation, and more efficient DNA repair— are thought to contribute to the increased lifespan and healthspan observed in organisms subjected to DR (Grabski, 2020). However, although extensive research has been done on the biological mechanisms by which

DR affects aging and lifespan, there are still many unknown mechanistic connections between diet and aging given their intricate and multifaced nature. Also, DR is a broad term, encompassing the reduction of total nutrients and/or of specific dietary macromolecules, therefore its effects can vary enormously depending on the restricted nutrients and/or the specific genetic response employed by different organisms (Green et al., 2022; McCracken et al., 2020; Wilson et al., 2020).

1.4 Amino acid restriction and longevity

Research has shown that the beneficial effects of DR can be replicated by restricting protein intake instead of reducing the overall caloric intake (Ferraz-Bannitz et al., 2022; Pamplona & Barja, 2006). Therefore, numerous studies have investigated the beneficial effects of altered protein signalling, which is triggered by the availability of specific amino acids (AAs), as a mediator of aging (Dato et al., 2019; Liu et al., 2021; Soultoukis & Partridge, 2016).

Interestingly, it has been shown that restricting especially AAs that cannot be directly synthetized by an organism but must be necessarily obtained from the diet (i.e. essential AAs) provoke significant effects on aging and lifespan (Grandison et al., 2009; Jonsson et al., 2022). Non-essential amino acid (NEAA) restriction, though less studied, also shows potential benefits (Kosakamoto et al., 2022).

In a recent study using *Drosophila Melanogaster*, Fulton et al. (2024) were able to replicate the lifespan and stress resistance benefits of DR without significantly reducing food intake, but instead temporarily depriving flies of the EAA isoleucine. Similarly, research has shown that long or short-term restriction of methionine can significantly extend lifespan in fruit flies, especially when total AA levels are reduced to 40% of the control diet (Kosakamoto et al., 2023). This extension of lifespan is attributed to the reduction of oxidative damage through the induction of longevity-related genes, such as Methionine sulfoxide reductase A (MsrA), which reduces oxidatively damaged methionine (Kosakamoto et al., 2023). Similar effects have been observed in rodent models, where EAA restriction extends both lifespan and healthspan, reducing the incidence of age-related diseases such as cancer and diabetes(Kitada et al., 2019). These effects are seen upon methionine restriction (Miller 2005 REF) and for restriction of isoleucine and the other branched-chain amino acids (BCAAs). For instance, reducing isoleucine intake in mice by two-thirds improved lifespan, reduced frailty,

and decreased the incidence of age-related diseases such as cancer(Hill & Kaeberlein, 2021) and lifelong restriction of the BCAAs leucine, isoleucine, and valine has been shown to extend lifespan in male mice(Richardson et al., 2021). Together, these data highlight the powerful benefits of single EAA restriction.

It is worth noting that while mild restriction of an EAA can be beneficial, a complete deprivation of any one of the EAAs should cause a reduction in lifespan. However, recent work in *Drosophila* has shown that organisms survive for varying amounts of time depending on the identity of the EAA being restricted, which must result from a combination of AA sensing and the organism's ability to initiate strategies to protect itself (Dick et al., 2011; Jin et al., 2020; Johnstone et al., 2023). Quite strikingly, the exclusion of Phe from flies' diet was unique in that it does not significantly affect lifespan when compared to a diet that includes all AAs (Johnstone et al., 2023). Thus, Phe deprivation may be a useful tool to uncover the somatic maintenance events that are required for organismal survival during AA restriction. Exactly how stress-response signals are generated and what the protective strategies are, has not been fully elucidated.

1.4.1 Amino acid sensing mechanisms

It is widely recognized that many fundamental aging pathways are influenced by nutrient levels and composition in an evolutionary-conserved manner (Longo & Anderson, 2022). Amino acid sensing is a fundamental biological process that is highly conserved across species, playing a crucial role in maintaining cellular and organismal homeostasis.

Eukaryotes can sense and adapt to the presence and scarcity of AAs through two major widely conserved sensing and signalling pathways, primarily involving the mechanistic target of rapamycin complex 1 (mTORC1) and the general control nonderepressible 2 (GCN2) kinase (Jonsson et al., 2022) (Figure 3). These pathways are essential for matching protein synthesis, cell growth, and metabolic processes to the availability of the AAs, which are required for their synthesis and progression.

The kinase mTORC1 senses the presence of AAs and functions as a controller of cellular growth (Albert & Hall, 2015). When mTORC1 is activated by the presence of AAs, it phosphorylates substrates that enhance anabolic processes, such as the translational suppressor 4EBP1, whose inhibition (by phosphorylation) stimulates translation initiation

factor eIF4E to initiate protein synthesis (Battu et al., 2017), while catabolic processes (e.g., autophagy) are suppressed (Wolfson & Sabatini, 2017). The TOR pathway integrates signals from AAs, energy status, and growth factors to ensure that cells grow and divide only when sufficient nutrients are available (Liu et al., 2019). The activation of mTORC1 by AAs involves several upstream sensors and regulators, including the Rag GTPases, which facilitate the translocation of mTORC1 to the lysosomal surface where it becomes activated (Hu & Guo, 2020).

Conversely, GCN2 is activated under conditions of AA deprivation. This sensor is especially activated by uncharged transfer RNAs (tRNAs) or when ribosomes stall, which reflects the scarcity of AAs (Gallinetti et al., 2013; Matějů & Chao, 2022). GCN2 activation leads to the phosphorylation of the eukaryotic initiation factor 2α (eIF2α) which drives the reduction of global protein synthesis and selectively enhances the production of stress resistance proteins (Lidsky et al., 2023; Pakos-Zebrucka et al., 2016). A recent study on *Drosophila* showed that GCN2 is required for maintaining flies alive when fed diets without the EAA phenylalanine (Phe) and this could be partially phenocopied by manipulating autophagy (Johnstone et al., 2023). The authors suggest that GCN2 plays a pivotal role by triggering autophagy to utilize stored AAs to enhance survival. This adaptive response helps cells to conserve resources and survive periods of nutrient scarcity.

The conservation of both TOR and GCN2 across different organisms underscores their evolutionary importance. For instance, studies in model organisms such as yeast, flies, and mice have demonstrated that the mTORC1 and GCN2 pathways are critical for regulating lifespan and healthspan. Inhibition of mTORC1, either through genetic manipulation or pharmacological intervention, has been shown to extend lifespan and delay the onset of age-related diseases in various species(Battu et al., 2017). Similarly, activation of GCN2 has been linked to increased stress resistance and longevity(Fulton et al., 2024). Both of these interventions phenocopy the beneficial effects of dietary AA restriction.

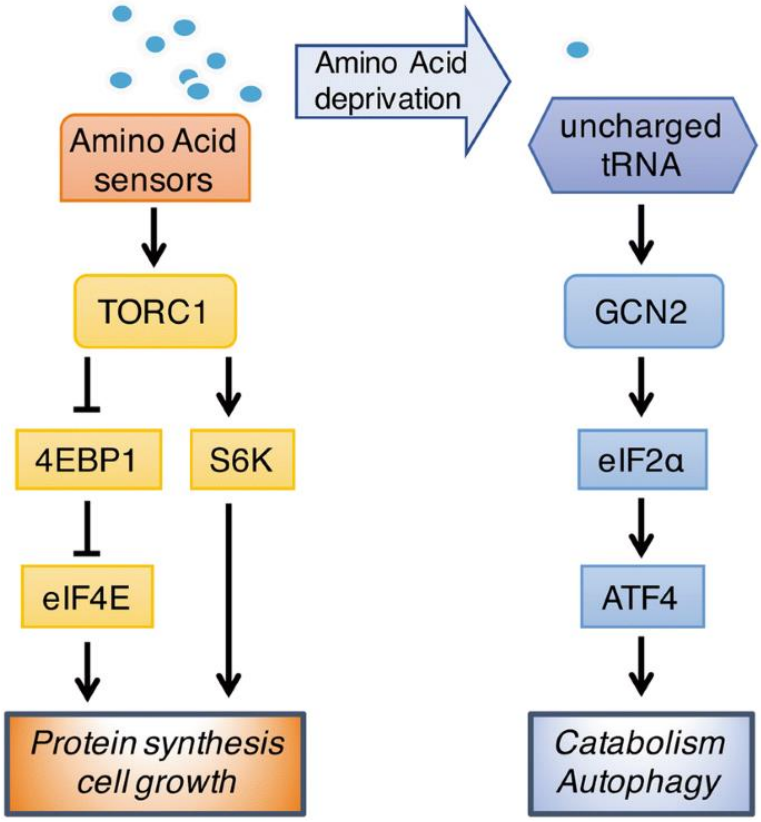

Figure 3. Schematization of GCN2 and mTORC1 pathways showing their role as key regulators that controls protein synthesis based on the varying availability of AAs. Source: Liu et al. (2019)

1.4.2 The GCN2/eIF2-alpha system under AA deprivation

The relationship between GCN2 and eIF2α is central to the so-called Integrated Stress Response (ISR), a complex signalling pathway activated in response to various environmental stimuli including AA deprivation, hypoxia, glucose scarcity, and viral infection, as well as internal factors such as endoplasmic reticulum stress, resulting from the accumulation of unfolded proteins (Matějů & Chao, 2022; Pakos-Zebrucka et al., 2016). Mammalian cells have four kinases that phosphorylate eIF2α and are regulated by different stimuli: HRI (hemin deprivation), PKR (double-stranded RNA in virus-infected cells), PEK or PERK (unfolded proteins in the ER), and GCN2 (serum starvation). Not all of these kinases are evolutionarily conserved. Mammalian GCN2 homologs have been identified in *N. crassa*, *D. melanogaster*, and *S. cerevisiae*, suggesting that the amino acid sensor GCN2 is the most widespread and founding member of the eIF2α kinase subfamily (Dong et al., 2000; Gallinetti et al., 2013).

The central event that links all of these stressors into a common ISR is the phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α) at a conserved serine residue (serine 51). This protein belongs to the eukaryotic initiation factor 2 complex, which is composed of three subunits: eIF2alpha, eIF2beta, and eIF2gamma (Hinnebusch, 2014). The function of eIF2alpha is particularly significant as it controls the initiation of translation by binding to GTP and the initiator methionyl-tRNA (Met-tRNAi), forming the ternary complex necessary for the recruitment of the ribosome to the mRNA. Specifically, phosphorylation of eIF2α inhibits eIF2B, the guanine nucleotide exchange factor (GEF) for eIF2, which normally catalyses the exchange of GDP for GTP on eIF2, thus decreasing the availability of the active eIF2-GTP-Met-tRNAi ternary complex (Figure 4) (Pavitt, 2005). This inhibition of eIF2B effectively reduces global protein synthesis, conserving resources in a manner appropriate for the stress condition (Dong et al., 2000). Moreover, this reduction in general translation allows for the selective translation of specific mRNAs, particularly those involved in stress responses, such as those coding for the transcriptional activator GCN4 in yeast (ATF4 in mammals) (Boye & Grallert, 2020; Castilho et al., 2014). GCN4 / ATF4 activates the transcription of genes involved in AA metabolism, antioxidant responses, and stress-related signalling pathways (Jonsson et al., 2022). This allows the cell to adapt to nutrient scarcity by promoting the synthesis of proteins that help restore AA levels, mitigate oxidative stress, and maintain cellular homeostasis.

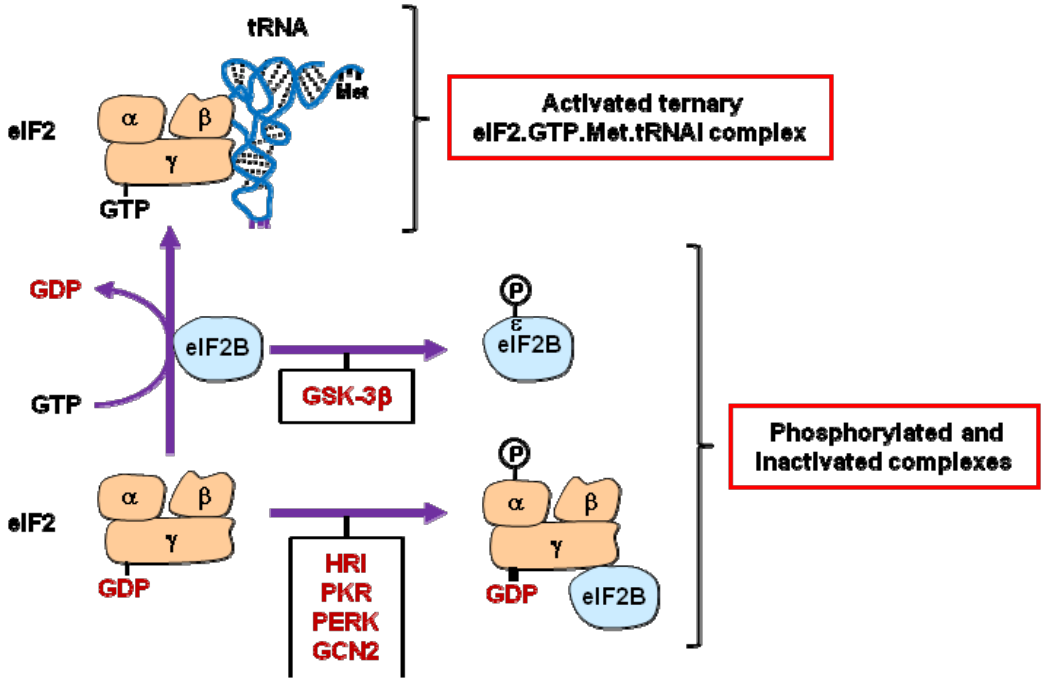

Figure 4. Regulation of eIF2 activity and related formation of the ternary eIF2–GTP–Met-tRNAiMet complex. Transcription factors eIF2 and eIF2B are inhibited by phosphorylation of their eIF2α by HRI, PKR, PERK or GCN2 kinases. Sources: Lasfargues et al. (2012).

1.5 *Drosophila* as a model organism to study diet and aging

Research in diet and aging is often challenging due to the necessity for numerous animals, invasive procedures, and ethical considerations, which are all limiting factors especially when using mammalian models (Longo & Anderson, 2022; Robinson et al., 2019). Additionally, the relatively long lifespan of mammals often restricts aging studies. On the other hand, invertebrate model organisms like yeast, worms, and fruit flies overcome many of these obstacles, allowing more feasible yet rigorous investigations of the connection between diet and longevity (Fontana et al., 2010; Ogienko et al., 2022).

The fruit fly *Drosophila melanogaster*, in particular, has been employed since the early 20th century as a model organism to study fundamental biological processes (Mariateresa et al., 2018). Indeed, the significant evolutionary conservation of signalling pathways between *Drosophila* and humans makes this organism extremely valuable for identifying pathways involved in the determinants of life (Ogienko et al., 2022; Roote & Prokop, 2013). *Drosophila*'s genome is relatively small (180 Mbp), containing approximately 14,000 genes (*FlyBase*, 2024). Moreover, *Drosophila* has homologs for approximately 75% of human disease genes and

about 60% of all fly genes are conserved in humans, along with a high proportion of shared mechanisms that regulate gene expression (Ogienko et al., 2022). Additionally, the fly model is suitable for conducting studies involving a large population of organisms and *Drosophila*'s short life cycle and lifespan of approximately 60-70 days further enhance its advantages over mammalian models for studying lifespan (Ogienko et al., 2022). Finally, the extensive array of genetic tools available to study the effects of specific genes in *Drosophila* facilitates thorough investigations of the interaction between genetics and diet in regulating lifespan (McCracken et al., 2020). The latter can in fact be easily manipulated in fly experiments as discrete modifications of dietary nutrients can be performed using completely defined synthetic diets (Evangelakou et al., 2019). In this regard, recent advancements have been made in deriving objective criteria for determining the quality of dietary AAs balance, allowing the design of optimised chemically-defined diets with advantages for the implementation of standardized AA restriction studies(Piper et al., 2017).

1.6 Literature gap

The GCN2 and mTOR pathways have co-evolved in eukaryotes, functioning as a key regulatory mechanism that controls protein synthesis based on the varying availability of AAs. While there is evidence supporting differential regulation of mTORC1 by specific AAs (Misra et al., 2021), research on AA-specific control of the ISR and subsequent gene expression remains limited (Jonsson et al., 2022). Moreover, although the GCN2/eIF2α system has been characterised at a molecular level in different organisms (Boye & Grallert, 2020; Dever et al., 1992), experimental confirmation of the evolutionary conservation of the GCN2/eIF2α system at the biochemical and genetic level in flies is lacking as no such research has been done *in vivo*. Therefore, it has not been demonstrated that the observed effects following stress, which are linked to eIF2α phosphorylation, rely entirely on this specific phosphorylation event in flies (Boye & Grallert, 2020). These data are critical for studying the role of AA restriction in lifespan.

Given the importance of *Drosophila* as a model organism to study the foundational biological mechanisms in the field of diet and aging, and the demonstrated implications of the GCN2/eIF2α system for an organism's lifespan (Wang & Proud, 2022), this issue needs to be clarified.

Furthermore, to better understand the downstream effects of the ISR and the major biological processes involved, it is important to characterise the proteomic response to specific AA deprivation, and how this is shaped by GCN2 activation. Doing so will help identify the major biological processes involved and expand the knowledge of how GCN2-mediated ISR modulates gene expression and cellular functions under nutrient stress conditions. This knowledge is essential for understanding the broader implications of AA sensing and ISR in aging and dietary interventions, potentially revealing novel targets for enhancing lifespan and healthspan in model organisms and, by extension, in humans.

2 Scope of the thesis

This project sought to elucidate the role of GCN2/eIF2α in ensuring lifespan under AA deprivation in flies. The specific aims of the study were:

1) to assess whether both GCN2 and eIF2α are essential in *Drosophila* for protection against phenylalanine (Phe) deprivation. This would validate the classical model where AA stress activates GCN2, leading to the phosphorylation of eIF2α, which subsequently inhibits protein synthesis to prioritize AA recovery mechanisms. We tested the effects of mutating GCN2 and eIF2α on lifespan under Phe deprivation, predicting that mutations in either gene would similarly compromise lifespan in response to this stress.

2) to identify the key proteome-related changes associated with the GCN2/eIF2α-mediated stress response during Phe deprivation, thereby uncovering the primary protective mechanisms activated to preserve lifespan under such conditions. We conducted a differential proteomic analysis between wild-type and *GCN2*-knockout flies in response to dietary Phe deprivation. We hypothesized that the proteome responses to Phe-deprivation in wild-type flies, buit not GCN2 nulls, would reveal candidate mechanisms to ensure survival under Phe deprivation in a GCN2-dependent manner.

3 Methods

3.1 Fly husbandry

An outbred, wildtype strain of *Drosophila melanogaster* called Dahomey, carrying a mutation in the *white* gene (wDah), was used as control genotype for all the experiments. In this genetic background, the effects of a *GCN2* mutation (Srivastava et al., 2022) and a phosphorylation-dead *eIF2α* mutation (S50A) (Roote & Prokop, 2013) were studied. All flies were raised under controlled population density conditions, utilizing eggs deposited by mothers of the same age, as reported in Linford et al. (2013), and grown to adulthood on a sugar-yeast (SY) medium according to the procedure outlined by Bass et al. (2007). Newly emerged adult flies underwent a two-day mating period on the SY medium to ensure uniform mating status among all flies. Subsequently, 48-hour-old adult female flies were sorted under CO_2 anaesthesia and allocated to their respective experimental diets. Throughout both the rearing and experimental phases, controlled environmental conditions were set at 25°C temperature, 70% humidity, and a 12-hour light/dark cycle was applied. Since female flies typically exhibit more pronounced phenotypic responses to nutrition than males, females have been employed in this study.

3.2 Diets

A SY medium was used to rear flies to adulthood in accordance with well-defined laboratory practices for flies(Bass et al., 2007; Roote & Prokop, 2013). All experimental synthetic diets have been prepared according to the exome-matched FLYAA formula outlined by Piper et al. (2014) and Piper et al. (2017). This diet is considered to have a low protein concentration (10.7 g/L) compared to other high-protein diets used in other *Drosophila* studies and was shown to provide a dietary optimum for adult reproduction, and lifespan (Piper et al., 2017). The base feed contains AAs (in proportions that match the AA profile of the *Drosophila* translated exome), carbohydrates (sucrose), lipids (short chain fatty acids and cholesterol), and a mix of vitamins and minerals that meets the nutritional requirements of the flies.

Three experimental diets were prepared which included:

- a nutritionally complete control diet containing all the AAs (All_AAs Diet)
- an experimental diet lacking the EAA Phenylalanine (no_Phe Diet)

- a diet lacking the non-essential AA Tyrosine (no_Tyr Diet). This served as an additional control to determine if any effect observed upon Phe deprivation were specific to loss of an EAA, or just a generalised worsening of fly health.

3.3 Lifespan experiments

After being reared and allowed to mate on SY food, 48-hour-old adult females were sorted under mild CO_2 anesthesia (less than 30 minutes) and allocated to the experimental diets (All_AAs Diet, no_Phe Diet and no_Tyr Diet) at a density of 10 flies per vial. We aimed at a sample size of n = 100 flies per condition, which is considered appropriate to minimize the impact of sample size and obtain enough statistical power in lifespan experiments with *Drosophila (Sun et al., 2013)*. However, for *GCN2*-null mutants and *eIF2alpha*-P-dead mutants, we couldn't reach 100 flies per condition due to a slightly lower egg deposition of these genotypes. Instead, we obtained 70, 70, and 60 flies for the *GCN2*-null mutants under the All AAs, No Phe, and No Tyr conditions, respectively. For the *eIF2alpha*-P-dead mutants, we obtained 90, 100, and 100 flies under the same conditions. These sample sizes were still appropriate for the field (Linford et al., 2013), and provided appropriate statistical power.
The method outlined by Linford et al. (2013) for lifespan studies with *Drosophila* was followed, which consisted of transferring flies to fresh food every 2 days throughout their life while also performing a census to record any deaths or censors with the dLife software(Linford et al., 2013). Censors include flies drowning or escaping during food changes (i.e. non-natural causes of death or exclusion from the experiment). By providing new food every 2 days we limited the chances of larvae hatching and degrading the food by burrowing, which could lead to adult flies drowning. Figure 5 schematizes the experimental setup of lifespan experiments.

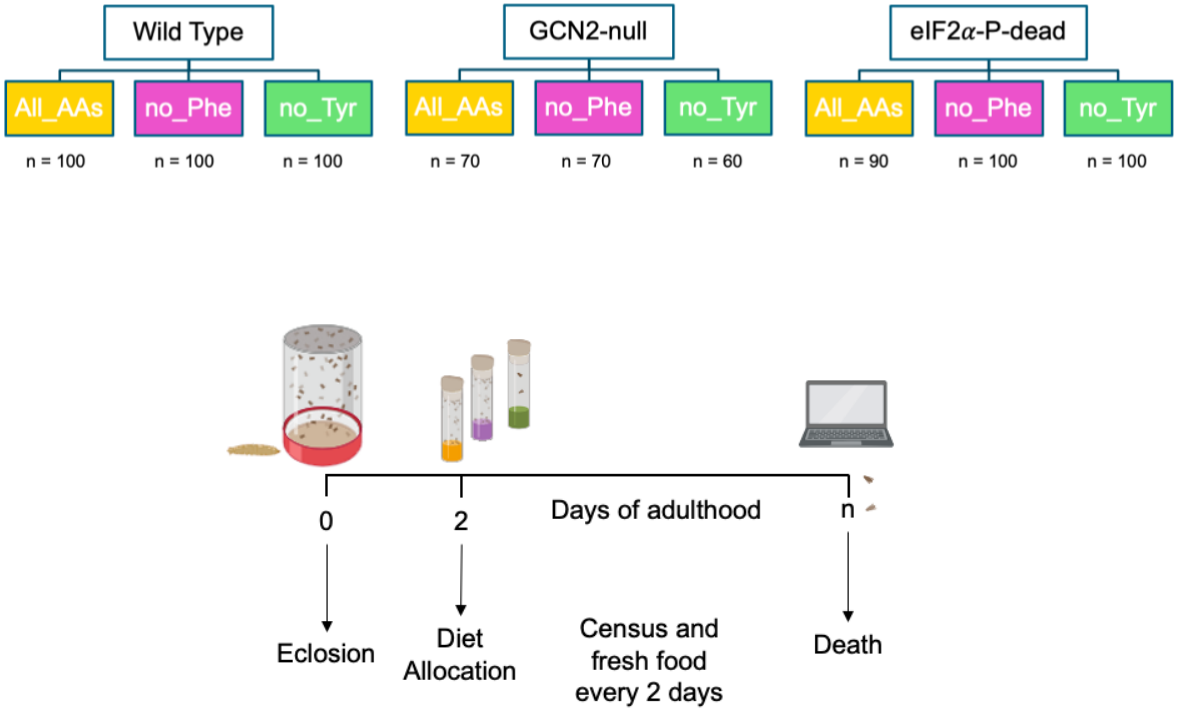

Figure 5. Experimental setup of lifespan experiments. The figure schematizes the experimental design and protocol used. Three genotypes (wild type, *GCN2*-null and *eIF2α*-P-dead) were reared to adulthood on a common duet whereupon they were subjected to three experimental diets (no_Phe and no_Tyr) and a control diet (All_AAs). Adult females were transferred to fresh food every 2 days until death. A census was also performed every 2 days recording any deaths or censors. The analysis included a total of 790 individuals, with 664 deaths recorded and 126 censors.

3.4 Proteomics experiments

For proteomic experiments, the same fly husbandry, rearing methods and diet preparation methods used for lifespan experiments were used (section 3.1 to 3.3 above). After 10 days of diet exposure (with or without Phe) flies were sampled by rapidly freezing in liquid nitrogen. Protein extraction and proteomic analysis were performed by the Monash University's Proteomics & Metabolomics Facility. Sodium deoxycholate (SDC) solubilization was used for protein extraction (Huang et al., 2020), and subsequent peptide separation was performed by nano-HPLC (Dionex UltiMate 3000 RSLCnano system). Finally, mass spectrometry (Orbitrap Fusion mass spectrometer, ThermoFisher Scientific) was used for the identification of protein identity and relative abundance. The acquired dataset were then analysed in R Studio(2021) (see section 3.5.2 on the statistical analysis of proteomic data). Figure 6 schematizes the experimental setup of the proteomic experiments.

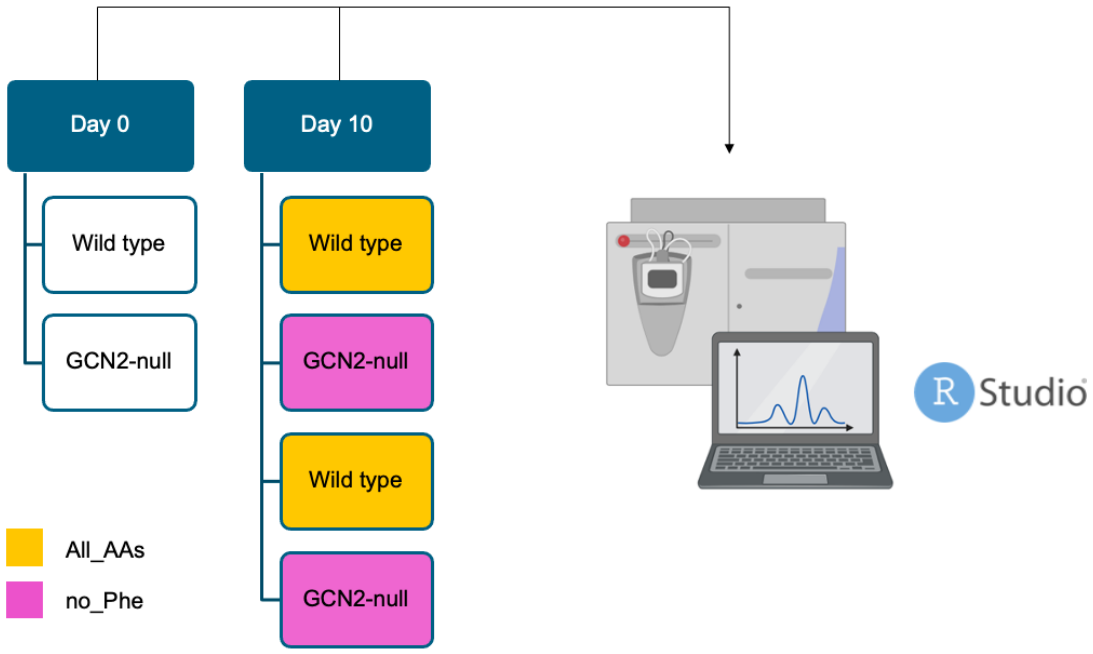

Figure 6. Experimental setup of proteomics experiments. Two genotypes (wild type and *GCN2*-null) were sampled (4 biological replicates per condition) at day 0 and day 10 after each were exposed to the two experimental diets (All_AAs and no_Phe). After protein extraction, peptide separation and protein identification were performed by nano-HPLC/MSMS and the raw dataset was further analysed with R Studio.

3.5 Statistical analysis

3.5.1 Lifespan Data

The raw data produced by the dLife software(Linford et al., 2013) were analyzed in R Studio(2021). The Cox proportional hazards (CoxPH) model was used to investigate the effects of genotype and diet on lifespan. The CoxPH models were fitted using the coxph function from the survival package in RStudio(Rickert, 2017). The significance of the factors and genotype by diet interactions were assessed through ANOVA on the CoxPH models using the Anova function from the car package(Fox et al., 2012). Pairwise comparisons were then conducted using the emmeans function with significance threshold adjusted using Tukey's method for multiple comparisons (Lenth & Lenth, 2018) to understand specific differences between pairs of diet groups or genotypes within each diet group. The results provided the estimated differences between diet and genotype groups on the log scale.

3.5.2 Proteomics

The raw data files coming from the HPLC/MS analysis were processed using the MaxQuant software suite version 1.6.2.10(Tyanova et al., 2016), along with its integrated Andromeda search engine(Cox et al., 2011), to identify proteins from a Uniprot *Drosophila* database. Principal Component Analysis (PCA) was performed to assess the variance and clustering of the data across different conditions. Then, the analysis focused on evaluating differential protein expression under the various experimental conditions. The statistical analysis was performed in R Studio(2021) using the R packages “tidyverse” (Wickham et al., 2019) for data manipulation, “ggpubr” (Kassambara, 2018) for visualization, and “clusterProfiler” (Yu et al., 2012) for bioinformatics analysis.

Data formatting

The data was first formatted into a "tidy" format, obtaining the $\log_2$ fold changes of identified proteins and relative p-values across different experimental conditions. Gene identifiers were converted to Entrez IDs using the “bitr” function from the “clusterProfiler” package, utilizing the “org.Dm.eg.db” database. This conversion was necessary to perform downstream gene ontology analysis. The resulting data was merged with the original dataset to include the Entrez IDs alongside the existing gene identifiers.

Filtering and Comparison

The analysis focused on filtering the data based on specific experimental comparisons, particularly examining differences between genotypes (wDah vs. GCN2null) across different diets (All_AAs and no_Phe) and time points (D0 and D10). To identify significant differences in protein expression, a $\log_2$ fold change cutoff was used (>0 for upregulation and <0 for downregulation), and a p-value threshold of <0.05 was applied. This filtering allowed for the identification of proteins that were upregulated or downregulated between the wDah and GCN2null genotypes on Day 10 under different dietary conditions (NoPhe or AllAAs). Specifically, proteins were filtered for the comparison between D10_wDah_NoPhe and D10_GCN2null_NoPhe, as well as between D10_wDah_AllAAs and D10_GCN2null_AllAAs.

Refinement and functional profile analysis

To refine the analysis, proteins that were uniquely upregulated or downregulated between genotypes in the D10_NoPhe condition but not in the D10_AllAAs condition were isolated. This was done by excluding proteins that showed upregulation or downregulation in both conditions, ensuring that the final list included only those proteins specifically altered in one genotype by the no_Phe diet.

Additionally, the analysis focused on proteins that were the same on Day 0 (p-value > 0.05) and uniquely altered on Day 10 under the no_Phe condition. This was achieved by intersecting the results from the Day 0 comparison (proteins not changed) with those from the D10_NoPhe condition, focusing on proteins that met the significance thresholds.

The over-representation of proteins from particular gene ontology categories was assessed using both the “enrichGO” and “enrichKEGG” functions within the “clusterProfiler” package. An adjusted p-value threshold of 0.05 was applied. These analysis highlighted groups of proteins that were significantly over-represented in the protein set compared to their occurrence in the *Drosophila* genome (Kanehisa & Goto, 2000).

4 Results and discussion

4.1 Lifespan experiments

The focus of the lifespan experiments was to test the requirement for the GCN2 and eIF2α in protecting *Drosophila* adults during dietary EAA deprivation. Although recognized and demonstrated in yeast and mice(Masson, 2019), this mechanism has never been experimentally verified in flies. Therefore, two mutant flies were used in which the expression of either GCN2 or eIF2α was knocked out, so that the activity of the two proteins could be differentially excluded.

We chose to study their requirement under three dietary conditions: a diet containing all amino acids (control), a diet without the essential AA phenylalanine (experimental) and a diet without the non-essential amino acid tyrosine (control). Choosing Phe to investigate the role of GCN2 and eIF2α in the ISR was strategic due to the fact that, unlike other AA deprivations, Phe deprivation has little effect on wild type lifespan, but a large effect on the lifespan of *GCN2* nulls (Johnstone et al., 2023).The use of Tyr as a second control alongside a full AA diet was also strategic, as Tyr is non-essential because to can be directly synthesized from Phe via hydroxylation. Thus, we could assess whether the effects observed under Phe deprivation were specific to the absence of Phe itself or if similar stress responses and lifespan effects occur when the closely related non-essential AA Tyr is deprived. If Phe deprivation uniquely activates the ISR and impacts lifespan, while Tyr deprivation does not, this would suggest that GCN2 and eIF2α are specifically responsive to Phe levels. Conversely, if Tyr deprivation also activates these pathways, it would indicate a broader role for these AAs in regulating the ISR.

We conducted a Cox proportional hazards regression analysis to investigate the effects of genotype (*WDah*, *GCN2_null*, and *eIF2alpha_null*) and diet (All_AA, no_Phe, and no_Tyr), and their interactions, on lifespan. The global CoxPH model indicated significant effects for both genotype (χ^2 = 9.531, p = 0.00852) and diet (χ^2 = 22.509, p = 1.295e-05), as well as their interaction (χ^2 = 269.361, p < 2.2e-16), therefore we also carried out pairwise comparisons within each genotype and diet. The results of statistical analysis are summarised in Appendix I (Table 1 to 4).

4.1.1 Chronic Phenylalanine deprivation shortens lifespan in the absence of GCN2 or eIF2α

Figure 7 shows that no significant changes in lifespan were observed between genotypes when flies were fed a complete diet containing all AAs. This demonstrates that the mutant lines are not exhibiting generalised detrimental effects, which is consistent with previous data that show under fully fed conditions, the ISR is not activated, and therefore not required to sustain lifespan (Postnikoff et al., 2017).

Similarly, wildtype flies (WDah) showed similar lifespan when fed with either a complete diet or a Phe-depleted diet (Figure 7A). However, there was a small, but statistically significant decrease in wildtype lifespan when Phe was absent compared to when all AAs were present ($p<0.01$, Tukey's HSD).

By contrast, when the activity of either GCN2 or eIF2alpha was knocked out flies were much shorter lived on Phe-depleted food ($p<0.001$, Tukey's HSD) (Figure 7B and 7C, respectively). This finding is critical because it confirms the necessity of both GCN2 and eIF2α in maintaining lifespan under conditions of Phe deprivation.

GCN2 and eIF2alpha are critical sensors and mediators of AA scarcity, with GCN2 detecting uncharged tRNAs that accumulate during AA deprivation, subsequently phosphorylating eIF2α to attenuate global protein synthesis and activate stress-responsive gene expression programs (Masson, 2019). We have demonstrated that without either protein, flies are unable to adequately respond to the absence of Phe, leading to shortened lifespan potentially due to reduced somatic maintenance. In particular, the activation of the ISR through GCN2 and eIF2α in wild type flies is likely to have determined adequate management of cellular resources enhancing the organism's ability to survive under AA stress (Malzer et al., 2013; Misra et al., 2024).

These findings suggest that Phe plays a relevant role in signalling the need for stress responses critical to sustain life.

4.1.2 Chronic Tyrosine deprivation does not shorten lifespan in the absence of GCN2 or eIF2α

To test if loss of lifespan for GCN2 or eIF2alpha nulls was a generalised sensitivity to amino acid deprivation, or specific to deprivation for an essential amino acid, we also measured lifespan of flies fed a diet lacking the non-essential AA Tyr.

Figure 7 (D-F) shows that removal of Tyr did not result in a significant decrease in lifespan for either of the genotypes compared to fully fed conditions, thus confirming that the GCN2/eIF2α system is required for protecting the flies against EAA depletion. The absence of a lifespan reduction under Tyr deprivation conditions in all genotypes demonstrates that Tyr is not a critical factor for the activation of the ISR pathway.

Tyrosine is a non-essential AA, meaning that flies can synthesise it *de novo* with no significant impacts on their lifespan (Kosakamoto et al., 2022). By contrast, the deprivation of Phe uniquely triggers a stress response that significantly impacts lifespan in the absence of functional GCN2 or eIF2α. This aligns with previous data supporting that the effects of reduced lifespan in flies are primarily attributed to EAAs, which are those AAs that the body cannot synthesize and must be obtained through the diet, rather than NEAAs (Hoedjes et al., 2017). Moreover, a comprehensive study using human hepatoma cells has demonstrated that individual AAs regulate gene expression differently (Palii et al., 2009). Although the mechanism by which this occurs is not known, the authors speculated that the intracellular concentration of the limiting AA may change depending on the transport and metabolic use of that AA, ultimately triggering different metabolic pathways. Accordingly, differential response to AAs is further supported by studies showing that various AAs can activate distinct signalling pathways, leading to varied cellular outcomes. For instance, leucine, an EAA, is well-known for its role in activating the mTOR pathway, a critical regulator of cell growth and metabolism, which does not respond similarly to other AAs like glycine or alanine (Saxton & Sabatini, 2017).

Our results further confirms that the GCN2/eIF2α system is specifically responsive to the deprivation of EAAs like Phe, rather than a general response to any AA deficiency, as has been observed in other organisms (Kosakamoto et al., 2022; Mazor & Stipanuk, 2016). This specificity is crucial because it suggests that not all AA deprivations equally stress the organism or activate the same molecular pathways.

Given the metabolic link between Tyr and Phe, with Phe being a precursor for Tyr, it is interesting that Tyr deprivation alone does not shorten the lifespan of the ISR deficient mutants. Because these flies have normal levels of dietary Phe, these data indicate that the synthesis of sufficient Tyr from Phe to sustain life does not invole the ISR. Phe is not only an EAA but also a precursor for the synthesis of tyrosine, dopamine, and other catecholamines, making it uniquely integral to various metabolic and signalling pathways (Matthews, 2007). The specific sensitivity to Phe deprivation may reflect its dual role as both a structural component and a signalling molecule, robustly triggering the ISR when absent. This well aligns with the ISR model where the GCN2/eIF2α system likely serves as a protective mechanism, prioritizing the conservation of resources and maintenance of cellular function under nutrient stress(Olson et al., 2020).

Overall, our findings corroborate the idea that the ISR tailors its response to the specific type of AA deprivation, ensuring longevity by activating distinct pathways critical for cellular homeostasis.

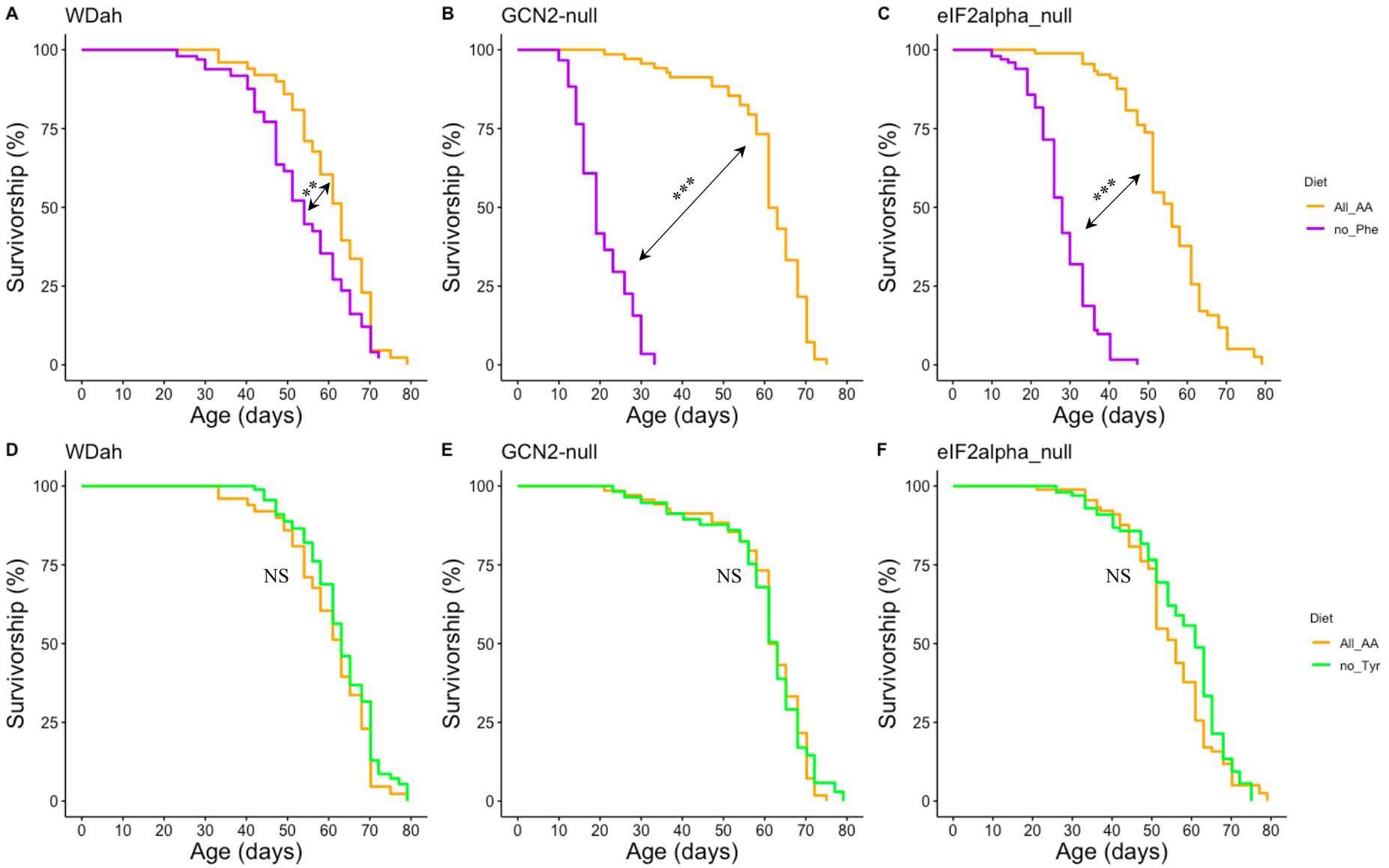

Figure 7. Lifespan outcomes for wildtype, GCN2-null and eIF2α phospho-dead mutants under All AAs, Phe-depleted (A-C) or Tyr-depleted (D-F) diet. Removal of Phe resulted in significant decrease in lifespan for GCN2-null and eIF2α phospho-dead mutants, while removal of Tyr did not result in a significant decrease in lifespan for any of the genotypes. *** significance ($p < 0.001$); ** significance ($p < 0.01$); NS means non significant

4.1.3 *GCN2*-null flies exhibit slightly shorter lifespan compared to *eIF2α* phospho-dead mutants under Phenylalanine deprivation

Replotting the data we obtained allows direct comparison of the lifespans of both *GCN2*-null and *eIF2α* mutants to wildtype flies under Phe deprivation (Figure 8). Interestingly, *GCN2*-null flies had a significantly shorter lifespan ($p < 0.001$, Tukey's HSD) compared to *eIF2α* mutants under Phe deprivation. If GCN2 signalling to eIF2α was the only route for protecting flies against Phe deprivation, these mutants should phenocopy each other. The difference observed might indicate that GCN2 has additional roles/array of targets beyond the phosphorylation of eIF2α that contribute to the stress response and lifespan maintenance. The absence of GCN2 could therefore lead to broader changes in cellular homeostasis compared to the absence of phosphorylated eIF2α alone, meaning that its loss results in shorter lifespan.

GCN2 inhibits translation initiation and selectively translates specific mRNAs by phosphorylating eIF2α. However, evidence suggests that other substrates may mediate GCN2's role in cellular responses to environmental stress (Dokládal et al., 2021; HP, 2003). In mammalian cells, GCN2 was shown to phosphorylate methionyl-tRNA synthetase (MRS), inhibiting its activity and reinforcing the inhibition of translation initiation (Murguía & Serrano, 2012). Moreover, it was shown that inactivation of GCN2-induced phosphorylation at either eIF2α or MRS enhanced the role of the other, indicating a crosstalk between MRS and eIF2α for effective translational inhibition (Kwon et al., 2011). The interaction between these pathways could explain the slightly longer lifespan observed in our *eIF2α* phospho-dead mutants compared to the *GCN2*-null mutants, where the beneficial effects mediated by MRS would also be lost.

Another recent study used phosphoproteomic techniques to analyse protein phosphorylation in yeast (Dokládal et al., 2021) and identified a new target for GCN2. Specifically, GCN2 was able to phosphorylate not only eIF2α, but also the beta subunit of eIF2. This would bypass the inhibited activity of eIF2α in our phospho-dead mutants, thus allowing for a partial restoration of eIF2 activity and therefore lifespan of eIF2α-null flies under Phe deprivation.

An additional explanation for the lifespan difference observed between *GCN2*-null and eIF2α-null mutants in our study may be the presence of other kinases, translation factors or signalling pathways that would compensate for the lack of eIF2α phosphorylation to some

extent, which might not be the case for *GCN2*-null mutants where the initiating signal of the ISR is entirely absent. The mTOR pathway is a central regulator of cellular growth and metabolism, which responds to nutrient availability, including AAs. Under conditions of AAs deprivation, the mTOR pathway is downregulated, which leads to reduced protein synthesis and growth. Given that both the GCN2/eIF2α and mTOR pathways are responsive to AA levels, it is plausible that there may be crosstalk between them. This is supported by studies in yeast, where the activation of GCN2 was found to be dependent on mTOR activity, and GCN2 was necessary for timely inactivation of the mTOR pathway under AA restriction (Elise Rødland et al., 2014). Similar evidence was also found in mice, indicating that GCN2 and mTOR pathways are coordinated in response to AA deprivation (Misra et al., 2021) and that mTORC1 repression is reverted in *GCN2*-null mice fed a leucine-deprived diet (Anthony et al., 2004). Interestingly, it has been shown that GCN2 contributes to mTORC1 inhibition by leucine deprivation through a mechanism independent of the downstream transcription factor ATF4, pointing to a novel role for GCN2 and phosphorylation of eIF2α in the control of mTORC1 by certain AAs (Averous et al., 2016). These findings suggest that the activity of GCN2 on the mTOR pathway, in addition to its effects on eIF2alpha, is likely to be relevant for assuring full lifespan under EAA deprivation.

Another interesting role of GCN2 was recently pointed out by Liu et al. (2021) in mice models. The authors demonstrated that, upon dietary tryptophan withdrawal stress, GCN2 intervenes as mediator of the immune response, boosting the production of proinflammatory cytokines and significantly intensifying the pathological response to systemic challenges (Liu et al., 2021). This study points out an interesting involvement of GCN2 in the immune response, showing that specific AA deprivation stress signals via GCN2 act in synergy with proinflammatory signals to increase innate immune responsiveness, potentially affecting health and lifespan of the organism involved.

A recent study using wild-type, *GCN2* knockout, and unphosphorylable *eIF2α* mutant mouse embryonic fibroblasts (MEFs), identified a novel pathway that upregulates the expression of CARE-containing genes in an ATF4-dependent manner, but independent of GCN2/eIF2α phosphorylation (Mazor & Stipanuk, 2016). CARE (Cis-acting Regulatory Element) is a transcription factor binding site in the promoter region of genes that leads to the upregulation of those genes particularly under stress conditions such as heat, oxidative stress, or nutrient deprivation(Shan et al., 2016). Mazor and Stipanuk (2016) showed that this pathway is

activated in MEFs lacking either *GCN2* or phosphorylatable eIF2α when exposed to methionine-deficient medium. This pathway then still converges with the traditional GCN2/eIF2α kinase-dependent pathway at the level of ATF4, leading to the upregulation of CARE-containing genes. It was hypothesized that the critical role of methionine-charged initiator tRNA in forming the ternary complex underlies the strong ability of methionine deficiency to induce ATF4 and the ISR, even without GCN2 or eIF2α kinase activity (Mazor & Stipanuk, 2016).

Besides corroborating our hypothesis that compensatory mechanisms between the GCN2/eIF2α system and other major signalling pathways exist, all these findings emphasize the complexity of signalling networks in biological systems and point out the difficulty in isolating specific gene/protein effects while trying to study complex interactions such as that between diet and aging.

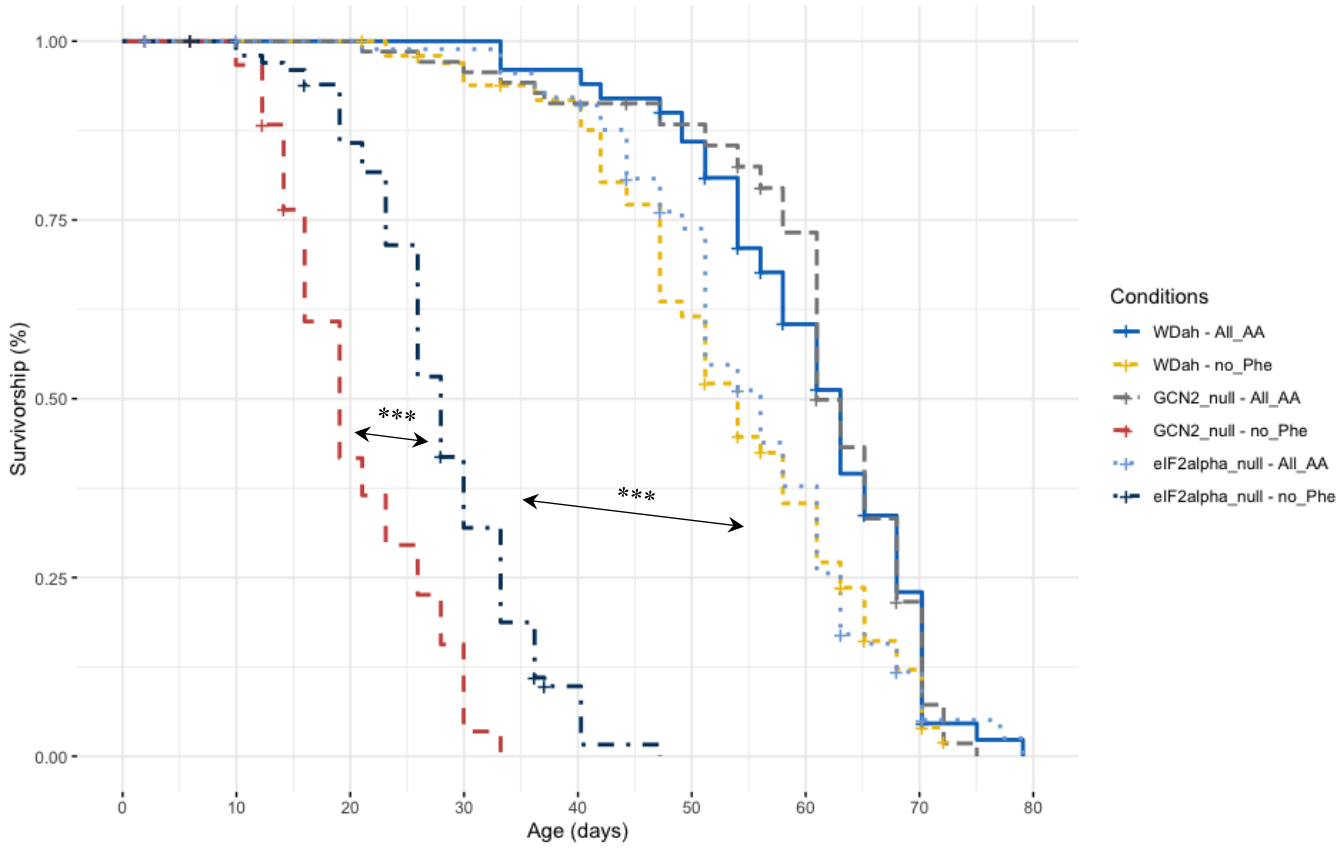

Figure 8. Comparison of lifespans of *GCN2*-null and *eIF2α* mutants to wildtype flies under Phe deprivation (no_Phe) vs fully-fed conditions (All_AA). Both mutants exhibit significantly shorter lifespan under a Phe-depleted diet compared to fully fed conditions. Additionally, *GCN2*-null flies have a shorter lifespan compared to *eIF2α* mutants upon Phe deprivation, suggesting additional roles of GCN2 besides the phosphorylation of eIF2α. *** significance ($p < 0.001$)

4.2 Proteomic experiments

Having confirmed the centrality of GCN2 in preserving lifespan of flies under Phe deprivation, our second research aim was to examine the differential proteomic response patterns in *Drosophila* between wild type flies and *GCN2*-null mutants under the same AA stress. This was done to try to identify molecular pathways, and possibly crucial genes, which are activated in flies in a GCN2-mediated manner to survive Phe deprivation, thus expanding the current knowledge about the ISR mediated by GCN2.

A Principal Component Analysis (PCA) was conducted using the abundance of 8,910 proteins identified across all six experimental conditions, which included two genotypes (GCN2null and

wDah), two diets (NoPhe and AllAAs), and two time points (D0 and D10). This analysis (Figure 9) provides a visual representation of how the different experimental conditions cause variation in protein expression. The first two principal components explained approximately 78% of the total variability, effectively capturing the key differences among the conditions. In the plot, the two genotypes at day 0 (GCN2null_AllAAs and wDah_AllAAs) show a tight cluster that is distinctly separated from the conditions at day 10. The two genotypes at day 10 under fully fed conditions (D10_GCN2null_AllAAs and D10_wDah_AllAAs) are well-separated from their day 0 counterparts along PC1. Furthermore, the data from D10_wDah_NoPhe and D10_GCN2null_NoPhe (both under Phe deprivation) are separated from the D0 samples along PC2, suggesting that diet-related differences over time produce more variation than genotype-related differences.

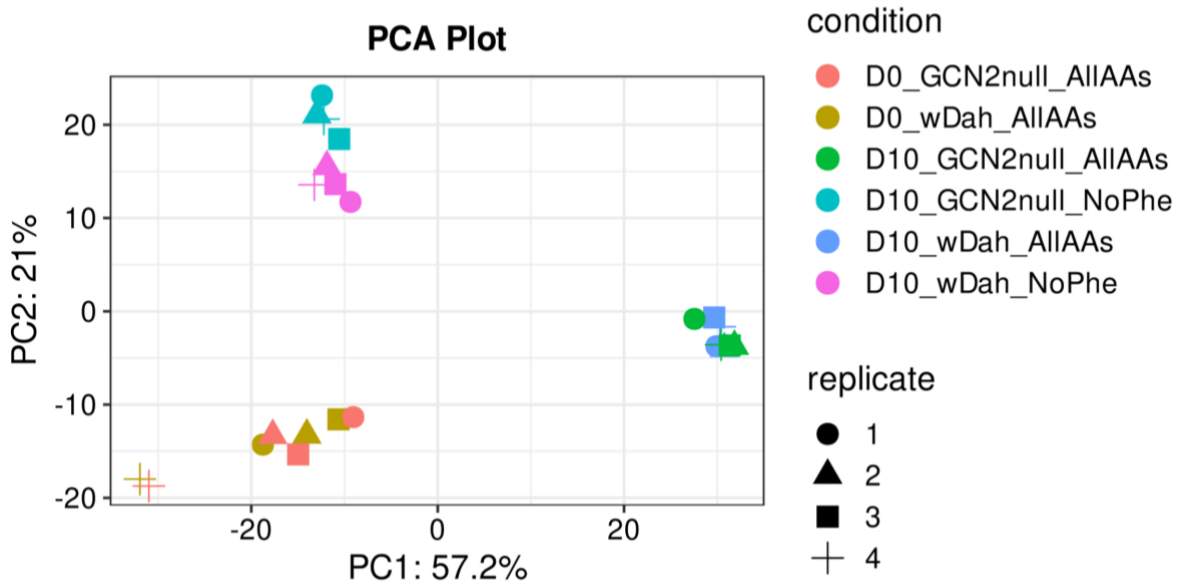

Figure 9: Principal Component Analysis (PCA) of proteomic data across experimental conditions. The PCA plot illustrates the separation of protein expression profiles based on genotype and dietary conditions. Principal Component 1 (PC1) accounts for 57.2% of the variability, while Principal Component 2 (PC2) explains an additional 21%, together capturing approximately 78% of the total variance. Clusters representing the two genotypes at day 0 (D0) are positioned at the bottom left, well-separated from day 10 (D10) conditions along PC1. The D10 groups under phenylalanine deprivation (NoPhe) cluster together in the upper region of the plot but show distinct separation along PC2, indicating a genotype-specific response to phenylalanine deprivation. Different shapes correspond to biological replicates.

4.2.1 *Drosophila* exhibits a characteristic *GCN2*-mediated gene expression in response to Phenylalanine deprivation

The proteomic data were mapped to their corresponding genes by linking the Protein IDs (UniProt IDs) to gene identifiers (Entrez IDs). Then we filtered the data to identify genes that exhibited consistent expression levels at Day 0 (D0) across the two genotypes, to ensure that subsequent analyses focused on changes specifically induced by dietary intervention and not by baseline genetic differences. Subsequently, we isolated proteins that exhibited genotype-dependent changes in expression in response to diet at Day 10 (D10), enabling the identification of genes uniquely responsive to Phe deprivation in the wDah genotype compared to the *GCN2*-null genotype. In this way, further analyses focus on proteins that were either significantly upregulated in wDah compared to *GCN2*-null under the NoPhe diet at D10 and proteins that were significantly downregulated in wDah compared to *GCN2*-null under the NoPhe diet. Both these group of genes were filtered to exclude those that were also up or downregulated under an All_AAs diet, thus ensuring that the upregulation was specific to the NoPhe condition. This allowed to identify 651 upregulated and 479 downregulated genes only in wild type flies under Phe deprivation that were not similarly changed in GCN2 nulls. These proteins were then subjected to Gene Ontology (GO) enrichment analysis to gain insights into the biological processes that are differentially up- or downregulated in wDah compared to *GCN2*-null genotypes under the NoPhe diet.

The GO enrichment for genes that were upregulated in wDah under Phe deprivation revealed significant enrichment in processes related to AA metabolism, cellular stress response, and protein folding (Figure 10A). This suggests that the absence of Phe triggers a GCN2-mediated compensatory mechanism in flies, likely to mitigate the effects of AA deprivation. In contrast, the enrichment amongst downregulated genes included pathways related to growth regulation, metabolic processes, and cellular proliferation (Figure 10B). The downregulation of these processes in wDah under NoPhe conditions may reflect a strategic shift away from growth and metabolism that the GCN2 nulls cannot make, allowing the wild type organism to conserve resources under nutrient-limited conditions. The differential enrichment in these biological processes highlights how wDah flies adapt their physiology to cope with the absence of an EAA, a response that is modulated by the presence of the GCN2 pathway, known for its role in AA sensing and stress response.

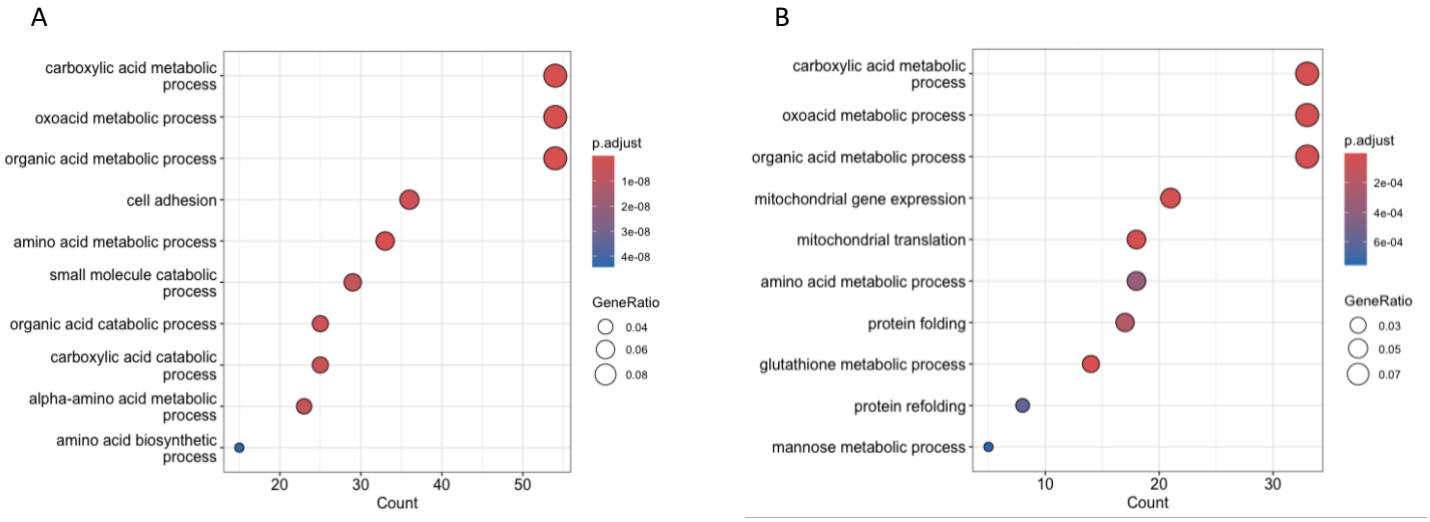

Figure 10: Gene Ontology (GO) enrichment Plot for upregulated (A) and downregulated (B) genes in wild type flies and not in *GCN2*-null flies under Phe deprivation. The GO enrichment analysis for genes upregulated in wDah flies under a NoPhe diet showed significant involvement in amino acid metabolism, cellular stress response, and protein folding, indicating a GCN2-mediated compensatory mechanism to counteract phenylalanine deprivation. Conversely, downregulated genes were associated with pathways governing growth, metabolism, and cellular proliferation, suggesting that under NoPhe conditions, wDah flies reduce these activities to conserve resources during nutrient scarcity. The size of the dots represents the proportion of the proteins attributed to each process (GeneRatio), while the colour indicates the adjusted p-value, highlighting the biological processes most associated with protein up or down regulation under conditions of Phe deprivation.

To further elucidate the pathways affected by dietary and genotype interactions, we performed KEGG pathway enrichment analysis on the same two filtered gene sets. The analysis for upregulated genes indicated significant enrichment in pathways related to valine, leucine, and isoleucine degradation (Figure 11A). This indicates that under Phe deprivation, wild type flies upregulate genes involved in the catabolism of branched-chain amino acids (BCAAs). The increased degradation of valine, leucine, and isoleucine could be a compensatory mechanism to provide alternative sources of AAs and energy (Holeček, 2018), offsetting the lack of Phe. This adaptation may be crucial for maintaining essential metabolic functions and cellular energy balance under nutrient stress. Additionally, the upregulation of genes involved in the extracellular matrix (ECM) receptor interaction suggests changes in cell-matrix communication, potentially as a response to altered cellular conditions caused by the absence of Phe. Modifications in the ECM and its interaction with cell surface receptors might reflect the flies' attempt to adjust cellular adhesion, signalling, and structural integrity under

stress, ensuring tissue stability and function during nutrient scarcity (Hughes & Jacobs, 2017; Sonbol, 2018).

On the other hand, several pathways related to drug metabolism, antioxidant defence, and other metabolic processes are prominently downregulated in wild-type, but not GCN2 nulls in response to Phe deprivation (Figure 11B). This broad downregulation likely reflects a shift in metabolic priorities, where energy and resources are diverted from detoxification and non-essential metabolic functions to focus on survival and stress adaptation in response to nutrient scarcity. This downregulation also aligns with the observed GO terms and supports the hypothesis that wDah flies prioritize survival and stress management over growth and proliferation under Phe deprivation. Overall, these findings are consistent with the role of the GCN2-mediate stress response pathway which is activated under AA deprivation (Dever & Hinnebusch, 2005).

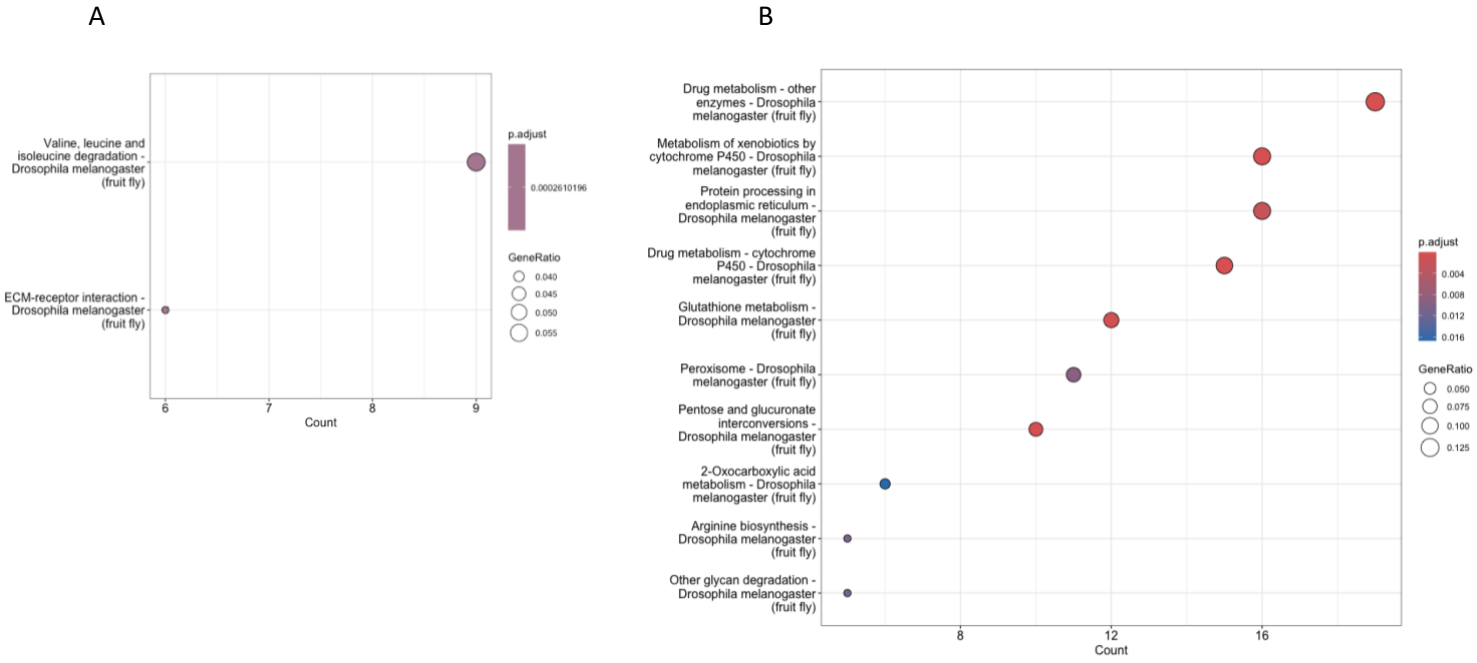

Figure 11: KEGG pathway enrichment analysis for upregulated (A) and downregulated (B) genes in wild type flies and not in GCN2-null flies under Phe deprivation. The plots show significant enrichment in pathways related to AA biosynthesis, oxidative phosphorylation, and stress-induced signal transduction, suggesting that wDah flies upregulate energy production and stress response mechanisms to cope with phenylalanine deficiency. Conversely, pathways associated with cell cycle progression, DNA replication, and ribosome biogenesis were significantly downregulated, indicating a shift in priority from growth and proliferation to survival and stress management under nutrient stress conditions.

4.2.2 Selected genes whose expression is GCN2-dependent under Phenylalanine deprivation

To streamline the analysis of proteomic data, and focus on biologically significant proteins, we filtered the two sets of 651 upregulated and 479 downregulated proteins found in the previous section for those that exhibited both a high $\log_2$ fold change (greater than 1 for upregulation and less than -1 for downregulation) in wild-type flies compared to *GCN2*-null mutants and within the top 50% in terms of abundance (Figure 12). This approach prioritizes proteins that are not only significantly up- or downregulated but also present in relatively high quantities with larger fold changes, under the assumption that larger changes in expression and greater abundance are more likely to have biologically meaningful effects. While this method has its limitations—primarily due to the assumption that greater changes in protein levels equate to more significant biological outcomes—it reduces data complexity to focus on larger effect sizes. This filtering allowed for the selection of 33 upregulated and 56 downregulated proteins for further in-depth study. These sets of proteins and their related genes are listed respectively in table 2 and 3 below.

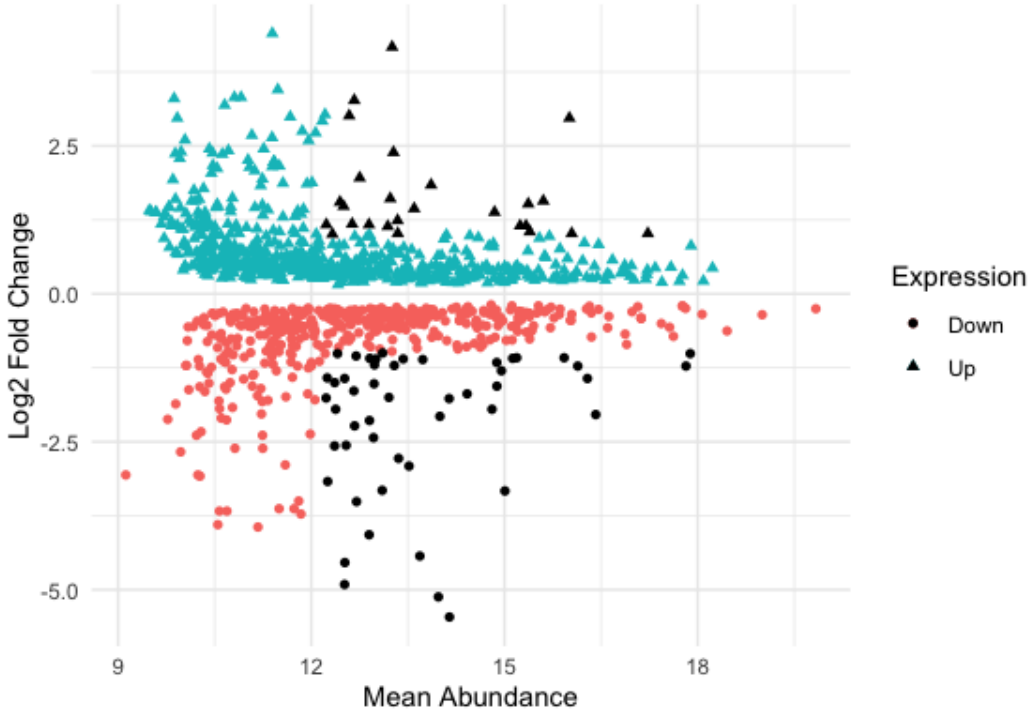

Figure 12: Scatter plot of Log_2 Fold Change versus Mean Abundance (as normalized intensity from MS), highlighting proteins that meet the filtering criteria for significant differential expression. Proteins with absolute Log_2 Fold Change greater than or equal to 1 and mean abundance above the 50th percentile are marked in black, indicating both up-regulated and down-regulated proteins that pass the threshold.

Looking at the upregulated genes (table 2), it appears that a coordinated effort to conserve energy, protect cellular structures, and enhance stress resilience, is elicited in Phe-deprived flies with functional GCN2 (wDah), all of which contribute to sustaining the organism's physiological functions. Under conditions of Phe deprivation, the activation of the ISR through GCN2 in wild-type flies is a strategic response that shifts the organism's priorities towards somatic maintenance and stress resistance, ultimately preserving normal lifespan.

For instance, the upregulation of many proteins involved in the maintenance of the chitin-based larval cuticle (e.g., Cpr64Ac, Dmel\CG8927, verm.1 and stw) may indicate the organism's response to strengthen physical barriers and ensure structural integrity of protective tissues for withstanding adverse conditions (Belle, 2013; Tajiri et al., 2017). This upregulation supports the shift towards somatic maintenance by prioritizing the preservation and reinforcement of vital structures, which may be critical for survival under AA deprivation, as energy and resources are redirected from growth and reproduction to repair and protection of existing tissues (Campisi et al., 2019).

The upregulation of Cytochrome P450 enzymes, a large class of proteins primarily involved in detoxification (Hossam Abdelmonem et al., 2024) may suggest a compensatory response to eliminate harmful by-products that accumulate as a result of impaired metabolism under Phe deprivation. This increased detoxification activity could reflect the organism's attempt to mitigate cellular stress and maintain homeostasis when EAA availability is disrupted, in line with an increased prioritization of somatic maintenance (Maitra et al., 2002).

Interestingly, the upregulation of Lsp2 (Larval serum protein-2) and Bcat (Branched-chain amino acid transaminase) suggests a strategy focused on efficient utilization of stored nutrients. Lsp2 is a major storage protein, crucial for supplying AAs necessary for adult protein synthesis (Valzania et al., 2023), while Bcat plays a role in the catabolism of branched-chain amino acids, which can be used as an alternative energy source during nutrient deprivation (Dimou et al., 2022). Additionally, the upregulation of proteolytic enzymes such as SP99 indicates an effort to break down proteins, facilitating the recycling of AAs to support essential functions during nutrient scarcity (Ross et al., 2003).

Table 2. List of selected proteins and genes that show increased expression in wild-type *Drosophila* compared to *GCN2*-null mutants under phenylalanine deprivation. Protein descriptions were obtained from *FlyBase* (2024), unless otherwise noted in the table.

N	Protein ID	Gene name	Protein description	Log_2 Fold Change	Mean Abundance
1	Q9W4N5	Dmel\CG6414	Predicted carboxylesterase activity, orthologous to human CES3 and CES4A, involved in drug metabolism and detoxification.	4.17	13.3
2	Q6NMY2	BEST:GH10831	Predicted carbohydrate-binding activity, expressed in adult head, related to human CLEC4F and CD209, involved in immune responses.	3.27	12.7
3	Q8IQU1	verm.1	Predicted chitin-binding activity, involved in cuticle and respiratory system development. Located in cell surface and extracellular matrix.	3.03	12.2
4	Q7JYV3	anon-WO0140519.63	Predicted metallocarboxypeptidase activity, involved in proteolysis, orthologous to human CPA5, part of M14 metallopeptidases.	3.01	12.6
5	Q24388	Lsp2	Larval serum protein-2, a major protein in third-instar larvae, involved in amino acid storage for adult protein synthesis (Valzania et al., 2023).	2.97	16.0
6	D3PFG8	LysB	Predicted lysozyme activity, involved in defense against Gram-negative bacteria, located in the extracellular space, expressed in midgut (Ragland & Criss, 2017).	2.92	12.2
7	Q8I099	Dmel\CG2663	Predicted phosphatidylinositol bisphosphate binding, involved in lipid transfer, related to human TTPA, expressed in adult head and trachea.	2.72	12.1
8	Q6IKG0	Dmel\CG34301	No information available.	2.59	12.0
9	Q9VYD5	Bcat	L-isoleucine, L-leucine, and L-valine transaminase activity, involved in amino acid catabolism, active in mitochondrion, related to human BCAT2 (Dimou et al., 2022).	2.39	13.3
10	P37161	LysX	May digest bacteria in food, potentially involved in bacterial clearance from larval gut before metamorphosis.	1.96	12.8
11	Q9VN75	plh	Involved in hypoxia response, located in membrane and mitochondrion, part of the Mpv17/PMP22 family, possibly forms ion channels/pores.	1.88	12.0
12	Q9VXC9	SP99	Enables serine hydrolase activity, involved in proteolysis.	1.86	11.9
13	Q9W168	Dmel\CG16837	Predicted dynein intermediate chain binding, involved in microtubule movement, part of cytoplasmic dynein complex, active in centrosome and cytoplasm.	1.84	13.9
14	Q9VIQ4	Hf	Involved in innate immune response.	1.61	13.2
15	Q9VZG0	Cpr64Ac	Predicted structural constituent of chitin-based larval cuticle, involved in cuticle development, located in extracellular matrix (FlyBase, 2024; Tajiri et al., 2017).	1.57	15.6
16	Q27593	Cyp6a8	Involved in insect hormone metabolism and insecticide breakdown, part of the Cytochrome P450 family, catalyzes monooxygenase reactions.	1.55	12.4
17	Q7K4Y0	Desat1	Fatty acid desaturase, involved in unsaturated fatty acid synthesis, important for sex pheromone production.	1.52	15.4
18	Q9W1R8	Dmel\CG3500	Predicted COPII receptor activity, involved in ER to Golgi transport.	1.48	12.5
19	Q9VSY6	aay	Phosphoserine phosphatase activity, involved in serine biosynthesis, related to human PSPH, part of the HAD hydrolase family.	1.44	13.6
20	Q8MRN5	CG5839	Predicted metalloaminopeptidase activity, involved in peptide catabolism and proteolysis.	1.38	14.8
21	Q9VEY0	Dmel\CG8927	Predicted structural constituent of chitin-based larval cuticle, active in extracellular matrix.	1.24	13.3
22	A4V3W1	sgg	Encodes glycogen synthase kinase 3, involved in Wnt, Insulin, Hedgehog, and BMP signaling pathways, key in cell polarity and development.	1.18	12.6

23	Q9VCK1	GILT2	No information available.	1.17	12.9
24	Q9W1F8	Orcokinin	Neuropeptide involved in male courtship behavior and oogenesis regulation.	1.17	12.2
25	M9PI96	Dmel\CG18135	Glycerophosphocholine phosphodiesterase, involved in lipid metabolism and sexual reproduction, no phenotypic data available.	1.15	15.2
26	Q9VRS6	Jon65Aiv	Enables serine hydrolase activity, involved in proteolysis, cleaves substrates following hydrophobic amino acids.	1.15	15.3
27	Q9VRS4	yip7	Similar to Jon65Aiv, serine hydrolase involved in proteolysis.	1.14	13.2
28	A1Z6F4	stw	Predicted catechol oxidase and tyrosinase activity, involved in cuticle development, active in plasma membrane, expressed in adult head.	1.05	15.4
29	A1Z7Z4	Dmel\CG1648	No information available.	1.02	17.2
30	M9NGA3	CkIIbeta	Regulates alpha subunit catalytic activity, involved in Wnt signaling, circadian rhythm, and developmental processes.	1.02	13.3
31	Q967S0	Prat2	Amidophosphoribosyltransferase, essential for de novo synthesis of inosine monophosphate (IMP), involved in purine nucleotide biosynthesis (Ji & Clark, 2006)	1.02	16.0
32	Q9VEQ3	Dmel\CG18622	No information available.	1.01	12.1
33	Q9VKQ5	Dmel\CG17124	Predicted protein serine/threonine phosphatase inhibitor activity.	1.01	12.3

With regards to downregulated genes (table 3), these reflects a strategic shift in the organism's physiological priorities, redirecting resources away from processes related to reproduction, detoxification, and certain metabolic functions. By scaling back some of these activities, the flies may better conserve energy and focus on somatic maintenance, enhancing their ability to cope with stress and ultimately sustaining normal lifespan.

For instance, an adaptive response to de-prioritize reproductive processes may be seen in the downregulation of genes involved in eggshell formation and chorion development (e.g., Cp15 and Cp16) and in that of genes affecting cell division and fertility (e.g., Ifrd1) (Lewis, 2019; Niepielko et al., 2013). This, once again, would confirm that in times of AA stress, reproduction is not vital for immediate survival, so these genes are downregulated likely to conserve energy and resources(Lu et al., 2020).

Furthermore, GCN2 is known to reduce global protein synthesis when AAs in the environment are scarce (Hu et al., 2018). The observed downregulation of the eukaryotic translation initiation factor 6 (eIF6) in wild-type flies may indicate a strategic decrease in protein synthesis to conserve resources. eIF6 is a multifunctional protein essential for ribosome biogenesis and assembly, significantly influencing the overall protein synthesis machinery (Biffo et al., 2014). Therefore, its downregulation is likely to be involved in the adaptive response to maintain cellular homeostasis under nutrient-limited conditions.

Table 3. List of selected proteins and genes that show decreased expression in wild-type *Drosophila* compared to *GCN2*-null mutants under phenylalanine deprivation. Protein descriptions were obtained from *FlyBase* (2024), unless otherwise noted in the table.

N	Protein ID	Gene name	Protein description	Log_2 Fold Change	Mean Abundance
1	P07185	Cp15	Structural egg chorion protein; involved in eggshell formation.	-5.46	14.1
2	P22977	Cp16	Egg shell protein; protects the egg from environmental factors.	-5.12	14.0
3	P02515	Hsp22	Heat shock protein 22 (Hsp22); chaperone involved in aging, stress response, and lifespan determination.	-4.91	12.5
4	Q9VG97	GstD3	Predicted to be involved in glutathione metabolism.	-4.54	12.5
5	Q9VG95	GstD5	Conjugates reduced glutathione to hydrophobic electrophiles; involved in detoxification.	-4.43	13.7
6	P10090	w	ABCG2 transporter; transports cyclic GMP, biogenic amines, and pigments; mutations cause white eyes in flies.	-4.07	12.9
7	Q9VYH8	Dmel\CG15721	Involved in chorion-containing eggshell formation; located in egg chorion.	-3.51	12.7
8	Q7K1U0	Arc1	Master regulator of synaptic plasticity; mediates RNA transfer from motorneurons to muscles; involved in energy balance.	-3.33	15.0
9	Q9VZK8	Dmel\CG12766	Predicted aldose reductase activity; implicated in 46,XY sex reversal.	-3.32	13.1
10	Q8IQS5	Dmel\CG32195	Ecdysteroid 22-kinase activity; involved in detoxification by phosphorylation.	-3.17	12.3
11	Q9VVR9	Cyp12c1	Cytochrome P450; involved in heme binding, iron ion binding, and monooxygenase activity; associated with various diseases.	-2.91	13.5
12	Q8IMT4	CG13665	Ecdysteroid 22-kinase activity.	-2.78	13.4
13	Q9W223	Cyp6d2	Cytochrome P450; involved in detoxification; responds to camptothecin.	-2.57	12.4
14	Q9VV61	CG10162	Predicted GTPase and ribosome binding activity; involved in cytosolic ribosome assembly.	-2.56	12.5
15	Q9VQI5	Ifrd1	Expressed in adult brain; orthologous to human IFRD2; affects cell division and fertility.	-2.43	13.0
16	Q9VQD2	Cyp309a1	Cytochrome P450; involved in heme binding, iron ion binding, and monooxygenase activity.	-2.37	12.0
17	Q7JYX2	Dmel\CG2065	Enables all-trans-retinol dehydrogenase activity; involved in retinal metabolism.	-2.23	12.7
18	Q9VTL5	RIOK1	Predicted serine/threonine kinase activity; regulates glial cell proliferation; located in cytoplasm and nucleus.	-2.14	12.9
19	Q9VLI0	LManV	Lysosomal alpha-mannosidase V; involved in mannose metabolism.	-2.07	14.0
20	Q9VNF3	Dmel\CG12171	Enables estradiol 17-beta-dehydrogenase and testosterone 17-beta-dehydrogenase activities.	-2.04	16.4
21	Q7KK90	GstE1	Glutathione S transferase E1; involved in glutathione metabolism and heat response.	-1.95	14.8
22	Q9VLI1	LManIV	Alpha-mannosidase activity; involved in mannose metabolism; located in lysosome.	-1.95	12.4
23	Q9VMW2	Rtnl1.3	Involved in endoplasmic reticulum organization and reticulophagy; phenotypic classes include abnormal behavior.	-1.79	12.1
24	P56538	eIF6	Eukaryotic translation initiation factor 6; involved in ribosomal subunit export and protein-RNA complex organization.	-1.77	14.1
25	Q9W2J3	Ugt49C1	UDP-glycosyltransferase activity; implicated in various diseases; involved in glucuronidation.	-1.76	12.2

26	Q9VS22	Acbp3	Long-chain fatty acyl-CoA binding activity; involved in fatty acid metabolism.	-1.75	13.2
27	Q9VCQ9	Dmel\CG6733	Aminoacylase activity; involved in amino acid metabolism.	-1.69	14.4
28	Q9V3A0	CT25774	No information available.	-1.69	11.9
29	Q9VBT2	Dmel\CG11893	Ecdysteroid 22-kinase activity; involved in caffeine response and detoxification.	-1.64	12.7
30	A1Z729	Rdh1	Enables all-trans-retinol dehydrogenase activity; involved in lipid droplet formation and retinal metabolism.	-1.56	14.9
31	Q9VGT8	Ugt35C1	UDP-glycosyltransferase activity; implicated in various diseases; involved in glucuronidation.	-1.52	13.0
32	Q9W274	Alp2	Alkaline phosphatase activity; implicated in inflammatory bowel disease; located on cell surface.	-1.5	12.4
33	O97479	Sodh1	L-iditol 2-dehydrogenase activity; involved in fructose and sorbitol metabolism.	-1.43	16.3
34	Q961D3	rdog	ATPase-coupled transmembrane transporter activity; involved in transmembrane transport.	-1.43	12.5
35	Q9VDU3	Acsx1R	Long-chain fatty acid-CoA ligase activity; involved in fatty acid metabolism.	-1.42	12.2
36	Q9VTY2	anon-WO0172774.89	Aldose reductase activity; involved in fructose and sorbitol biosynthesis.	-1.3	15.0
37	Q9VCW1	Cyp6d4	Cytochrome P450; involved in heme binding, iron ion binding, and monooxygenase activity.	-1.25	12.0
38	Q9VXI1	Had1	L-gulonate 3-dehydrogenase activity; involved in fatty acid metabolism.	-1.22	17.8
39	Q9VSP9	Acbp5	Long-chain fatty acyl-CoA binding activity; involved in fatty acid metabolism.	-1.22	16.1
40	A0A1Z1CH25	Dmel\CG11891	Ecdysteroid 22-kinase activity.	-1.22	12.0
41	Q9VE26	Dmel\CG14292	No information available.	-1.21	13.3
42	A1ZBR1	Dmel\CG16898	Ecdysteroid 22-kinase activity.	-1.2	13.0
43	Q9VLG9	Argl	Argininosuccinate lyase activity; involved in arginine biosynthesis.	-1.16	14.9
44	Q9VRD1	Dmel\CG1304	Serine-type endopeptidase activity; involved in proteolysis.	-1.16	12.0
45	Q7K1R6	Dmel\CG10916	Zinc ion binding protein with ubiquitin-protein transferase activity; involved in lifespan determination and signal transduction.	-1.14	12.1
46	Q9VBT8	CT33083	Ecdysteroid 22-kinase activity; expressed in adult head.	-1.11	13.7
47	Q9VET9	Decay	Caspase related to Apopain/Yama; involved in apoptotic processes and programmed cell death.	-1.1	13.4
48	Q9VF56	obe	Ski2-family helicase; regulates mRNA splicing and cell polarity; involved in adherens junction organization.	-1.1	13.0
49	Q9VF53	AOX1	Aldehyde oxidase 1; involved in pyridoxal metabolism.	-1.09	15.1
50	Q9VES7	Dmel\CG17562	Alcohol-forming very long-chain fatty acyl-CoA reductase activity; involved in fatty-acyl-CoA metabolism.	-1.09	12.9
51	Q9U1L2	EG:BACR7A4.14	Enables estradiol and testosterone 17-beta-dehydrogenase activities; expressed in various tissues.	-1.08	15.9
52	Q9VGF3	Dmel\CG18547	Enables estradiol and testosterone 17-beta-dehydrogenase activities; involved in fatty acid beta-oxidation.	-1.08	15.2
53	Q9VG96	GstD4	Glutathione transferase activity; involved in glutathione metabolism and detoxification.	-1.05	12.7

54	Q9V3W0	UK114	Molecular chaperone with HSP70-like function; involved in protein folding and protection during heat shock.	-1.01	17.9
55	A1Z8D3	Hao	(S)-2-hydroxy-acid oxidase activity; involved in fatty acid alpha-oxidation.	-1.01	12.4
56	M9NDW0	Tango2	Involved in Golgi and ER organization; depletion causes Golgi-ER fusion; associated with metabolic and cardiac disorders.	-1.01	12.2

Overall, the activation of ISR in response to Phe deprivation prompts a suite of gene regulations that collectively prioritize somatic maintenance over reproduction. The focus on maintaining cellular integrity, energy efficiency, and stress resistance likely explain, at least in part, how flies can survive the AA deprivation without a significant reduction in lifespan. By conserving resources and enhancing protective mechanisms, the flies effectively mitigate the potential damage caused by Phe deprivation, thereby maintaining normal physiological function and longevity.

4.2.3 A GCN2-mediated strategy to maintain lifespan of flies under Phenylalanine deprivation

A growing body of literature identifies GCN2 as a pivotal regulator in maintaining cellular homeostasis during AA deprivation (Liu et al., 2021). Once activated, GCN2 initiates a signalling cascade that modulates protein synthesis, cellular metabolism, and stress responses to adapt to the nutrient-deprived state. Additionally, GCN2 activation is closely linked to the induction of autophagy, a cellular process that degrades and recycles intracellular components to access stored AAs. This mechanism provides a critical means of replenishing essential AAs during periods of deficiency, thereby supporting cellular function and survival.

The recent work of Johnstone et al. (2023) introduces a model where GCN2 sustains somatic maintenance in flies during Phe deprivation by accessing stored AAs through the activation of autophagy, thereby preventing the detrimental effects of AA scarcity on lifespan. The study highlights that Phe, unlike other EAAs, does not limit lifespan when deprived, indicating that flies store sufficient amounts of Phe during development to meet the demands of adulthood. However, the depletion of this Phe reserve, such as through the additional deprivation of Tyr, which is synthesized from Phe, can shorten lifespan. This suggests that the balance between

stored Phe and its somatic use is critical for lifespan maintenance. The upregulation of specific genes involved in somatic maintenance, as identified in our study, supports this model. In particular, we found that Larval Serum Protein 2 (Lsp2) is continuously synthesized even under Phe deprivation, showing levels approximately 8-fold changes higher (2.97 Log_2 fold change) in wild-type flies compared to *GCN2*-null mutants.

Lsp2 is abundantly expressed during the larval stages of *Drosophila* and is known to store large amounts of EAAs like Phe(Roberts et al., 1991). In the context of the proposed model, Lsp2 serves as a vital reservoir that the organism can draw upon when external sources of Phe are limited, such as during adult Phe deprivation. The observed upregulation of Lsp2 in wild-type flies under Phe deprivation suggests that the organism is actively enhancing its capacity to store or mobilize Phe from internal reserves to meet ongoing metabolic demands, especially for processes critical to somatic maintenance. This upregulation could enhance the efficiency of the autophagic process by ensuring that sufficient storage capacity for Phe is available for release later on when needed. In contrast, *GCN2*-null flies, which lack the ability to activate this stress response pathway effectively, would not exhibit the same upregulation of Lsp2, leading to a more rapid depletion of Phe and a consequent reduction in lifespan.

To test the hypothesis that Lsp2 plays a key role in the adaptive response to Phe deprivation by maintaining EAA reserves, further research could involve generating *Lsp2* knockout flies and assessing their lifespan under a Phe-deprived diet. In addition to monitoring survival rates, advanced proteomic techniques, such as stable isotope labelling, could be employed to track changes in amino acid reserves over time. By comparing these data to wild-type flies, we could determine whether the absence of Lsp2 impairs the ability to store or mobilize Phe during deprivation. This approach would help clarify whether Lsp2 directly contributes to maintaining AA homeostasis and somatic maintenance under nutrient stress, providing crucial insights into the molecular mechanisms involved. This would either validate Lsp2 as a critical factor in Phe storage or highlight alternative pathways that ensure survival during amino acid deprivation.

5 Conclusions

Our study provides evidence that the GCN2/eIF2α system is essential for maintaining lifespan of flies under conditions of EAA deprivation, specifically Phe deprivation. The differential responses to Phe versus Tyr deprivation highlight the specificity of the GCN2/eIF2α system in responding to certain types of AA stress. The observed differences in lifespan reduction between *GCN2*-null and *eIF2α* phospho-dead mutants suggest additional roles for GCN2 beyond the traditional ISR pathway that warrant further investigation. Understanding these mechanisms in greater detail could have broader implications for improving stress resistance and longevity in other organisms, including humans.

Using proteomic techniques to identify genes and proteins which are heavily regulated for lifespan maintenance under Phe deprivation, we observed a coordinated response to conserve energy, protect cellular structures, and enhance stress resilience. The downregulation of genes involved in reproductive processes also supports the idea that the ISR and GCN2-mediated pathways prioritize somatic maintenance over reproduction under AA deprivation, which is a well-known evolutionary strategy to enhance survival under nutrient stress.

Interestingly, the upregulation of Lsp2 in our study provides strong support for the model proposed by Johnstone et al. (2023). It highlights the critical role of stored Phe in sustaining somatic maintenance and suggests that wild-type flies are better equipped to manage Phe deprivation through enhanced storage and mobilization of this EAA. This upregulation aligns with the proposed mechanism where GCN2 activation leads to autophagy and the utilization of internal AA stores, ultimately supporting longevity under conditions of nutrient scarcity.

In summary, our findings underscore the significance of the GCN2/eIF2α system in modulating lifespan under AA deprivation, highlighting its potential as a target for interventions aimed at enhancing stress resilience and longevity. Future research should delve deeper into the non-canonical roles of GCN2 and explore how these insights can be translated to other species, paving the way for novel strategies in age-related health management.

6 References

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7 Appendix

Table 1. Cox Proportional Hazards Model with Genotype and Diet Interactions

Predictor	*Coefficient (coef)*	*Exp(coef)*	*Std. Error (se(coef))*	*z-value*	*p-value*	*95% CI Lower*	*95% CI Upper*
GenotypeGCN2_null	-0.01089	0.98916	0.17088	-0.064	0.9492	0.7076	1.383
GenotypeeIF2alpha_null	0.44029	1.55315	0.15995	2.753	0.0059 **	1.1352	2.125
Dietno_Phe	0.51736	1.67760	0.15678	3.300	0.00097 ***	1.2338	2.281
Dietno_Tyr	-0.22609	0.79765	0.16202	-1.395	0.1629	0.5806	1.096
GenotypeGCN2_null	4.34132	76.80913	0.29835	14.551	< 2e-16 ***	42.8009	137.839
GenotypeeIF2alpha_null	2.76985	15.95621	0.25827	10.725	< 2e-16 ***	9.6181	26.471
GenotypeGCN2_null	0.16058	1.17419	0.25116	0.639	0.5226	0.7177	1.921
GenotypeeIF2alpha_null	-0.06325	0.93871	0.22617	-0.280	0.7798	0.6026	1.462

Model Statistics:

- Concordance: 0.746 (SE = 0.012)
- Likelihood Ratio Test: 602.8 on 8 df, p < 2e-16
- Wald Test: 521.4 on 8 df, p < 2e-16
- Score (Logrank) Test: 1192 on 8 df, p < 2e-16

Table 2. Analysis of Deviance (Type III tests)

Effect	*LR Chisq*	*Df*	*p-value*
Genotype	9.531	2	0.00852 **
Diet	22.509	2	1.295e-05 ***
Genotype x Diet	269.361	4	< 2.2e-16 ***

Notes:

- **LR Chisq**: The likelihood ratio test statistic measures how much better the model with the specified term fits compared to the null model.
- **Df**: Degrees of freedom associated with the test. Represents the number of parameters tested.
- **p-value**: Indicates statistical significance of the term. Significance codes: *** p < 0.001, ** p < 0.01.

Table 3. Pairwise Comparisons for Diet Within Each Genotype

Genotype	*Comparison*	*Estimate*	*SE*	*p-value*
WDah	All_AA vs. no_Phe	-0.516	0.157	0.0029 **
	All_AA vs. no_Tyr	0.226	0.163	0.3488
	no_Phe vs. no_Tyr	0.742	0.160	< 0.0001 ***
GCN2_null	All_AA vs. no_Phe	-4.277	0.446	< 0.0001 ***
	All_AA vs. no_Tyr	0.059	0.196	0.9511
	no_Phe vs. no_Tyr	4.336	0.451	< 0.0001 ***
eIF2alpha_null	All_AA vs. no_Phe	-3.171	0.254	< 0.0001 ***
	All_AA vs. no_Tyr	0.275	0.159	0.1931
	no_Phe vs. no_Tyr	3.445	0.259	< 0.0001 ***

Notes:

- **Estimate**: The log hazard ratio estimate for the comparison.
- **SE**: Standard error of the estimate.
- **p-value**: Significance level of the comparison. Significance codes: *** p < 0.001, ** p < 0.01.

Table 4. Pairwise Comparisons for Genotypes within Each Diet

All_AA Diet

Comparison	*Estimate*	*SE*	*p-value*
WDah vs. GCN2_null	0.0257	0.171	0.9876
WDah vs. eIF2alpha_null	-0.4521	0.160	0.0133 **
GCN2_null vs. eIF2alpha_null	-0.4778	0.171	0.0144 **

no_Phe Diet

Comparison	*Estimate*	*SE*	*p-value*
WDah vs. GCN2_null	-4.10	0.307	< 0.0001 ***
WDah vs. eIF2alpha_null	-3.01	0.269	< 0.0001 ***
GCN2_null vs. eIF2alpha_null	1.09	0.179	< 0.0001 ***

no_Tyr Diet

Comparison	*Estimate*	*SE*	*p-value*
WDah vs. GCN2_null	-0.161	0.185	0.6579
WDah vs. eIF2alpha_null	-0.396	0.161	0.0371 *
GCN2_null vs. eIF2alpha_null	-0.235	0.182	0.4015

Notes:

- **Estimate**: The log hazard ratio estimate for the comparison.
- **SE**: Standard error of the estimate.
- **p-value**: Significance level of the comparison. Significance codes: *** p < 0.001, ** p < 0.01.