Early-adulthood intermittent fasting and reduced insulin/IGF-1 signalling additively increase lifespan and slow down reproductive ageing

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Abstract

Intermittent fasting (IF) and reduced insulin/IGF-1 nutrient-sensing signalling (rIIS) increase lifespan via partially shared downstream genetic pathways. The relationship between IF and rIIS is unclear because IF reduces reproduction, while adulthood-only rIIS does not. Here, we show that early-adulthood (but not late-adulthood) IF and adulthood-only rIIS additively increase lifespan and slow reproductive ageing in *C. elegans*. Mechanistically, we show that in the combined IF + rIIS treatment, nuclear localisation of DAF-16 transcription factor is primarily driven by IF in early-life, and primarily driven by rIIS in late-life. Full factorial genome-wide RNA-seq across the life course demonstrates that early-adulthood IF and rIIS modulate the age-specific expression of pro-longevity genes. Early-adulthood IF, rIIS and combined IF + rIIS treatment downregulated genes involved in peptide metabolism in early life and differentially regulated immunity genes in later life. Importantly. combined IF + rIIS treatment uniquely regulated a large cluster of genes in mid-life that are associated with immune response. These results suggest that the combined impact of different treatments aimed at healthy ageing can achieve better outcomes by targeting different ages.

Keywords: ageing, intermittent fasting, lifespan extension, senescence, reproduction

Introduction

Intermittent fasting (IF) is a form of dietary restriction (reduced nutrient intake without malnutrition) which involves cycling periods of fasting and food consumption (Mattson et al., 2017). Different IF regimes have been shown to extend lifespan in model organisms and reduce age-related pathologies (Baumeier et al., 2015; Catterson et al., 2018; Honjoh et al., 2009; Liu et al., 2019; Ulgherait et al., 2021; Uno et al., 2013). Several studies also suggest that IF (including so-called fasting-mimicking diets) may have clinically important beneficial effects in humans (Azevedo et al., 2013; Patterson et al., 2015; Tinsley & La Bounty, 2015). Recent work in *Drosophila* suggests that even short-term early-life IF can have long-lasting effects on lifespan and gut health in later life (Catterson et al., 2018). This is promising because transient IF regimes are easier to comply with compared to long-term ones (Wegman et al., 2015). Understanding the underlying mechanisms of IF-mediated effects on late-life health is important to maximise the benefits and avoid potential costs associated with such interventions.

IF is hypothesised to act via inhibiting insulin/IGF-1 signalling (IIS) in *C. elegans*, and previous work suggested that IF does not markedly increase lifespan in *daf-2* mutants (Honjoh et al., 2009; Uno et al., 2013). This could be in part due to the behaviour of the DAF-16 transcription factor, which translocates from the cytoplasm to the nucleus in response to fasting but relocates back to the cytoplasm under prolonged food shortage (over 24 hours). Therefore, long-term fasting treatments likely bypass IIS and act via different pathways, including DAF-16 independent pathways such as AMPK (Greer & Brunet, 2009; Henderson & Johnson, 2001; Sun et al., 2017). Similarly, it is possible that different IF regimes also act via different pathways and vary in their effect on late-life health (Greer & Brunet, 2009).

Furthermore, the timing and duration of IF are likely to have a profound effect on lifespan and age-related pathologies. Despite the growing popularity of IF, the optimal timing for its application in model organisms remains unclear. Because dietary restriction has detrimental effects on growth and reproduction when applied during development (Runagall-Mcnaull et al., 2015; Tu & Tatar, 2003), research on IF mainly focused on its application during adulthood (Honjoh et al., 2009; Ulgherait et

al., 2021; Uno et al., 2013). In *C. elegans,* whole adulthood IF allows up to 57% increase in lifespan (Honjoh et al., 2009), while in *Drosophila melanogaster*, IF increases lifespan when applied during early-adulthood but not late-adulthood (Ulgherait et al., 2021). This highlights the importance of understanding how IF applied at different times across the life cycle affects late-life performance – survival and reproduction – and which molecular and physiological processes underlie these effects.

Here, we first explored age-specific effects of IF and showed that IF during early-adulthood, but not late-adulthood, additively increases the lifespan and late-life reproduction of *C. elegans* subjected to rIIS. Then, we investigated the impact of early-adulthood IF and rIIS on age-specific nuclear localisation of DAF-16 transcription factor and found that combined IF + rIIS treatment has a stronger effect across the life course because different treatments are more effective at different ages. IF leads to DAF-16 nuclear translocation during early-adulthood while rIIS causes a more moderate DAF-16 nuclear localisation during both early and late-adulthood. Therefore, when IF and rIIS are combined, this results in a more pronounced DAF-16 nuclear localisation from early to late-adulthood. Finally, we investigated the effects of both treatments on genome-wide gene expression in full-factorial design across three different ages (young, mid-life and old) and found that both similar and distinct pro-longevity physiological processes are modulated by early-adulthood IF and rIIS treatments at different ages. Early in life, shared mechanisms, such as suppressing translation, were activated in IF, rIIS and their combination. In contrast, late in life, more distinct mechanisms, such as the ones involved in immunity, were differentially regulated by IF, rIIS and their combination.

Methods

Experimental population

Three *C. elegans* wild-type strains: N2 (UK, Bristol), JT11398 (Portugal), EG4725 (USA) and the mutant strain: zIs356 [daf-16p::daf-16a/b::GFP + rol-6(su1006)] were obtained from Caenorhabditis Genetics Center (University of Minnesota, USA, funded by NIH Office of Research Infrastructure Programs, P40 OD010440) and stored at −80°C until use. Prior to the setup, the defrosted *C. elegans* strains were propagated for two generations on NGM agar supplemented with nystatin (100 µg/mL),

streptomycin (100 μg/mL), and ampicillin (100 μg/mL) to prevent infection, following standard recipes (Lionaki & Tavernarakis, 2013). The agar plates were seeded with the antibiotic-resistant *Escherichia coli* bacterial strain OP50-1 (pUC4K, from J. Ewbank at the Centre d'Immunologie de Marseille-Luminy, France). To standardize parental age and eliminate any effects from defrosting, we bleached the eggs from the grandparents of experimental individuals before conducting the experiments.

To decrease the expression of the insulin/IGF-1 signalling receptor homolog, *daf-2*, during adulthood, we fed late-L4 larvae with *E. coli* bacteria that expressed *daf-2* double-stranded RNA. As a control, we used RNase-III deficient, IPTG-inducible HT115 *E. coli* bacteria containing an empty vector plasmid (L4440) (Dillin et al., 2002; Lind et al., 2019). The RNAi clones were obtained from the Vidal feeding library (Source BioScience), which was created by M. Vidal lab at Harvard Medical School, USA. Prior to delivery, all clones were verified through sequencing.

Experimental treatments

To investigate the effects of intermittent fasting (IF) and reduced insulin signalling (rIIS) on *C. elegans* survival, early and late-life reproduction, we designed four distinct treatments (Figure 1). **"Control"** treatment group consisted of worms kept on standard NGM agar plates with an *E. coli* lawn that produced empty vector RNAi. **"IF"** treatment group consisted of worms exposed to a novel IF regimen during one week of their lifespan by being fed with empty vector RNAi. In this IF regime, we kept worms on foodless plates for 9 hours on alternate days. These foodless plates were not seeded with *E. coli* and contained no peptone, a crucial ingredient for bacterial growth. **"rIIS"** treatment group included worms treated like control treatment but maintained on *daf-2* RNAi producing *E. coli* lawn. Finally, **"IF + rIIS (combined)"** treatment group included worms treated like IF treatment but kept on *daf-2* RNAi producing *E. coli* lawn. Thus, we had a fully factorial design with two different feeding treatments and two different RNAi treatments. This approach allowed us to examine the combined effects of IF and rIIS on survival and reproduction.

Survival assays

To investigate the effects of IF and rIIS on worm survival, we explored how IF early and late in adulthood influences lifespan of worms in the absence and presence of rIIS. For early-adulthood IF, we kept the worms individually during their self-reproductive period. After reproduction ceased, we grouped the worms into cohorts of approximately 10 individuals. For late-adulthood IF, we always kept the worms in groups until they died. We monitored survival until the death of each worm, censoring any worms that were lost during the experiment.

Reproductive assays

We monitored self-reproduction during the first week of each worm's lifespan. For the Control and rIIS treatment groups, worms were transferred to new plates every 24 hours. For the IF and IF + rIIS treatment groups, worms were transferred to new plates twice on fasting days and every 24 hours on ad libitum (adlib) days. To ensure that the eggs laid during the 7-day reproductive period developed into larvae, we allowed the eggs to hatch and develop for two days. After this period, the hatched larvae were killed at 42°C for 3.5 hours and stored for counting later.

We monitored late-life reproduction by mating hermaphrodites with males after the self-reproductive period ended. Males were produced by exposing around 50 L4 hermaphrodites to 30°C heat shock for 5 hours on NGM plates and then incubating them at 20°C. On days 8 and 12, two males were paired with a single hermaphrodite for 24 hours, after which the males were discarded. The hermaphrodites were then transferred to fresh plates daily until the cessation of reproduction on day 14. Hermaphrodites were maintained on their corresponding RNAi treatments until the end of reproduction. We replicated this experiment by maintaining all experimental worms on empty vector RNAi producing *E. coli* during late reproduction (days 8-14). This was done to control for any possible effect that *daf-2* RNAi might have on male fertility and reproductive behaviour (Gems et al., 1998). In order to control for these effects, we report the results from the second replicate in the main text. The results from the first replicate are included in the Supplementary Material.

Visualisation of DAF-16 nuclear localisation

We used the zIs356 [daf-16p::daf-16a/b::GFP + rol-6(su1006)] strain to observe DAF-16 localisation. Synchronised worms expressing DAF-16::GFP were maintained under four different treatments (control, IF, rIIS, and combined treatments; see Figure 1) until days 1 and 7 when visualisation took place. For IF and combined treatments, visualization occurred following a 9-hour fasting period. Worms were anesthetized using 25 mM levamisole on a 2% agarose pad, with approximately 20-25 worms per slide. Data on DAF-16 localisation were collected as binomial outcomes. We classified worms exhibiting predominant nuclear DAF-16 localisation as '1', and those with primarily cytoplasmic localisation as '0'. This binary categorization was chosen to quantitatively assess the influence of different treatments on DAF-16 translocation dynamics.

RNA extraction

For gene expression analysis, we collected worms from four different treatments on days 1, 7 and 13. On days 1 and 7, IF and rIIS + IF worms were collected right after the 9-hour fasting period, and the Control and rIIS worms were collected around the same time for consistency, although they were not fasted. We had three biological replicates with a sample size of 100. In total, we collected 1200 worms (4 treatments x 3 timepoints x 3 biological replicates). RNA extraction was performed using TRIsure (Bioline, Cat. No. BIO-38032) following standard phenol-chloroform RNA extraction protocol. The purity of the isolated RNA was checked with Nanodrop, and the integrity was quantified using Agilent 2200 TapeStation System. RNA concentration was determined with Qubit using RNA HS Assay (Invitrogen™ Q33224).

Illumina RNA Sequencing

500 ng of total RNA was used to perform mRNA isolation using NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB Cat. No. E7490). Isolated mRNA material was used to prepare the libraries using NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina® (NEB, Cat No. E7760S) following the manufacturer’s instructions. The resulting libraries were quantified using Qubit dsDNA HS Assay (Invitrogen™ Q32851).

Data processing

Standard adapter sequences, common contaminants were removed from the paired-end reads using BBduk from the BBtools package version 37.62 (Bushnell, 2020). Positions with a Phred quality score of less than 15 were trimmed, and only reads with a length greater than 34 were retained. The quality of the reads was assessed using FastQC version v0.12.1 and MultiQC version 1.13 (Andrews, 2010; Ewels et al., 2016). The reads were aligned to the WBcel235 Caernohabditis elegans reference genome using STAR version 2.7.8a (Dobin et al., 2013) with default parameters, and a splice junction database was generated from the Ensenbl release 107 reference annotation. Gene-level read counts were quantified using feature Counts version v2.0.3 (Liao et al., 2014) in paired-end mode.

Differential expression analysis

Differential gene expression analysis was performed using DESeq2 version 1.32.0 (Love et al., 2014). Pairwise comparisons were conducted using a Wald test, comparing different combinations of genotypes (empty vector or *daf-2* RNAi), diets (ad libitum or intermittent fasting), and time points (days 1, 7, and 13) against each other. The independent filtering feature was used to eliminate weakly expressed genes. P-values were corrected for multiple testing using the false discovery rate (FDR) computed using the Benjamini-Hochberg method. Log fold change shrinkage with the "ashr" shrinkage estimator (Stephens, 2017) was utilized to control the log2 fold change estimates of genes with little information or high dispersion. Differentially expressed genes were identified based on log2 fold change cutoffs of 1 and FDR cutoff of 0.05.

Gene Ontology analysis

Gene Ontology enrichment analysis was performed separately for uniquely and commonly up- and down-regulated genes using clusterProfiler version 4.2.0 (Wu et al., 2021) together with org.Ce.eg.db version 3.17.0 (Carlson, 2019). P-values were corrected for multiple testing using the false discovery rate (FDR) computed using the Benjamini-Hochberg method. A 0.1 (10%) FDR threshold was applied to identify enriched biological processes relative to the background. Biological processes were deemed redundant if their GO terms consisted of overlapping sets of genes and belonged to the same branch.

To avoid redundancy, only the top parent terms (superset of child term) were preserved, and all child terms (subset of parent term) were filtered out from the results.

Statistical analysis

Self-fertilised reproduction - We fit generalized linear mixed-effects models using the glmmTMB package in R, with reproduction as the response variable, and food (AL vs. IF), RNAi (empty vector RNAi vs. rIIS) and their interaction as fixed effects. We also included batch as a fixed effect and the worm id as a random effect to account for any potential batch and worm id effects. We first used different families of models including Poisson, negative binomial type 1 and 2 and generalized Poisson distribution. Then, we compared the models using Akaike information criterion (AIC) and selected the best model based on the lowest AIC score. Finally, we used ANOVA on the best model to further investigate the significance of the fixed effects. This was done for all three populations (N2, USA and Portugal) separately.

Late-life mated reproduction – We fit generalized linear mixed-effects models using the glmmTMB package in R, with reproduction as the response variable, and food, RNAi and their interaction as fixed effects. We compared models with error distributions from four different families of models (Poisson, negative binomial type 1 and 2 and generalized Poisson distribution) and additional zero-inflation parameters. We selected the best model based on lowest AIC and performed ANOVA on it.

DAF-16 localisation – We used a generalized linear mixed-effects model with a binomial family to analyse the relationship between DAF-16 nuclear localisation and the interaction between RNAi, food, and day, while controlling for the worm id. We used ANOVA to test for the significance of fixed effects in our glmmTMB model.

We used the 'glmmTMB' package to fit generalized linear mixed-effects models using the 'glmmTMB' function, 'car' package to perform ANOVA analysis, 'ggplot2' package for data visualization, and 'MuMIn' package for model selection and inference (Barton & Barton, 2015; Fox et al., 2012; Magnusson et al., 2019; Wickham, 2011). All analyses were performed in R v. 3.6.2 (R Core Team, 2019)

Results

Early-adulthood IF and rIIS additively increase survival and slow down reproductive ageing

Considering that the timing of dietary interventions matters, we examined how early and late-adulthood IF influenced survival (Figure 2, Figure S1). Moreover, to understand whether IF operates through IIS as previously suggested, we explored the effects of IF in the absence and presence of rIIS. We found that early-adulthood IF increased survival both in the absence and presence of rIIS for wild-type N2 strain (Figure 2a). In contrast, late-adulthood IF did not affect survival, regardless of whether rIIS was present or not (Figure 2b). We also assessed the impact of early-adulthood IF on various strains of *C. elegans* to evaluate the generalisability of our findings beyond N2, the commonly used laboratory strain. For USA strain, early-adulthood IF increased survival both in the absence and presence of rIIS (Figure 2c). For Portugal strain, there was no effect of early-adulthood IF on the survival of control worms but it increased survival in the presence of rIIS (Figure 2d). In summary, our findings showed that early-adulthood IF increases survival when combined with rIIS and this was consistent across different worm strains.

Knowing that various dietary restriction can reduce reproduction, we also investigated how IF affects self-fertilised reproduction. As expected, IF significantly reduced reproduction (Figure 3a, IF: χ^2 = 76.41, df = 1, $p < 0.001$), whereas rIIS had no effect on reproduction in the N2 strain (rIIS: χ^2 = 0.65, df = 1, p = 0.421; IF x rIIS interaction: χ^2 = 2.41, df = 1, p = 0.121). These effects did not change when matricides were censored and were similar in two other *C. elegans* strains (Figure S2). In brief, we showed that intermittent fasting, like other dietary restriction regimens, decreases reproduction during its application.

To test whether IF slows down reproductive ageing similar to other dietary regimens and whether it is through the IIS pathway, we explored the effects of IF and rIIS on late-life mated reproduction. Our study revealed that both IF and rIIS independently led to increased late-life reproduction (IF: χ^2 = 16.5049, df = 1, $p < 0.001$; rIIS: χ^2 = 6.9117, df = 1, p = 0.009; IF x rIIS interaction: χ^2 = 0.0122, df = 1, p = 0.912). When IF and rIIS were combined, late-life reproduction was higher than all other

treatments (Figure 3b, Figure S3). Altogether, these data emphasize that IF and rIIS additively delay reproductive ageing.

Both IF and rIIS enhance DAF-16 nuclear localisation but in an age-specific manner

To delve into the potential mechanisms underlying the effects of IF and rIIS on ageing, we explored the nuclear localisation of DAF-16 transcription factor. DAF-16 activates longevity genes when it moves into the nucleus (Sun et al., 2017). We observed that IF significantly increased DAF-16 nuclear localisation during early-adulthood (Figure 4a, b), but this effect diminished gradually with advancing age (Figure 4c, d). On the other hand, rIIS also caused DAF-16 to move into the nucleus, but this effect was weaker compared to IF during early-adulthood (Figure 4a, b). Interestingly, unlike IF, rIIS continued to enhance DAF-16's localisation into the nucleus as worms grew older (Figure 4c, d). These varying effects at different ages led to a significant interaction between IF and rIIS ($\chi^2 = 5.12$, df = 1, $p = 0.02$) with marginally nonsignificant interactions between rIIS and age ($\chi^2 = 3.18$, df = 1, $p = 0.07$) and between IF and age ($\chi^2 = 3.10$, df = 1, $p = 0.08$). Thanks to the early-life effect of IF and the sustained impact of rIIS during both early and late in life, the combined treatment benefited from higher DAF-16 nuclear localisation throughout the lifespan (Figure 4).

IF, rIIS and their combined treatment exert age-dependent effects on gene expression.

We analysed gene expression changes in animals subjected to IF, rIIS or both at day 1, day 7 and day 13 by sequencing polyA(+) RNA in triplicate for each condition and time point. The principle component analysis (PCA) of samples shows that the largest variation in gene expression changes is due to the ageing of animals, as PC1 can separate all Day 1 animals from Day 7 and Day 13 (Figure S4). Next, we calculated the differential gene expression changes in worms exposed to rIIS, IF and combined treatments compared to control worms (Figure 5a). The largest gene expression changes are observed in day 1 animals, and the number of differentially expressed genes between conditions go down as the animals age for day 7 and day 13 samples.

To understand how DAF-16 activation contributes to the differential gene expression across treatments and time points, we analysed the presence of previously identified direct targets of DAF-16 (Schuster

et al., 2010), among the differentially expressed genes. In Day 1 samples, all treatments lead to the differential expression of many DAF-16 targets. On Day 7 and Day 13 samples, only a small number of DAF-16 targets are differentially expressed, suggesting that DAF-16-dependent regulation of gene expression changes from early to late life. Interestingly, on Day 13, IF treatment alone does not lead to differential expression of any DAF-16 targets, which aligns with our observation that IF treatment doesn't maintain DAF-16 nuclear localisation in late life.

Next, we analysed if different treatments lead to differential expression of unique or overlapping sets of direct DAF-16 targets (Figure 5b). During early adulthood (day 1), the combined treatment included direct DAF-16 target genes expressed both uniquely and shared with other individual treatments. As the worms aged (days 7 and 13), the response changed. For example, there were no direct DAF-16 targets shared between IF and the combined treatment in old worms. In contrast, several direct DAF-16 target genes were shared between rIIS and the combined treatment. There were also some DAF-16 targets that only responded to the combined treatment in older worms (Figure 5b). To conclude, when IF and rIIS were combined, they upregulated specific DAF-16 target genes that did not respond when those treatments were applied individually.

IF, rIIS, and their combination have age-specific effects on diverse biological functions later in life, particularly those related to immune response

We conducted a Gene Ontology analysis to explore the functions of genes differentially expressed with age across different treatments. First, we looked at genes differentially expressed in the combined treatment compared to the control (Figure 6, see Figure S5 & S6 for IF and rIIS treatments). During early adulthood (day 1), the combined treatment enhanced the expression of genes involved in developmental processes and simultaneously reduced the expression of genes associated with peptide metabolism (Figure 6a). Conversely, in late adulthood (days 7 and 13), distinct groups of genes associated with immune response exhibited varied expression patterns, some upregulated and others downregulated (Figure 6b, c).

To understand the shared and unique mechanisms between the combined treatment and the other two treatments, we used Venn diagrams for Gene Ontology (Figure 7-9). On day 1, the combined

treatment and IF together upregulated genes involved in processes such as nervous system development, cell fate commitment and cell projection. The combined treatment and rIIS together upregulated genes involved in processes such as ncRNA metabolic process, developmental process and organelle localisation. The combined treatment, IF and rIIS, upregulated genes associated with various developmental processes (Figure 7a). On the other hand, the combined treatment and IF together downregulated genes linked to immune response and metal ion transport on day 1. Interestingly, 27% of total downregulated genes were shared between all three treatments. These genes were involved in processes like peptide metabolism, ATP metabolic process and Ribonucleoprotein complex biogenesis (Figure 7b). In summary, during early adulthood, all treatments upregulated genes involved in developmental processes while downregulating genes associated with functions like peptide metabolism.

On day 7, the combined treatment differentially regulated several immune genes together with IF, rIIS, or both (Figure 8). Specifically, the combined and IF treatments together upregulated immune genes cnc-1, cnc-5 and clec-52, which are known to function through pathways beyond IIS, such as TGF-β and p38 MAPK pathways (Huang et al., 2020; Zhang & Zhang, 2009) (Figure 8a). However, the combined treatment also uniquely downregulated a substantial number of genes related to immunity (Figure 8b). Intriguingly, among them, cdr-4, previously reported to be upregulated by rIIS (Weng et al., 2023), exhibited the opposite response in the combined treatment. In brief, the combined treatment shares some immune genes with rIIS, but it also influences other genes not connected to insulin signalling, potentially through the effects of intermittent fasting.

On day 13, similar to the patterns observed on day 7, the combined treatment exhibited differential regulation of various immune genes, often together with either IF, rIIS, or both (Figure 9). Specifically, the combined and IF treatments jointly upregulated immune genes lys-8, spp-1 and ilys-2 (Figure 9a). Among them, lys-8 has been reported to be influenced by IIS, as well as TGF-β and p38 MAPK pathways (Alper et al., 2007). Interestingly, both spp-1 and ilys-2 were found to be downregulated by rIIS which is the opposite direction of how combined treatment and IF regulates them (Weng et al., 2023). So, only lys-8 was regulated in the same direction as rIIS. The combined

treatment and rIIS together upregulated genes involved in several processes including immune response, response to ROS and amino acid metabolism while downregulating mechanisms associated with immune response, organic acid metabolism and response to inorganic substance (Figure 9b). Intriguingly, no genes were downregulated by IF and the combined treatment together. In short, at older ages, the combined treatment shares the up and downregulation of certain immune genes with rIIS while also influencing genes typically not regulated by IIS, largely due to the presence of IF.

Discussion

In this study, we found that early-adulthood intermittent fasting (IF) and adulthood-only reduced insulin signalling (rIIS) additively increase lifespan and delay reproductive ageing. By examining the nuclear localisation of DAF-16 across different ages, we showed that the combined effects of early-adulthood IF and rIIS led to higher total nuclear localisation of this transcription factor, potentially maximizing its activity. Specifically, DAF-16 localisation in the combined treatment was mainly driven by IF instead of rIIS in early life, while the opposite was true later in life. Genome-wide RNA sequencing showed that IF and rIIS shape gene expression in an age-dependent manner. The GO terms analyses suggest that genes involved in immunity are potential candidates to explain the additive effects of IF and rIIS on ageing.

We found that IF during early-adulthood, but not late-adulthood, increases survival. This result aligns with previous work in *D. melanogaster* fruit flies, suggesting that IF has a greater impact on survival when applied early, but not late, in life (Catterson et al., 2018; Ulgherait et al., 2021). In fruit flies, 5 days fasting-2 days eating IF regimen increased lifespan when applied during early or mid-adulthood. However, when applied during late-adulthood, it shortened lifespan (Catterson et al., 2018). Similarly, 18 hours fasting-6 hours eating IF regimen also increased lifespan in fruit flies when applied during early, but not late, adulthood (Ulgherait et al., 2021). Our findings in worms further support the importance of the timing of the treatment, with early-adulthood being more beneficial.

Crucially, early-adulthood IF increased survival both in the presence and absence of rIIS, suggesting that IF may be working at least in part through a different route than IIS. Although early or

late-adulthood IF were not previously compared in *C. elegans*, it was shown that applying IF throughout the entire lifespan increases longevity (Honjoh et al., 2009; Uno et al., 2013). In these studies, a 2-day fasting-2-day eating regimen increased survival in *C. elegans* through an IIS-dependent mechanism, contrasting with our findings (Honjoh et al., 2009; Uno et al., 2013). Our IF regimen differs from previous studies, which used a 2-day intermittent fasting and eating cycle (Uno et al., 2013), whereas in our experiment, we applied 9 hours of fasting on alternate days. In fact, our IF regimen is perhaps more similar to the time-restricted feeding regimen used in fruit flies, which was suggested to work independently of inhibition of insulin-like signalling (Ulgherait et al., 2021).

Importantly, the impact of IF and rIIS on reproductive ageing was similar to their effect on survival. Both IF and rIIS increased late-life reproduction, leading to an additive effect when combined. However, early-adulthood IF decreased self-fertile reproduction while adulthood-only rIIS had no impact. These findings are consistent with previous research where various forms of dietary restriction, including intermittent fasting, reduced reproduction during food shortage while retarding reproductive ageing (C. Li et al., 2023; G. Y. Li & Zhang, 2019; Mendenhall et al., 2011; Sultanova et al., 2021). They also align with earlier findings indicating that knocking down IIS during adulthood does not adversely affect reproduction (Dillin et al., 2002; Lind et al., 2019). Likewise, the positive effect of rIIS on reproductive ageing is in line with previous studies showing that rIIS can decelerate reproductive ageing in *C. elegans* (Luo et al., 2009, 2010; Templeman et al., 2018). In contrast, in fruit flies, mutations impairing the IIS pathway can accelerate reproductive ageing (Hsu & Drummond-Barbosa, 2009). However, it is important to note that these mutants deactivate the IIS pathway throughout the entire lifespan, whereas we selectively applied rIIS during adulthood (Dillin et al., 2002), thus avoiding adverse effects on development, body size and reproduction (Carlsson et al., 2021; Gems et al., 1998; Lind et al., 2019). Additionally, it is possible that reducing IIS via RNAi does not deactivate it to an extent that it would impact reproduction. In fact, directly targeting DAF-2 receptor proteins using the auxin-inducible degradation system has been shown to decrease reproduction even when applied only during adulthood (Venz et al., 2021). In summary, the observed additive effect of IF and rIIS on both survival and late-life reproduction implies that these two

treatments either: i) synergistically engage common mechanisms (potentially at distinct time points or to different extents); ii) operate through distinct pathways; or iii) exhibit a combination of both.

To gain further insights into the mechanisms underlying our results, we first explored DAF-16 nuclear localisation. DAF-16 is a key transcription factor which acts downstream of nutrient-sensing signalling and germline signalling pathways (Sun et al., 2017). As a response to signals related to food availability or germline maintenance, it enters the nucleus and transcriptionally activates pro-longevity genes (Sun et al., 2017). In our experiment, DAF-16 is expected to remain in the cytoplasm in the presence of food and when the IIS pathway is active (control). However, when food is scarce (IF treatment), when the IIS is inactivated (rIIS treatment), or both (combined treatment), DAF-16 is expected to relocate to the nucleus (Henderson & Johnson, 2001). Our findings indicate that in early-adulthood, fasting causes DAF-16 to enter the nucleus more than rIIS. Conversely, fasting is no longer that effective in later adulthood, while rIIS worms maintain the same DAF-16 nuclear localisation as observed in their youth. DAF-16 has been shown to rapidly respond to changing food availability by moving into the nucleus shortly after fasting initiation and returning to the cytoplasm upon resuming a normal diet (Henderson & Johnson, 2001). Hence, in our IF treatment, DAF-16 is expected to be in the nucleus only during fasting periods (9 hours on alternate days) and back in the cytoplasm during non-fasting periods. In the rIIS treatment, DAF-16 is expected to be in the nucleus constantly, at least during the first week of lifespan, because IIS is constantly reduced by RNAi treatment. In fact, deactivating the IIS pathway has been shown to cause DAF-16 to stay in the nucleus even until later ages, as late as day 11 (Weinkove et al., 2006). In the combined treatment, worms experience both intermittent nuclear localisation due to IF and the constant nuclear localisation due to rIIS, and benefit from both treatments. These findings support the hypothesis that IF and rIIS are modulating DAF-16 localisation in an age-dependent manner, leading to potentially increased DAF-16 activity in combined treatment. This is further supported by the fact that some direct DAF-16 targets are only expressed in the combined treatment at later ages.

To better understand the mechanisms driving the effects of the combined treatment, we explored the function of genes that were differentially expressed by IF, rIIS and their combination at

different ages. Our findings revealed that IF and rIIS activate both similar and distinct mechanisms depending on the age of the worms. For example, during early adulthood, all treatments downregulate numerous genes involved in peptide metabolism, a change known to promote longevity (Hansen et al., 2007; Syntichaki et al., 2007). Likewise, in later adulthood, they all downregulate a gene involved in xenobiotic response, cyp-33C8, whose downregulation increases survival under oxygen deprivation (Ladage et al., 2016). This implies that similar mechanisms could contribute to extending lifespan across IF, rIIS, and combined treatments, particularly early in life.

However, later in life, the combined treatment uniquely combines distinct and shared immune mechanisms originating from both IF and rIIS. It upregulates certain immune response genes while downregulating others. Notably, numerous immune genes upregulated in the combined treatment together with IF are not related to the IIS pathway, indicating that IF can trigger additional immune responses through alternative mechanisms (Huang et al., 2020; Zhang & Zhang, 2009). Although immune response is crucial for survival, excessive production of antimicrobial peptides can actually harm the host due to elevated inflammation (Licastro et al., 2005). Therefore, finetuning the age-specific expression of immunity genes has an important role in regulating lifespan. In fruit flies, selection for late-life reproduction and longevity led to enrichment of immunity genes, suggesting that age-specific regulation of these genes contributes to extended lifespan (Fabian et al., 2018). The simultaneous control of various immune response pathways by the combined treatment could provide insight into its additive effect on survival and late-life reproduction.

Nevertheless, it is important to note that the downregulation of certain genes involved in immunity by the combined treatment could be detrimental and pose challenges in the presence of infectious threats. For example, we found that lys-3, a lysozyme induced in response to pathogenic bacteria (Boehnisch et al., 2011), is consistently downregulated in all three treatments during late adulthood, despite evidence that its overexpression can extend lifespan in *C. elegans* (Gallotta et al., 2020). Therefore, it becomes evident that not all mechanisms regulated by the combined treatment are universally beneficial. Finetuning immune response genes is indeed challenging but it could be a powerful way to effectively slow down ageing.

In conclusion, our study sheds light on the complex interplay between intermittent fasting and reduced insulin signalling in modulating lifespan and reproductive ageing. We observed that early-adulthood IF and adulthood-only rIIS additively enhance both survival and late-life reproduction. The dynamic regulation of DAF-16 nuclear localisation in an age-specific manner suggests a finely tuned mechanism through which IF and rIIS exert their effects. Our exploration of age-specific gene expression patterns reveals shared and unique regulatory pathways that may contribute to the additive effects of IF and rIIS. Notably, the presence of distinct mechanisms controlling immune response genes suggests that IF and rIIS can also modulate separate processes. Overall, our findings provide novel insights into the temporal dynamics of these interventions and their potential mechanisms of action. They also highlight the complex interactions between factors influencing ageing and lifespan. Further investigations into the specific roles of identified genes and pathways are necessary to unravel the full extent of their contributions to the observed additive effects. Particularly, the immune response mechanisms that are modulated by IF and rIIS are potential areas for ageing research. Furthermore, to broaden the applicability of these findings, future research should extend its focus to explore the impact of IF and rIIS on ageing across different organisms, including humans.

Acknowledgements

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Author Contributions

ZS, AS, KH, AA and AAM conceived and designed the experiments and wrote the draft manuscript. ZS conducted the experiments and microscopy. ZS and KH collected animals for RNA extraction. KH extracted and purified RNA and prepared sequencing libraries. AS analysed RNA-Seq data and prepared figures. HC, ZC and DC helped with data collection.

Figures

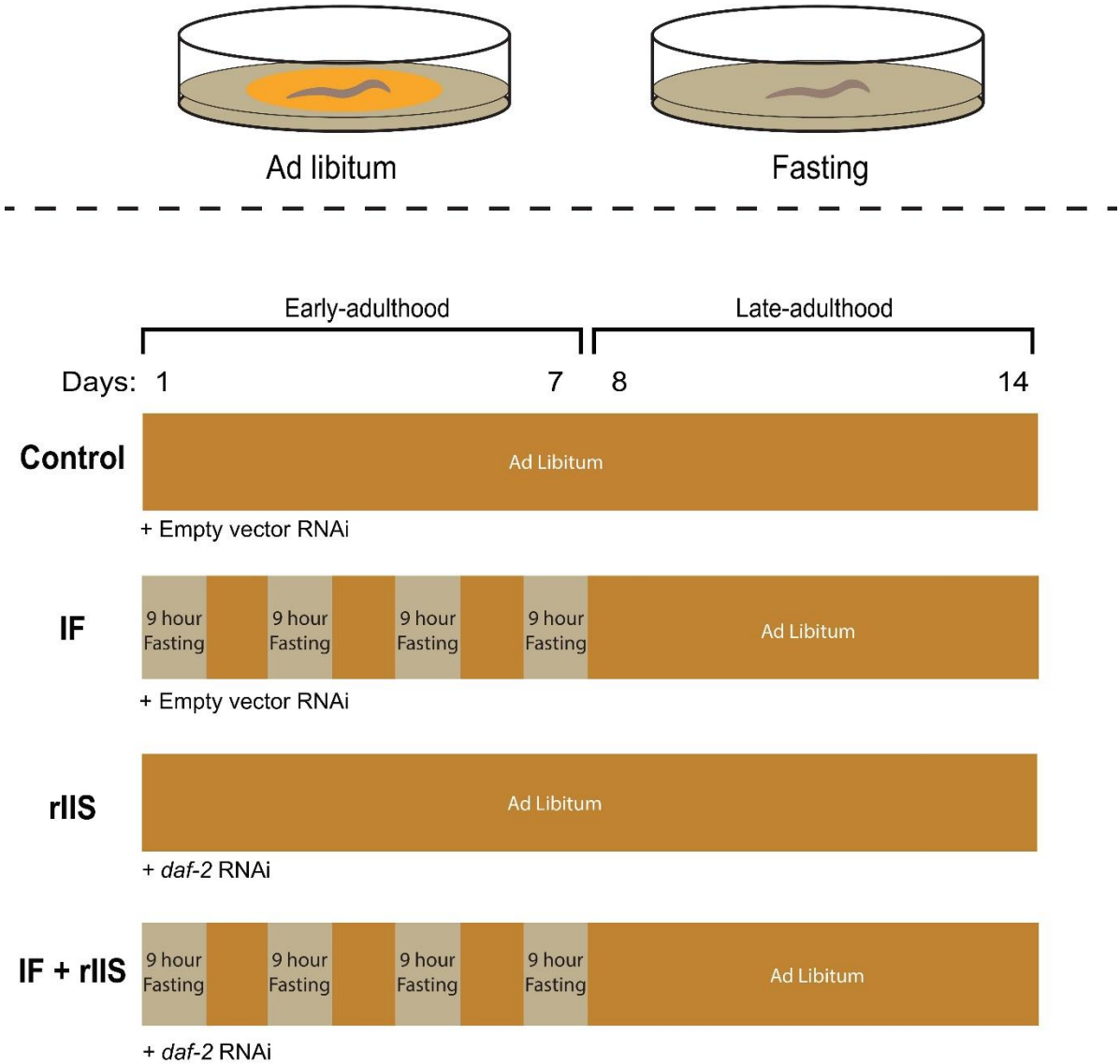


Figure 1. Schematic illustration of the experimental treatments. Late L4 stage worms were subjected to four different experimental treatments (Control, IF, rIIS and combined). Early-adulthood intermittent fasting involved fasting for 9 hours on alternate days during the initial week of lifespan. Late-adulthood intermittent fasting involved fasting for 9 hours on alternate days during the second week of lifespan. Control and IF treatments were fed with *E. coli* that produces empty vector RNAi; rIIS and combined treatments were fed with *E. coli* that produces *daf-2* RNAi.

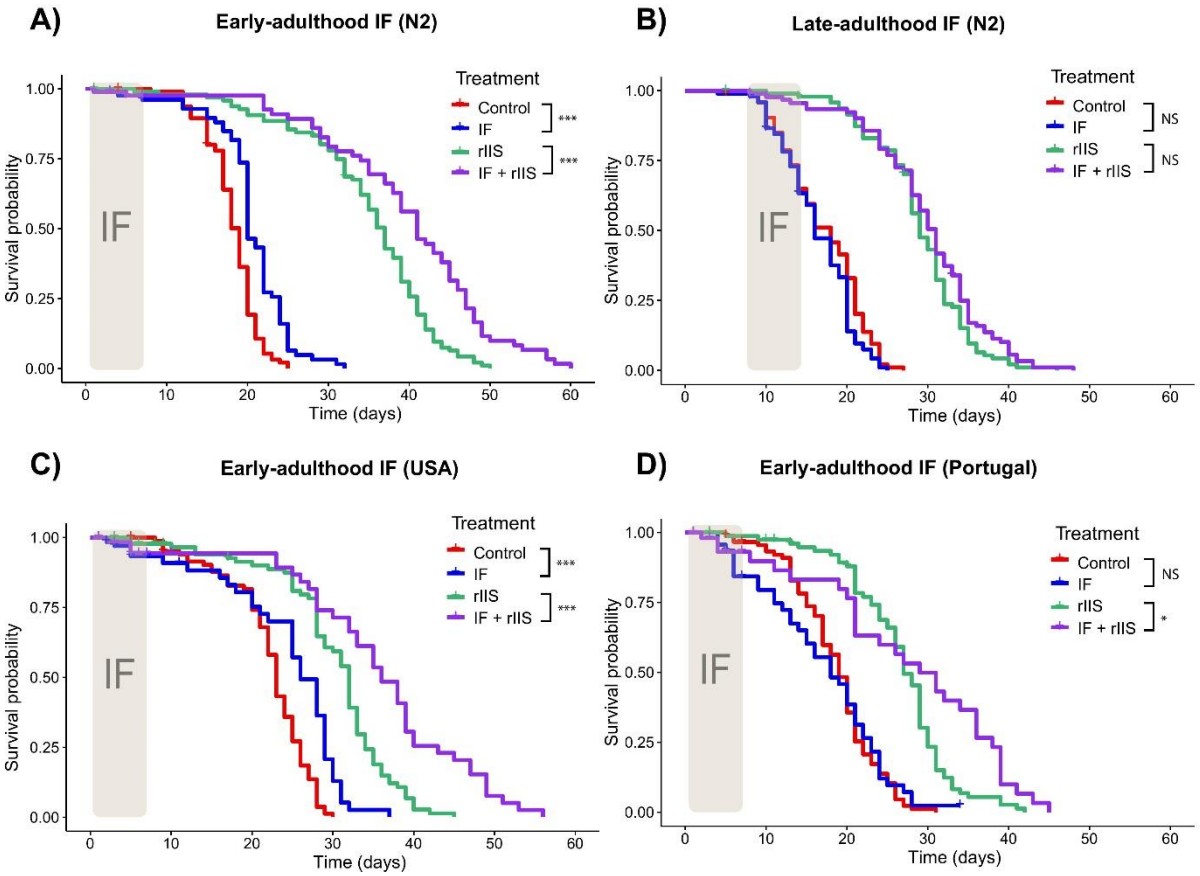


Figure 2. Effect of IF and rIIS on survival. Effect of early and late-adulthood IF on survival of N2 worms with and without rIIS (A and B, respectively). Effect of early-adulthood IF on survival of USA and Portugal strains with and without rIIS (C and D, respectively). Significance in the divergence of survival curves was assessed using log-rank tests, with statistical significance represented as follows: NS indicates $P > 0.05$, * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, and *** indicates $P \leq 0.001$.

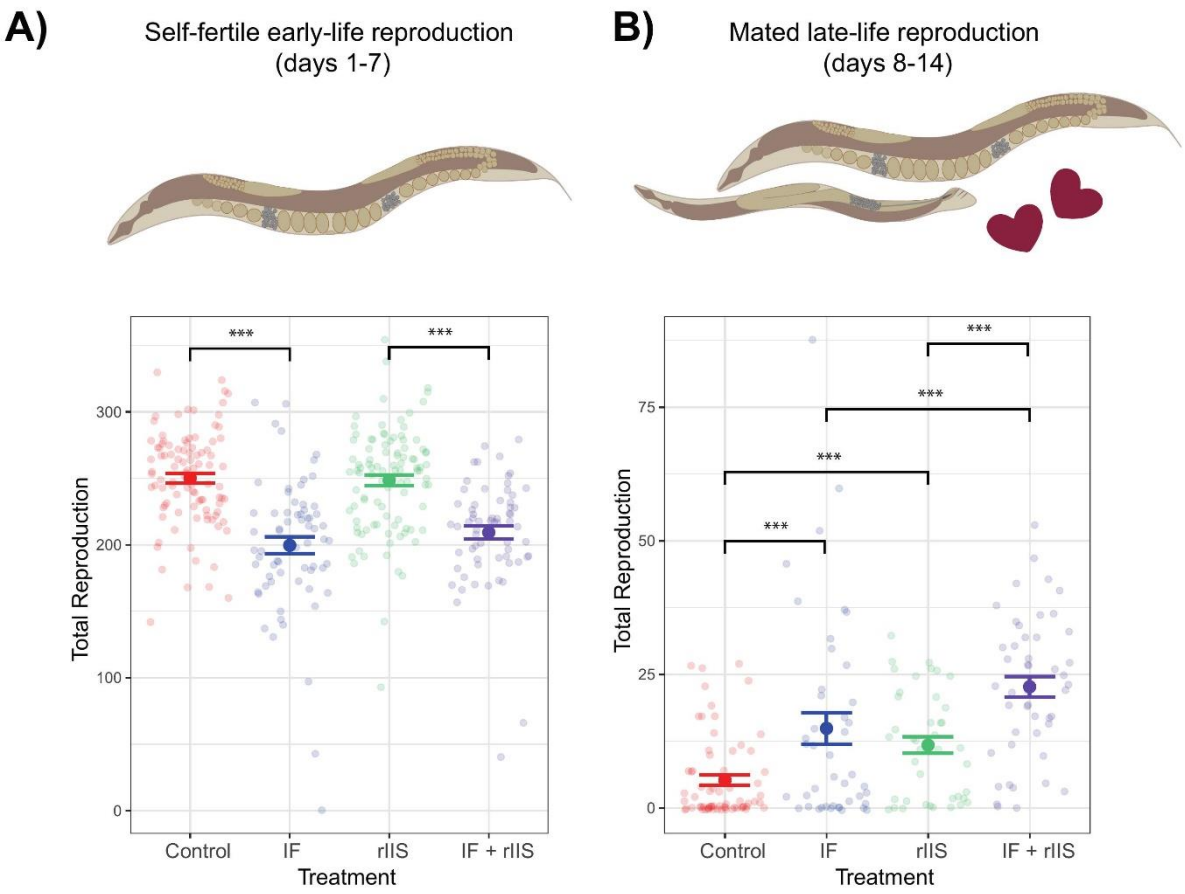


Figure 3. Effect of IF and rIIS on reproduction. For early-life self-fertile reproduction (A), differences between treatments were assessed by Tukey's HSD test with Benforini-Hochberg correction for multiple testing. For late-life mated reproduction (B), differences between treatments were assessed by Dunn test with Benforini-Hochberg correction for multiple testing. NS indicates $P > 0.05$, * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, and *** indicates $P \leq 0.001$.

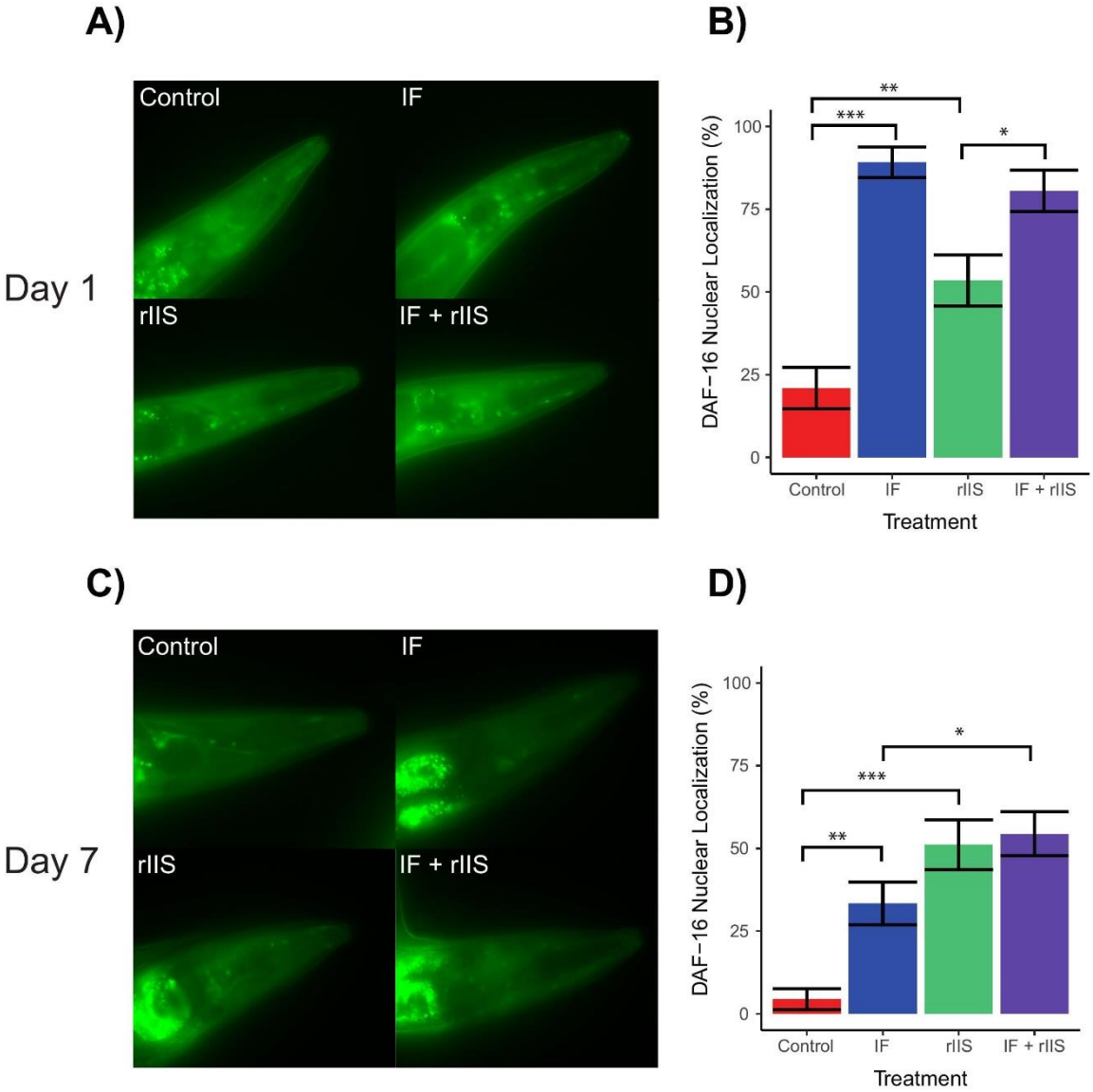
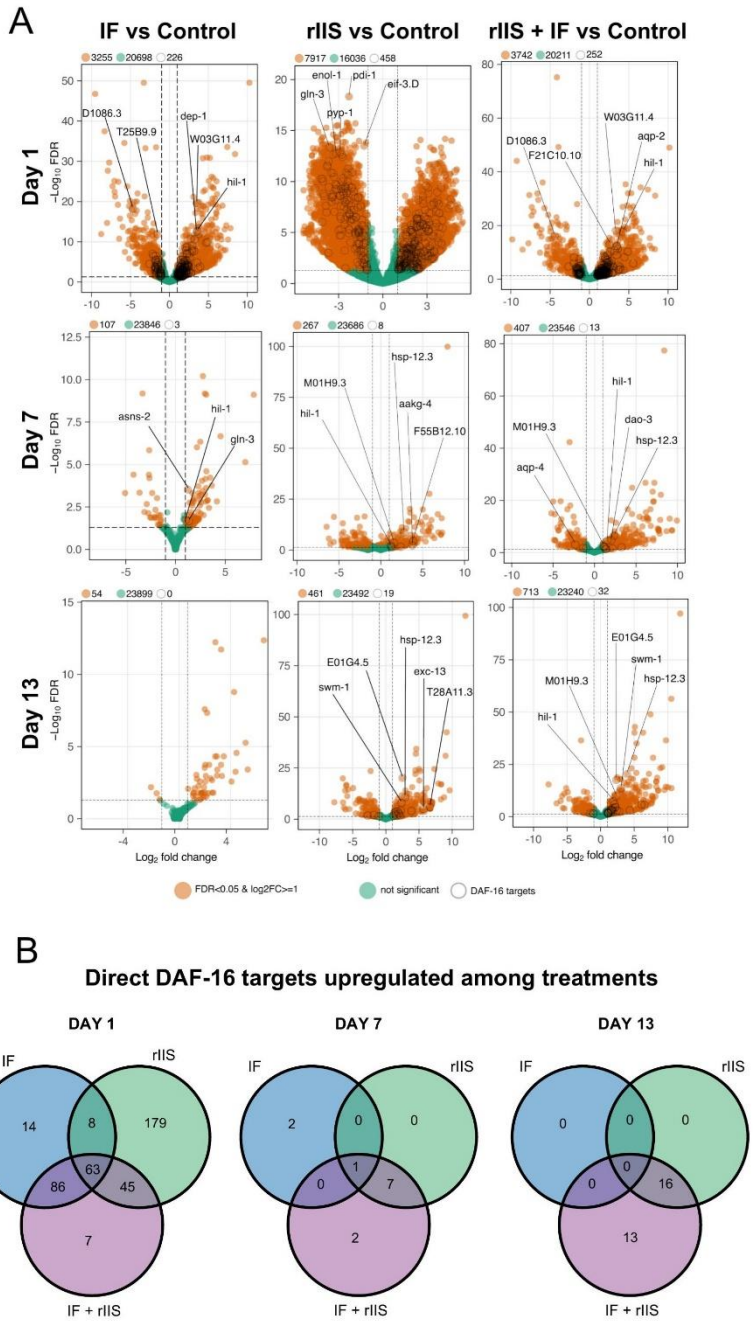


Figure 4. Effect of IF and rIIS on DAF-16 nuclear localisation. Photographs and corresponding bar plots illustrating the effect of experimental treatments on the nuclear localisation of the DAF-16 transcription factor tagged with GFP, assessed on day 1 (panels A and B) and day 7 (panels C and D). The differences between treatments were evaluated using chi-square tests with Benforini-Hochberg correction for multiple testing. NS indicates $P > 0.05$, * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, and *** indicates $P \leq 0.001$.



485 **Figure 5. Effect of IF and rIIS on age-specific gene expression.** Volcano plots comparing
486 differentially expressed genes in combined treatment compared to control. Top-5 direct DAF-16
487 targets that were differentially expressed in each treatment with respect to statistical significance were
488 shown on each plot. DAF-16 direct targets were taken from a previous study that identified them by
489 using chromatin profiling by DNA adenine methyltransferase identification, DamID (Schuster et al.,
490 2010) (A). Venn diagrams showing the number of direct DAF-16 targets that are differentially
491 regulated across IF, rIIS and combined treatments compared to control (Schuster et al., 2010) (B).

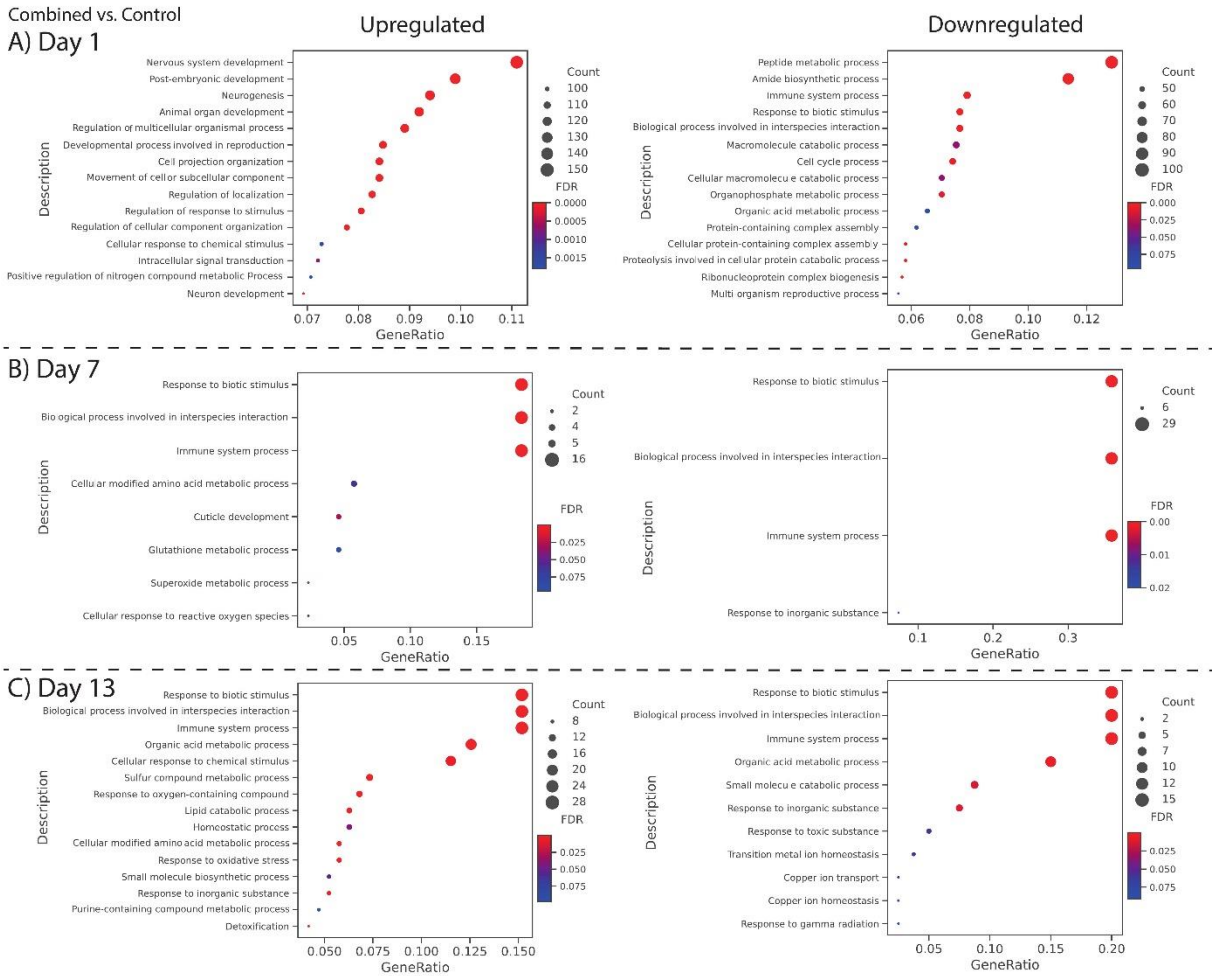


Figure 6. Gene Ontology analysis of differentially expressed genes in the combined treatment. GO term analyses showing the functions of genes up and down-regulated by the combined treatment compared to control across day 1 (A), day 7 (B) and day 13 (C).

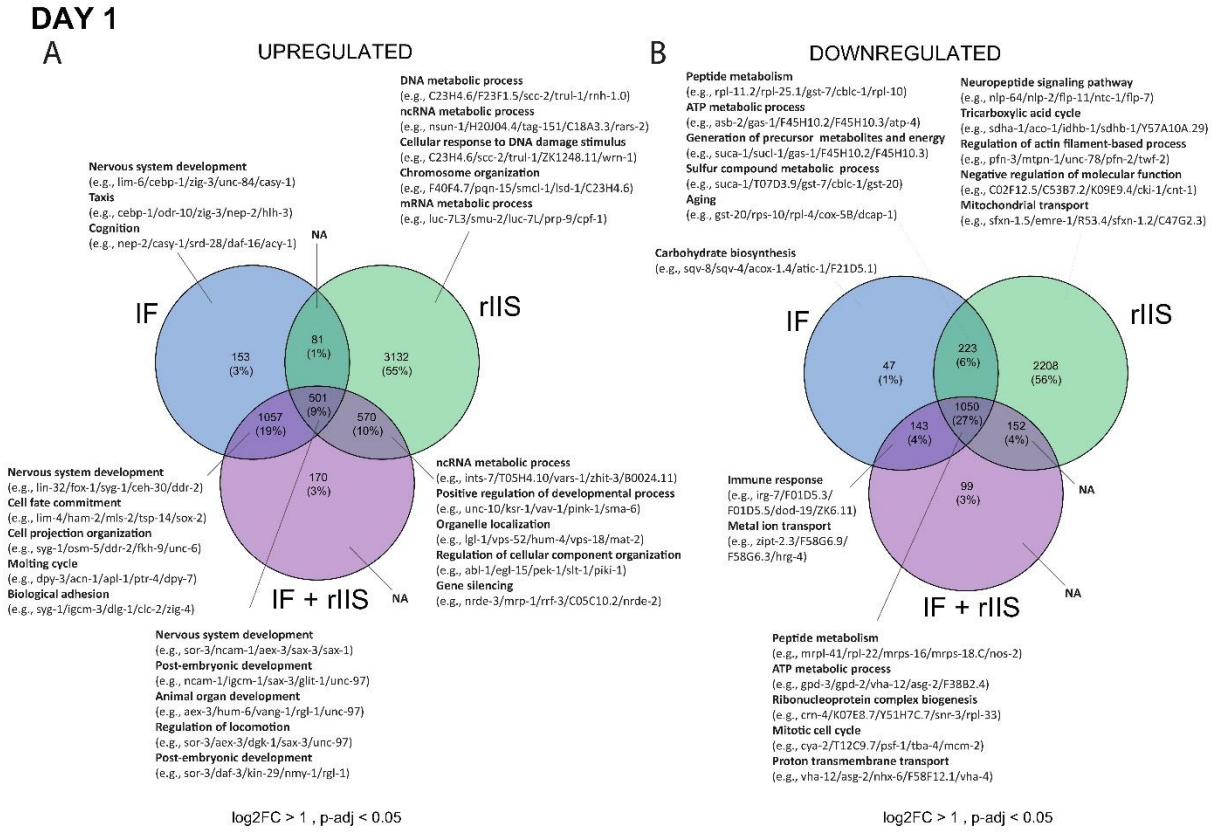


Figure 7. Gene Ontology analysis of differentially expressed genes across IF, rIIS and combined treatments on day 1. GO term analyses showing the functions of genes up-regulated (A) and down-regulated (B) by IF, rIIS and combined treatments compared to control on day 1.

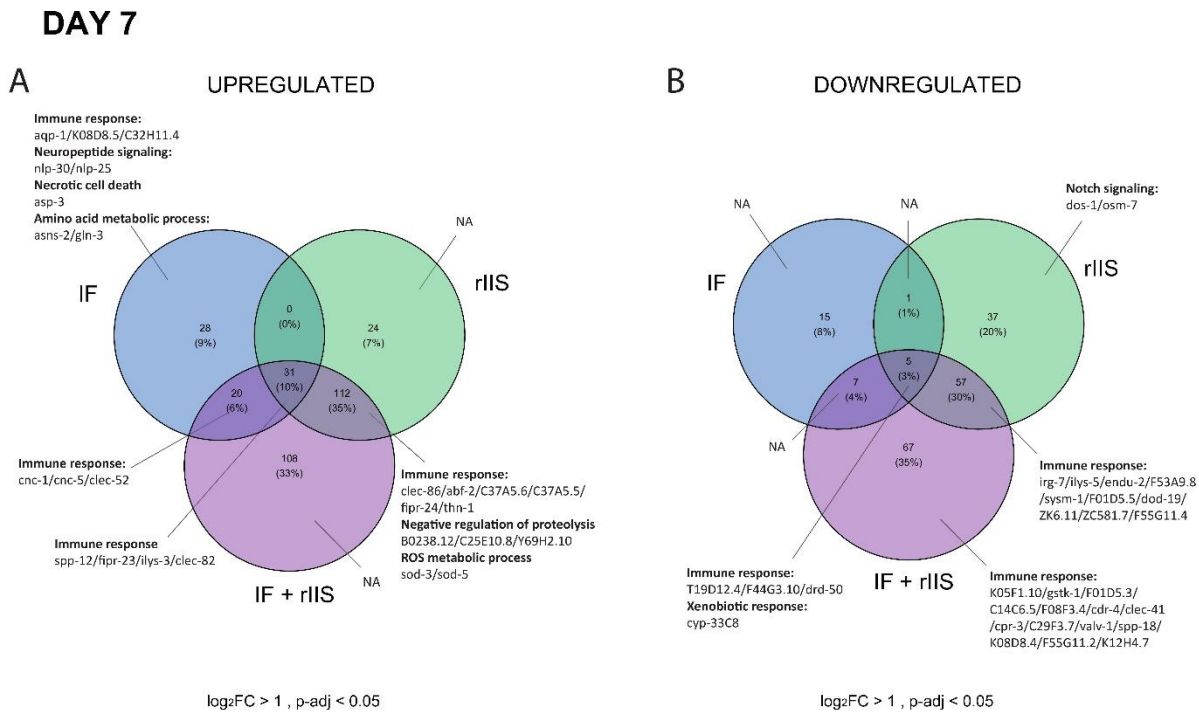


Figure 8. Gene Ontology analysis of differentially expressed genes across IF, rIIS and combined treatments on day 7. GO term analyses showing the functions of genes up-regulated (A) and down-regulated (B) by IF, rIIS and combined treatments compared to control on day 7.

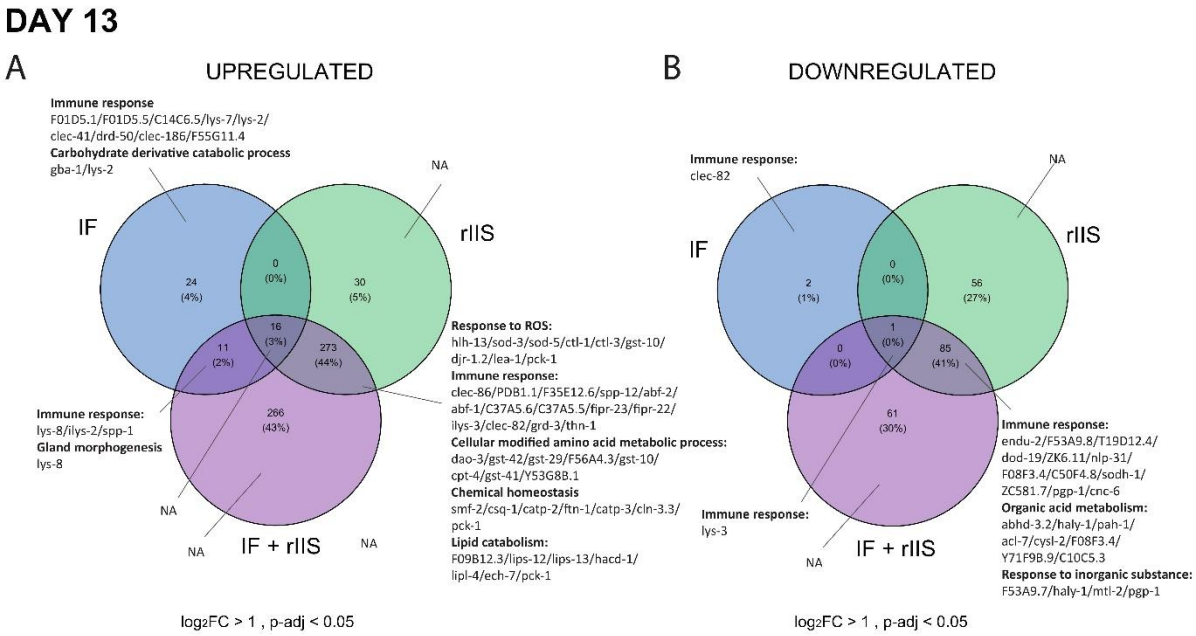


Figure 9. Gene Ontology analysis of differentially expressed genes across IF, rIIS and combined treatments on day 13. GO term analyses showing the functions of genes up-regulated (A) and down-regulated (B) by IF, rIIS and combined treatments compared to control on day 13.

Figure Legends

Figure 1. Schematic illustration of the experimental treatments. Late L4 stage worms were subjected to four different experimental treatments (Control, IF, rIIS and combined). Early-adulthood intermittent fasting involved fasting for 9 hours on alternate days during the initial week of lifespan. Late-adulthood intermittent fasting involved fasting for 9 hours on alternate days during the second week of lifespan. Control and IF treatments were fed with *E. coli* that produces empty vector RNAi; rIIS and combined treatments were fed with *E. coli* that produces *daf-2* RNAi.

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shown on each plot. DAF-16 direct targets were taken from a previous study that identified them by using chromatin profiling by DNA adenine methyltransferase identification, DamID (Schuster et al., 2010) (A). Venn diagrams showing the number of direct DAF-16 targets that are differentially regulated across rIIS, IF and combined treatments compared to control (Schuster et al., 2010) (B).

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Figure 7. Gene Ontology analysis of differentially expressed genes across IF, rIIS and combined treatments on day 1. GO term analyses showing the functions of genes up-regulated (A) and down-regulated (B) by IF, rIIS and combined treatments compared to control on day 1.

Figure 8. Gene Ontology analysis of differentially expressed genes across IF, rIIS and combined treatments on day 7. GO term analyses showing the functions of genes up-regulated (A) and down-regulated (B) by IF, rIIS and combined treatments compared to control on day 7.

Figure 9. Gene Ontology analysis of differentially expressed genes across IF, rIIS and combined treatments on day 13. GO term analyses showing the functions of genes up-regulated (A) and down-regulated (B) by IF, rIIS and combined treatments compared to control on day 13.

Figure S1. Effect of IF and rIIS on survival including matricides. Effect of early and late-adulthood IF on survival of N2 worms with and without rIIS (A and B, respectively). Effect of early-adulthood IF on survival of USA and Portugal strains with and without rIIS (C and D, respectively). Significance in the divergence of survival curves was assessed using log-rank tests, with statistical significance represented as follows: NS indicates $P > 0.05$, * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, and *** indicates $P \leq 0.001$.

Figure S2. Effect of IF and rIIS on early-life reproduction. Early-life reproduction including matricide across three different populations (A), early-life reproduction without matricide across three different populations (B), daily reproduction across three different populations (C).

Figure S3. Effect of IF and rIIS on late-life reproduction. Late-life reproduction including matricide (A), late-life reproduction without matricide (B), daily reproduction (C) in worms fed with empty vector RNAi producing *E. coli* during mating. Late-life reproduction including matricide (D), late-life reproduction without matricide (E), daily reproduction (F) in worms fed with empty vector and *daf-2* RNAi producing *E. coli* during mating.

Figure S4. Principal Component Analysis (PCA) of RNA-seq Samples. PCA plots illustrating the gene expression profiles for samples under four different treatments: Control, IF, rIIS, and Combined (IF + rIIS). The plots compare the gene expression patterns at three distinct time points – days 1, 7, and 13 – to demonstrate how the treatments influence genetic activity over time.

Figure S5. Gene Ontology analysis of differentially expressed genes in the IF treatment. GO term analyses showing the functions of genes up and down-regulated by the IF treatment compared to control across day 1 (A), day 7 (B) and day 13 (C).

Figure S6. Gene Ontology analysis of differentially expressed genes in the rIIS treatment. GO term analyses showing the functions of genes up and down-regulated by the rIIS treatment compared to control across day 1 (A), day 7 (B) and day 13 (C).

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