Age deceleration and reversal patterns in *Caenorhabditis elegans* dauer diapause

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List of Abbreviations

ABC transporters	ATP-binding cassette transporter
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
AP	antagonistic pleiotropy
ARD	adult reproductive diapause
ATP	Adenosine triphosphate
BCAA	branched-chain amino acid
BER	base excision repair
BP	biological process
C. elegans	*Caenorhabditis elegans*
CoA	coenzyme A
CPD	cyclobutane pyrimidine dimer
CpG	cytosine-phosphate-guanine
D1	day 1 dauer
Daf-c	dauer-constitutive
DDR	DMA damage response
DEG	differentially expressed gene
DNA	Deoxyribonucleic acid

DR	dietary restriction
DREAM complex	dimerization partner, RB-like, E2F and multi-vulval class B
DS	disposable soma
GG-NER	global-genome NER
GLTD	gene-length-dependent transcriptional decline
GO	Gene Ontology
GPCR	G protein-coupled receptor
GSEA	gene set enrichment analysis
h	hours
H3K9	histone H3 lysine 9
HR	homologous recombination
ICL-R	interstrand cross-link repair
IGF-1	insulin-like growth factor-1
IIS	insulin/insulin-like growth factor-1signaling
KEGG	Kyoto Encyclopedia of Genes and Genomes
MA	mutation accumulation
MAPK	Mitogen-activated protein kinase
MPTR	maturation phase transient reprogramming
mRNA	messenger RNA
mTOR	mechanistic/mammalian target of rapamycin
mTORC1	mechanistic/mammalian target of rapamycin complex 1

mTORC2	mechanistic/mammalian target of rapamycin complex 2
NADH	nicotinamide adenine dinucleotide
ncRNA	non-coding RNA
NER	nucleotide excision repair
NHEJ	non-homologous end joining
NMR	naked more-rat
ORA	overrepresentation analysis
OSKM	Oct4, Sox2, Klf4, c-Myc
PBA	predicted biological age
PC	principal component
PCA	principal component analysis
PI3K	phosphatidylinositol 3-kinase
RNA	ribonucleic acid
RNAPII	RNA polymerase II
rRNA	ribosomal RNA
TC-NER	transcription-coupled NER
TF	transcription factor
TGF-beta	Transforming growth factor beta
tRNA	transfer RNA
UPR	unfolded protein response
UPS	ubiquitin-proteasome system

UV	ultraviolet
V-ATPase	vacuolar H^+-ATPase
WT	wild-type

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Summary

While the aging process affects almost all species, some organisms show extraordinary ability to halt the aging process or even undergo a decrease in biological age, or rejuvenation. The nematode *Caenorhabditis elegans* has several diapause states including dauer diapause. *C. elegans* larvae enter dauer diapause during unfavorable conditions such as overcrowding or starvation. Dauer was originally termed as “non-aging” because of the lack of negative consequences in postdauer animals.

Here, we establish an experimental setup in which we test effects of dauer of different duration on postdauer fitness and report no changes in recovery dynamics, lifespan, or brood size. Using the BiT Age transcriptomic clock, we observe an increase in predicted biological age as the diapause progress and a reversal during recovery from dauer, although only worms arrested for up to 4 days revert their biological age completely.

We characterize transcriptomic patterns occurring in dauer and during recovery in a duration-dependent manner, highlighting the importance of protein homeostasis and the role of V-ATPase in dauer exit. We also compare aging patterns in dauer and adult worms with the same genetic background. Furthermore, we investigate the impact UV-treatment has on normal and NER-deficient worms and gene-length-dependent gene expression.

Taken together, our results suggest that *C. elegans* dauer diapause is an example of slow aging and dauer exit is a naturally occurring rejuvenation process.

Chapter 1 Introduction

1.1. The phenomenon of aging

Aging, its mechanisms, and evolution have long remained difficult to grasp, describe and disentangle from one another, and, importantly, make predictions or address the root causes. Aging can be defined as a progressive decline of functional capacity within adult organisms that leads to an increase in mortality rate and a fertility decline (Kirkwood & Shanley, 2010). From the perspective of the organism's survival, it is not apparent why aging would be permitted by natural selection. However, the natural selection argument only makes sense under the assumption that an organism will survive long enough to experience aging when according to the classical evolutionary theories of aging, extrinsic mortality is a much stronger predictor of organismal mortality than aging (Hamilton, 1966; Kirkwood, 1997; Medawar, 1952; Williams, 1957).

We will discuss classical theories of aging and their implications, evidence that supports or contradicts them, and some of the newly emerging aging theories.

1.1.1. Theories of aging

There are three theories that are typically referred to as classical aging theories. All three of them rely on similar premises and are complementary rather than mutually exclusive. First of them is the mutation accumulation (MA) theory proposed by Medawar in 1952 (Medawar, 1952). It states that most organisms do not survive long past sexual maturity due to the high rate of extrinsic mortality, therefore, there is no evolutionary pressure to select against mutations that would lead to negative

consequences later in life (selection shadow) which allows such mutations to accumulate without resistance. In 1957, Williams developed the antagonistic pleiotropy (AP) theory that expanded on MA theory proposing that the selection shadow does more than creating lack of evolutionary resistance. In fact, it creates conditions in which mutations that are beneficial in early life but detrimental in late life will be selected for (Williams, 1957). The disposable soma (DS) theory was formulated originally in 1977 by Kirkwood (Kirkwood, 1977) and developed further in Kirkwood's later works and focuses more on the interplay between resource availability, environmental hazards, the cost of reproduction and maintenance (Kirkwood, 1993). It emphasizes that in dangerous conditions and resource scarcity an organism would benefit more from allocating available resources into reproduction rather than maintenance of somatic tissues as the latter might never pay off due to high extrinsic mortality rates and detracts from the resources that could have been invested into more efficient reproduction.

All the classical theories suggest that an increase in extrinsic mortality will lead to selection of shorter life spans, and AP and DS theories imply an existing trade-off between long lifespan and fecundity.

A large amount of research has since been conducted to further investigate the dynamics between aging and external factors and most of the results support the prediction that follows from the classical theories.

Flying animals are a great subject to research the interplay between aging and extrinsic mortality as they occupy a less populated ecosystem which is associated with lower predation and disease levels, and have a unique ability to escape dangerous

situations as well as access a wider range of environments and, therefore, have access to more resources (A. A. Johnson et al., 2019). Birds live approximately three times longer than non-flying eutherian mammals that are maintained in the same captive conditions despite having increased metabolic rates compared to mammals of the same size as well as blood sugar and temperature levels (Holmes & Austad, 1995). A comparative study of 271 bird species revealed a strong correlation between survival rate and longevity after adjusting for body mass and variation due to sampling effort (Møller, 2006).

The notion that living in safer conditions increases life span is further supported by a study on arboreality that demonstrates an increase in life span for arboreal mammals compared to terrestrial mammals (Shattuck & Williams, 2010). This observation is applicable to all subgroups with two exceptions: Euarchonta and Metatheria subgroups. It's proposed that arboreal history of currently terrestrial species allowed them to develop an increased life span.

In *Caenorhabditis elegans* (*C. elegans*), a mutation in *age-1* gene increases adult lifespan by up to 80% but leads to a decrease in self-fertility (Friedman & Johnson, 1988). Additionally, *age-1* mutants demonstrate reduced fitness compared to wild-type worms when subjected to starvation intended to mimic natural conditions (Walker et al., 2000).

However, some studies demonstrate results that contradict predictions of the classical theories. Namely, the studies on guppies (Reznick et al., 2004) and nematodes (H. Chen & Maklakov, 2012) demonstrate that lower rates of aging and longer life spans can be selected for by high extrinsic mortality. Chen & Maklakov further emphasize

that although high random mortality leads to the evolution of the shorter life span, this is reversed when the mortality is condition-dependent. Moreover, in a stochastic modelling studdy (Shokhirev & Johnson, 2014), extrinsic mortality had a different effect on the lifespan depending on the cost of mating and energy resources available, further highlighting the true complexity of the matter.

One would be remiss to not mention the idea of programmed aging and its critiques. It was originally formulated by Weismann in 1889 and although it still receives some support (Pamplona et al., 2023), the arguments and reasoning that is used to support this idea are often flawed. A detailed examination of pro-programmed aging arguments demonstrated them to be mathematically or conceptually wrong or even if programmed death did evolve in certain models, it was based on other factors and conditions (Kowald & Kirkwood, 2016).

Recently emerging aging theories include hyperfunction, developmental (Gems, 2022) and deleteriome (Gladyshev, 2016) theories. According to the hyperfunction theory, aging is a quasi-program, an aimless derivative of growth program, that is itself is not programmed but can me modulated (Blagosklonny, 2006, 2022a). de Magalhães' developmental theory posits that aging occurs partially due to genetic programs that evolved to regulate development but persist into adulthood causing dysfunction and partially due to stochastic factors such as DNA damage and junk accumulation (Magalhães, 2012). Finally, the concept of deleteriome attempts to unify different aging theories including AP, MA, programmed, and others by proposing imperfectness as a basis of aging: since all biological molecules and processes are imperfect (Gladyshev, 2013), deleterious changes and substances will inevitably ensue that include but are not limited to molecular damage (Gladyshev, 2016).

While there is no one theory that everyone in the scientific community can agree on, one should keep in mind the variety of ideas and interpretations of the aging process in order to access results of their research in the most comprehensive and the least biased manner.

1.1.2. Special cases in aging

While aging is a process that affects almost all species, some organisms demonstrate negligible senescence (Ruby et al., 2018; Stenvinkel & Shiels, 2019; Yun, 2021) or an ability to modulate their rate of aging (Pinho et al., 2022).

1.1.2.1. Extreme longevity and negligible senescence

Naked mole-rat (NMR) is the longest-lived rodent and an outstanding model organism for aging research (Buffenstein, 2005). In captivity, NMR's lifespan exceeds 30 years (Lewis & Buffenstein, 2016) which is approximately 5 times longer than expected for their body size (Edrey et al., 2011). Even in the wild, breeding females were reported to remain in the same colony for over 15 years, while non-breeding individuals remain for 2 to 3 years (Hochberg et al., 2016). NMR do not show an age-related decline in fertility and no signs of menopause (Lewis & Buffenstein, 2016). Unlike for human, horse, and mouse, the levels of mortality hazard for NMR do not increase for at least up to 30 years, making them a unique, supposedly, non-aging species among mammals (Ruby et al., 2018). Other studies proposed conserved splicing regulation, stable alternative splicing patterns, and Nrf2 signaling as contributors to NMR's negligible senescence (B. P. Lee et al., 2020).

Turtles are popularly characterized as non-aging and resilient to the passage of time. While such description is not accurate for every testudines species (Warner et al., 2016), it does hold true for approximately 75% of 52 species that show slow or negligible senescence at least in protected environments (Da Silva et al., 2022).

Salamanders exhibit many extraordinary traits the most studied of which is their extreme regenerative capacity that made them an invaluable model in regenerative biology field (Yun, 2021). Additionally, their regenerative capacity persists into old age (Eguchi et al., 2011; Sousounis et al., 2015), they demonstrate remarkable cancer resistance (Yun, 2021), and extreme lifespans for their size and negligible senescence (Sousounis et al., 2014; Yun, 2021). A study that compared three salamander species concluded that while their survival decreased slowly with age, their mortality rates remain stable regardless of age (Cayuela et al., 2019), falling in line with negligible senescence. The mechanisms behind an apparent lack of aging are nebulous but some of the evidence suggests that lack of senescent cells accumulation (Walters & Yun, 2020; Yun et al., 2015), an activation of DNA damage response (DDR) upon injury (Sousounis et al., 2020), and large genomes (Nowoshilow et al., 2018; Poetsch et al., 2018) might play a role (Yun, 2021).

It is worth noting that the idea of negligible senescence has not gone unquestioned. First of all, as is apparent by the previous sections, it goes against all recognized evolutionary aging theories. Secondly, our data on the lifespan of wild animals might be not completely accurate as it is difficult to measure and recovery rates of animals with high dispersal ability are usually rather low (Xia & Møller, 2022). While this critique does not apply to lifespans of animals in captivity, it is certainly worth a consideration in the context of the lifespan of wild animals.

1.1.2.2. Hibernation and aging

Hibernation is probably the most known one out of various dormancy states such as torpor, diapause, etc. It is characterized by metabolic rate depression, steep decrease in body temperature, and metabolic shift to using lipids as a primary energy resource (C.-W. Wu & Storey, 2016). As a consequence, hibernating animals also face increased cellular stress (Storey, 2010). A hypothesis that hibernation can reduce rate of aging (Al-attar & Storey, 2020) was tested in a recent study (Pinho et al., 2022). Pinho et al. used yellow-bellied marmots as their model to demonstrate that epigenetic aging rate was drastically decreased during hibernation compared to active seasons.

1.1.3. Consequences of aging

The process of aging and, importantly, its consequences are often described in the context of the so-called hallmarks of aging (López-Otín et al., 2013, 2023) that were based on the seminal hallmarks of cancer (Hanahan & Weinberg, 2000, 2011). According to the latest version, the hallmarks of aging are separated into three groups based on the nature of their contribution to aging: primary (genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, and disable macroautophagy), integrative (dysbiosis, chronic inflammation, altered intercellular communication, and stem cell exhaustion), and antagonistic (cellular senescence, mitochondrial dysfunction, and deregulated nutrient sensing) (López-Otín et al., 2023). While the hallmarks provide a useful framework for scientists within aging field to relate their research to the big picture and a helpful overview of the consequences of aging, one should keep in mind that not all of the hallmarks have the same explanatory power

neither do they provide a robust structure of the aging process (Gems & De Magalhães, 2021).

Additional shortcomings include a lack of hallmarks on the organismal level, lack of clarity on the relationship between different hallmarks, and the implication that aging is caused by accumulation of molecular damage that is made by inclusion of genomic instability as one of the hallmarks (Blagosklonny, 2022b). Regarding the latter, while this is not inconceivable, it does not represent a scientific consensus and currently there is none. The role of DNA damage in particular in the aging process is discussed in more detail in section 1.1.4.

Overall, the hallmarks of aging (López-Otín et al., 2013, 2023) provide a useful point of reference for aging research but fall short of creating a paradigm with an explanatory power for causes of aging and their interactions, separation between life-liming and non-life-limiting phenotypes, and readouts for anti-aging, or pro-longevity interventions (Blagosklonny, 2022b; Gems & De Magalhães, 2021; Keshavarz et al., 2023).

1.1.4. DNA damage and aging

Oxidative damage and its accumulation throughout lifetime were once regarded as the cause of aging (Harman, 1956; Sohal & Weindruch, 1996). However, recently more studies emerged that contradict it (Blagosklonny, 2008; Cabreiro et al., 2011; Gems & Doonan, 2009; Gladyshev, 2014; Van Raamsdonk & Hekimi, 2012). As a consequence, it fell out of favor of the scientific community and was replaced by the damage theory (Gladyshev, 2014; Gladyshev et al., 2021). Justifying its name, the damage theory presents damage as being of critical importance in the aging process

at least and as the primary cause of aging at most (Gladyshev et al., 2021; Schumacher et al., 2021).

Because DNA has limited chemical stability (Lindahl, 1993), is affected by endogenous (De Bont, 2004; Swenberg et al., 2016) and exogenous (Swenberg et al., 2016) genotoxic insults and is prone to erroneous replication (Schumacher et al., 2021), instability is its inherent characteristic. DNA damage leads to an array of negative consequences including genome instability, epigenetic alteration, proteostatic stress, cellular senescence, and mitochondrial and telomere dysfunction (Gladyshev et al., 2021; Schumacher et al., 2021). DNA integrity is maintained via damage dilution and constant activation of repair pathways such as base excision repair (BER), nucleotide excision repair (NER), interstrand cross-link repair (ICL-R), non-homologous end joining (NHEJ), and homologous recombination (HR) (Niedernhofer et al., 2018). Defects in DNA repair pathways lead to accelerated aging and majority of progeroid diseases have a genome instability or intolerance to genotoxic stress component suggesting that DNA damage places an integral role in aging (Gladyshev et al., 2021; Niedernhofer et al., 2018; Schumacher et al., 2021). Based on this, most of the contemporary aging theories acknowledge the fact that damage place an important role in aging even if it is not its primary cause (Blagosklonny, 2021, 2022b; Gladyshev et al., 2021).

Another important question is: what is the role of DNA repair in rejuvenation? Several papers have summarized the current knowledge on the topic (Y. Chen et al., 2020; Ji et al., 2023) but the question of whether DNA repair is a common mechanism that mediates rejuvenation remains unanswered.

1.1.5. Rejuvenation and reprogramming

Until now, we discussed aging as a phenomenon that can be modulated in certain special cases but is mostly universal, inevitable, and irreversible. However, there are examples of both naturally occurring and artificially produced exceptions. We will now discuss a couple of the most important of such examples.

1.1.5.1. Naturally occurring rejuvenation events

Early embryonic development is thought to be an example of naturally occurring rejuvenation process since oocytes are formed during fetal development, are maintained in meiotic arrest until puberty (Jamnongjit & Hammes, 2005), and stay in the metabolically active state until fertilization. Thus, oocytes are aging with the rest of the organism albeit slower and accumulating damage that can only be partially repaired before fertilization (Gladyshev, 2021). For an oocyte to successfully give rise to the next embryo, it needs to undergo damage removal and rejuvenation. Indeed, based on DNA methylation data and results of epigenetic clocks, a rejuvenation event is thought to occur during early embryonic mouse development between E4.5 and E10.5 (Kerepesi et al., 2021). A similar phenomenon was described to take place around gastrulation phase of *Xenopus laevis'* development (Zhang et al., 2022).

Newts are also a candidate rejuvenation model. In a transcriptomic study from 2015, Sousounis et al. compared transcriptomes of lenses, irises and tails that underwent lens regeneration 19 times to the transcriptomes of the respective tissues from young newts that had never undergone lens rejuvenation (Sousounis et al., 2015). Although

the authors did not state this explicitly, an observation that an old lens regenerated multiple times has a transcriptional program comparable to young lens unlike an old tail compared to a young one, suggests that either lens regeneration co-occurs with rejuvenation, or a lens is extremely resistant to age-related changes. However, this study did not include a transcriptome of a lens from an organism that is comparably old to the one that underwent multiple lens regenerations. Therefore, the second explanation of old lens' youthful transcriptomic signature cannot be ruled out (Yun, 2021).

A commonly used model organism in aging research, *C. elegans* was reported to accumulate age-related changes during L1 arrest, a form of larval quiescence, that were reversed upon recovery and return to normal development (Roux et al., 2016).

1.1.5.2. Reprogramming and rejuvenation strategies

Research on induced pluripotency, that was trailblazed by in 2006 by Takahashi and Yamanaka (Takahashi & Yamanaka, 2006), provides examples of the possibility to artificially manipulate the aging status of cells (Lapasset et al., 2011) and at least partially reverse the altered structure of the nuclear envelope (G.-H. Liu et al., 2011) and mitochondrial function (Suhr et al., 2010).

Reprogramming has a high oncogenic potential as undifferentiated proliferating cells will form tumors (Abad et al., 2013; Hentze et al., 2009; Moradi et al., 2019). Therefore, to utilize the rejuvenative potential of cellular reprogramming, one must decouple it from the loss of somatic identity (Simpson et al., 2021). To this end, Ocampo et al. demonstrated that age-related hallmarks can be ameliorated by *in vivo* partial reprogramming via short-term expression of the Yamanaka factors (Oct4, Sox2, Klf4,

c-Myc, or OSKM) (Ocampo et al., 2016). The same reprogramming approach was later shown to prevent age-dependent reduction in H3K9 trimethylation in mouse neurons of the dentate gyrus (Rodríguez-Matellán et al., 2020). Furthermore, a novel method called maturation phase transient reprogramming (MPTR) was developed that restores youthful states of transcriptome and epigenome after a brief loss and reacquisition of cellular identity (D. Gill et al., 2022), demonstrating the possibility of divorcing rejuvenation from complete reprogramming. Another study (Kriukov et al., 2022) claims to have decoupled longevity and rejuvenation effects of reprogramming from loss of somatic identity which could aid greatly in disentangling these two often co-occurring processes and devising approaches to induce only the desired rejuvenation effect.

Overall, partial cell reprogramming was reported to revert some of the hallmarks of aging, such as mitochondrial dysfunction, increased inflammation, decreased autophagy, loss of proteostasis, epigenetic alterations (Sarkar et al., 2020), and increase level of senescent cells (in some cell types) (López-Otín et al., 2023; Manukyan & Singh, 2014; Yücel & Gladyshev, 2024), but had no effect on telomere attrition (Sarkar et al., 2020).

1.1.6. The biomarkers of aging and aging clocks

In recent years, there has been a growing interest in identifying biomarkers of aging that would aid in quantifying biological age. These efforts took shape of aging clocks based on various biomarkers among which are changes in phenotype, epigenome, transcriptome, proteome, metabolome, and circadian rhythms (Aging Biomarker

Consortium et al., 2023). Henceforth, we will discuss the utility of such clocks, particularly, epigenomic and transcriptomic clocks.

Broadly speaking, epigenetics includes all the processes and changes that do not alter DNA sequence but can impact gene expression and cause a phenotypical change. During aging, such changes accumulate and tend to lead to gradual degeneration (López-Otín et al., 2023). DNA methylation is one of the epigenetic changes that has been predominantly focused on in the context of epigenetic aging clocks. 5-methylcytosine is the most common methylation type and it usually occurs on CpG-sites (cytosine-phosphate-guanine) (Aging Biomarker Consortium et al., 2023). DNA methylation is evolutionary conserved (Zemach et al., 2010) and its levels are known to change during aging from yeast to humans (A. A. Johnson et al., 2012; Romanov & Vanyushin, 1981; Sedivy et al., 2008; Singhal et al., 1987; Wilson et al., 1987)

Famously, the first multi-tissue epigenetic clock was developed by Steve Horvath (Horvath, 2013, 2015) in which the analyzed CpG sites showed strong association with aging. It was later complemented by the Skin & Blood Clock (Horvath et al., 2018). An array of various aging, both chronological and biological, clocks followed including but not limited to Peters' transcriptomic clock (Peters et al., 2015), PhenoAge (Levine et al., 2018), GrimAge (Lu et al., 2019), and DunedinPACE (Belsky et al., 2022).

Transcriptomic data is a point of convergence for epigenetic modifications and chromatin structure changes which makes it a useful readout for building a predictor of age-related changes (Lai et al., 2019). Moreover, certain model organisms, such as *C. elegans*, have very low levels of DNA methylation which precludes usage of epigenetic clocks. To this end, a binarized transcriptomics clock (BiT Age) was

developed by Meyer in 2021 (Meyer & Schumacher, 2021) that combats the noise problem in previous clocks by applying binarization and age scaling strategies.

1.1.7. Length-dependent gene expression patterns

Gene expression patterns, their change in aging and their relationship to gene length have recently became a topic of interest after it was shown that genes downregulated in DNA repair-deficient mice are longer than those in WT mice (Vermeij et al., 2016). The same pattern was shown in aging *Drosophila* (H. Hall et al., 2017) and humans (Lopes et al., 2021). It was later shown that the decline in gene expression that is dependent on gene length occurs across species, tissues, and cell types not only in normal aging but also as a response to genotoxic insults such as UV, smoke exposure and in progeroid diseases such as Cockayne syndrome and trichothiodystrophy (Ibañez-Solé et al., 2023; Stoeger et al., 2022). The term gene-length-dependent transcriptional decline (GLTD) was coined to summarize this phenomenon (Soheili-Nezhad et al., 2024). The explanation for it is thought to be RNAPII stalling caused by transcription-blocking DNA damage (Gyenis et al., 2023) which links to it to NER, in particular, to its transcription-couple branch.

1.2. *C. elegans* as a model for aging research

C. elegans was first established as a model organism by Sydney Brenner (Brenner, 1974). It is a small (1 mm in adulthood), transparent, free-living nematode. Its transparency allows for easy visualization of cells and subcellular structures and usage of fluorescent proteins. *C. elegans* has a short life cycle taking it 3 days to develop from egg to egg-laying adult at 25°C. Most of its population is comprised of self-fertilizing hermaphrodites with males occurring at a frequency of <0.2% (Corsi et

al., 2015). Moreover, *C. elegans* is extremely well characterized: it consists of 959 somatic cells in hermaphrodites and 1031 cells in males, and every somatic cell and its fate has been tracked from fertilization to adulthood (Kimble & Hirsh, 1979; Sulston et al., 1983; Sulston & Horvitz, 1977). ~40% of *C. elegans* protein-coding genes have predicted human orthologs and 60 to 80% of human genes have a *C. elegans* ortholog (Corsi et al., 2015; Kaletta & Hengartner, 2006; Shaye & Greenwald, 2011).

1.2.1. Lifespan-regulating genes in *C. elegans*

While the topic of genes that regulate lifespan in *C. elegans* is vast and can be discussed in great detail, we will focus on a few key pathways that are known to contribute to the lifespan regulation, namely, insulin/insulin-like growth factor-1 (IGF-1) signaling (IIS) and mTOR signaling pathways and the role of autophagy.

1.2.1.1 Insulin/insulin-like growth factor-1 signaling pathway

IIS pathway was the first one to be established as regulating aging process (Kenyon, 2011) with the first long-lived mutant being *age-1* (Friedman & Johnson, 1988), where AGE-1 is the *C. elegans* homolog of phosphatidylinositol 3-kinase (PI3K). A mutation in *daf-2* – the homolog of the insulin/IGF-1 receptor – causes adult worms to double their lifespan compared to WT and this extension in dependent on *daf-16* activity (Kenyon et al., 1993; Kimura et al., 1997). DAF-16 is a homolog of the forkhead box O (FoxO) transcription factor (TF) and together with AGE-1 and DAF-2 they constitute three key components of IIS pathway (Uno & Nishida, 2016). Moreover, studies have shown that inhibition of IIS results in increased stress resistance and lifespan in *Drosophila* and mice (Blüher et al., 2003; Clancy et al., 2001; Tatar et al., 2001). JNK-1 and CST-1 were shown to regulate post-translational modification of DAF-16 and

their overexpression promotes lifespan extension in DAF-16-dependent manner (Lehtinen et al., 2006; Oh et al., 2005). Taken together with observations that overexpression of DAF-16 alone has only mild effect on lifespan (Henderson & Johnson, 2001), this suggests that DAF-16 requires other factors to exhibit its lifespan-extending effect. Other genes that were demonstrated to be involved in IIS include *rle-1* (loss increases lifespan, (W. Li et al., 2007)), *math-33* (suppresses extended lifespan in *daf-2* mutants, (Heimbucher et al., 2015)), hsf-*1, skn-1,* and *pqm-1* (all suppress extended lifespan in *daf-2* mutants, (Hsu et al., 2003; Tepper et al., 2013; Tullet et al., 2008)).

1.2.1.2 mTOR pathway

mTOR is a serine/threonine kinase which consists of two complexes, namely, mTORC1, which mainly regulates cell growth and metabolism, and mTORC2, which mainly regulates cell proliferation and survival (Kennedy & Lamming, 2016; Unni & Arteaga, 2019; Zou et al., 2020). Similarly to *daf-2* mutants, deficiency in mTOR activity more than doubles worms' lifespan compared to WT in a DAF-16-dependent manner (Vellai et al., 2003). Its effect is mediated by PHA-4/FoxA TF which, in turn, regulates autophagy that has been implicated in aging and rejuvenation processes (Aman et al., 2021; Hansen et al., 2008, 2018; Ho et al., 2017; Lapierre et al., 2011; Meléndez et al., 2003; Palmisano & Meléndez, 2019). Importantly, mTOR seems to play the mediating role in lifespan extension via dietary restriction (DR) which falls in line with its role in nutrient sensing (Blackwell et al., 2019; S. C. Johnson et al., 2013).

1.2.1.3 Autophagy

Autophagy is a fundamentally important process across species from yeast to mammals including humans, deficiencies in which lead to various defects and pathologies (Palmisano & Meléndez, 2019). Autophagy not only plays a role during *C.elegans* development such as removal of aggregate-prone proteins, paternal mitochondrial, and apoptotic cell corpses (Palmisano & Meléndez, 2019), but is also involved in several longevity pathways as mentioned above. Specifically, autophagy is required for IIS-mediated and DR- or mTOR-mediated lifespan extension as well as longevity induced by reduced mitochondrial respiration (Hansen et al., 2008; Tóth et al., 2008).

1.2.2. *C. elegans* and alternative developmental

The short life cycle and the ability to generate large number of progeny means that *C. elegans* populations can experience a sudden expansion to the point where resources

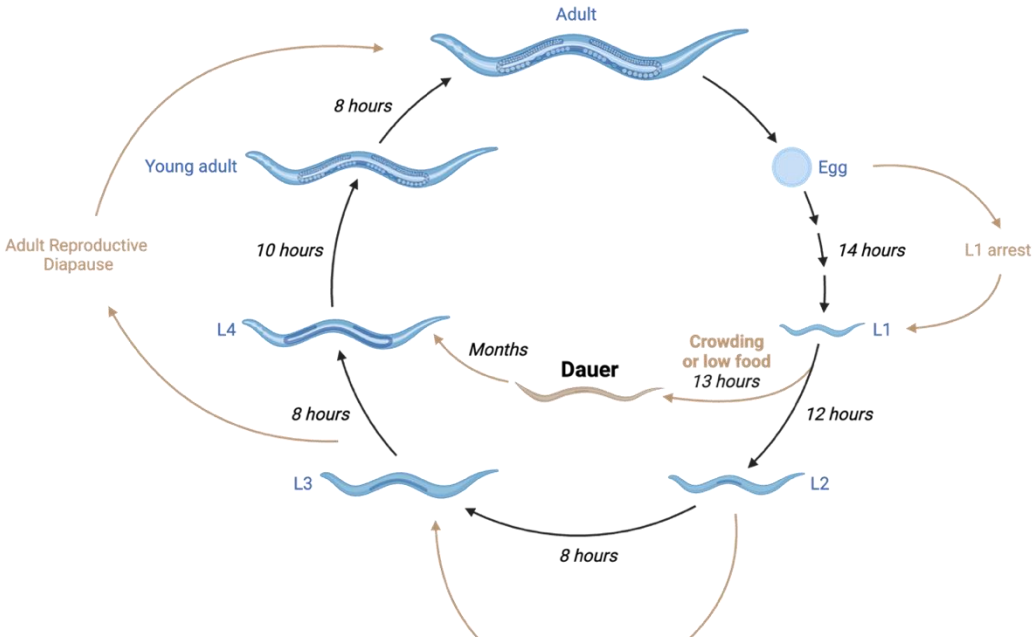

Figure 1.1 A schematic representation of C. elegans life cycle and its best described diapause stages

become limiting (Baugh & Hu, 2020). Therefore, responses to starvation that the worms encounter in the wild were developed and we will cover three of the most prominent and well-described ones: L1 arrest, dauer diapause, and adult reproductive diapause (ARD).

1.2.2.1. L1 arrest

Larvae that hatch in the absence of food arrest immediately in L1 stage without undergoing any morphological change in L1 arrest, or L1 diapause. Such arrested larvae can survive for weeks and continue with mostly normal development synchronously upon refeeding (Baugh, 2013; Baugh & Hu, 2020). Due to this, L1 arrest is often used in the lab as a convenient way to synchronize worm populations. Worms arrested in L1 have slowed metabolism and increased resistance to environmental stress which is reflected by their gene expression patterns (Baugh et al., 2009; Maxwell et al., 2012).

IIS and AMPK signaling are major regulators of the L1 arrest with *daf-16* and *aak-2* being required for L1 arrest as in their absence worms become sensitive to starvation and die during L1 arrest or display L1 arrest-defective phenotype, respectively (Baugh & Sternberg, 2006).

While originally it has been reported that post-arrest lifespan is normal, comparable to worms that never arrested (T. E. Johnson et al., 1984), mutations that affect lifespan were reported to also affect the length of L1 arrest (Baugh & Sternberg, 2006; I. Lee et al., 2012; Muñoz & Riddle, 2003). Due to this connection and the fact that genes modulating lifespan also modulate the duration of L1 arrest, Roux et al. propose to view L1 arrest as a stage where worms experience decline similar to aging that is

reversed upon recovery rather than a true diapause (Roux et al., 2016). Indeed, they found that L1 worms acquire aging-like features that are reverted afterwards.

1.2.2.2. Dauer diapause

If worms are exposed to unfavorable conditions such as overcrowding or lack of resources during early larval development, they enter an alternative larval stage named dauer diapause (sometimes, L3d) in which they can remain for many months (Cassada & Russell, 1975; Fielenbach & Antebi, 2008; Golden & Riddle, 1984). Entry into dauer is triggered by a large dauer-pheromone-to-food ratio and exacerbated by high temperatures (Riddle & Albert, 1997). Before worms develop into dauer, L1 larvae enter a predauer L2d stage during which they accumulate fat and carbohydrates (Fielenbach & Antebi, 2008; Golden & Riddle, 1984). At this stage, worms can either commit to dauer diapause or develop into L3 larvae if environmental conditions improve before the molt (Baugh & Hu, 2020; Golden & Riddle, 1984; Schaedel et al., 2012).

After committing to dauer arrest, worms undergo radial constriction and tissue remodeling (Cassada & Russell, 1975), they develop a thickened cuticle and seal all orifices (Riddle et al., 1981), their pharynx is constricted and does not pump (Cassada & Russell, 1975; Vowels & Thomas, 1992). Thus, throughout the diapause, the worms do not feed and rely on their internal lipid storage for energy, converting fat to glucose via glyoxylate cycle (Riddle & Albert, 1997). They are also resistant to stress, in particular, starvation, heat and oxidative stress (Fielenbach & Antebi, 2008).

After the conditions have become favorable again, worms arrested in dauer recover and resume reproductive growth (Cassada & Russell, 1975; S. E. Hall et al., 2010).

The reports on the effect of dauer diapause on postdauer fitness vary on almost all parameters, the only seemingly consistent one being the recovery time and success: they were shown to increase and decrease, respectively, i.e. it takes worms longer to recover from dauer and fewer of them succeed depending on the amount of time spent in dauer (S. Kim & Paik, 2008; Klass & Hirsh, 1976). Additionally, Kim & Paik, 2008 reported postdauer worms to experience sterility and reproductive organ defects, degree of which correlated with the amount of time spent in dauer. Lifespan, reportedly, is either not affected (S. Kim & Paik, 2008; Klass & Hirsh, 1976), increased after dauer (S. E. Hall et al., 2010), increased after X-irradiation during dauer (Onodera et al., 2010), or increased in F3 (Webster et al., 2018). Brood size was shown to not change (Klass & Hirsh, 1976), decrease (S. Kim & Paik, 2008) or increase (S. E. Hall et al., 2010), and even that the progeny is smaller and starvation-sensitive (Webster et al., 2018) in postdauers.

Additionally, postdauer animals exhibit transcriptomic and proteomic differences when compared to adults that underwent normal development (S. E. Hall et al., 2010; S. Kim et al., 2016). Moreover, even the trigger that induced dauer entry – starvation or dauer pheromone – seems to have an effect on the postdauer gene expression patterns and reproductive plasticity (Ow et al., 2018)

1.2.2.3. Adult reproductive diapause

Another alternative developmental stage that is starvation-induced is adult reproductive diapause (ARD). It occurs in late larval development, mostly between L3 and L4. Of note, ARD entry does not require crowding unlike entry into dauer diapause (Seidel & Kimble, 2011). Worms can remain in ARD for up to 30 days and still have a

normal adult lifespan afterwards (Angelo & Van Gilst, 2009) and up to 70 days with 22% lifespan reduction (Gerisch et al., 2020). Animals arrested in ARD are described as being smaller than normal adults, having relatively short gonads, experiencing slowed ovulation and increased apoptosis, the changes that are reversed upon refeeding (Carranza-García & Navarro, 2019; Seidel & Kimble, 2011) positioning ARD as another aging and rejuvenation model.

1.3. Aims of the thesis

The dauer diapause phenomenon poses a lot of interesting questions in the context of the nature of aging and how it can be halted, the rejuvenation process that was observed in nature and induced artificially, and sheer variance in fitness postdauer animals seems to exhibit. Therefore, in this thesis we aim to:

1. Assess the impact of dauer diapause on main life history traits, such recovery form dauer, lifespan, and brood size.
2. Characterize transcriptomic patterns of worms arrested in dauer for prolonged periods of time and recovering from dauer after being arrested for different amounts of time.
3. Compare transcriptomic patterns of dauer-arrested worms that are subject to chronological aging to those of aging non-dauer worms.
4. Assess the impact of UV-treatment during dauer on the recovery patterns and gene expression patterns in the context of gene length.

Chapter 2 Results

The manuscript in preparation with the working title “Age deceleration and reversal patterns in dauer diapause” is integrated into this chapter and, in parts, quoted verbatim. Additional figures were added for context and ease of comprehension. The manuscript is co-first-authored with Dr. João C. V. V. Barata, therefore, the thesis includes some of the results produced by Dr. Barata. All the contributions are explicitly specified in the respective chapter.

2.1. Dauer diapause has no negative consequences on postdauer fitness

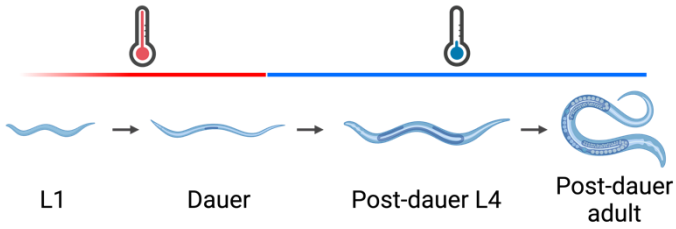

Figure 2.1 A schematic depiction of dauer entry and exit protocol
Red and blue lines indicate plate incubation at 25°C or 15°C, respectively.

Since the reports about consequences of dauer on postdauer fitness vary drastically, first, we wanted to examine the impact of the diapause on dauer recovery time, postdauer lifespan and brood size. For this and all following experiments, we chose to use a dauer-constitutive (Daf-c) mutant strain of *C. elegans daf-2(e1370)* whose entry into dauer is temperature-sensitive to achieve greater dauer entry efficiency and synchronization. We induced dauer entry by incubating synchronized populations of *daf-2(e1370)* L1 larvae at 25°C (Fig. 2.1.1). After 72

N.B.: throughout this thesis terms (dauer) “exit” and “recovery” are used interchangeably, as well as (dauer) “arrest” and “diapause”.

hours at 25°C, all nematodes established dauer arrest, and we termed them day 1 dauers (D1). Dauer recovery was induced by transferring worms to freshly seeded plates and shifting incubation temperature to 15°C.

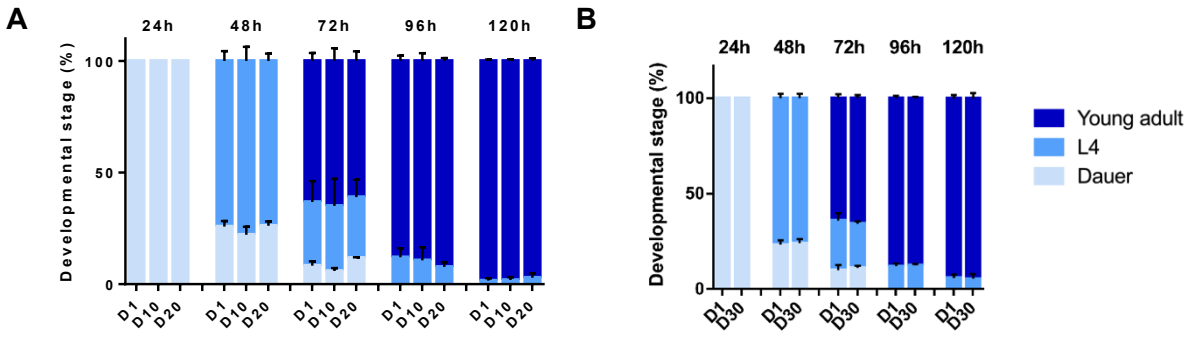

Figure 2.2 Increasing duration of dauer arrest does not affect dauer exit timing
Developmental resumption assay of *daf-2* animals arrested in dauer for 1, 10 or 20 days (A) and 1 or 30 days (B). The summary of 3 independent experiments is shown as mean ± SD; Mantel-Cox Log Rank test (B, comparisons with N2 are shown), one-way ANOVA with Dunnett's multiple comparison test (F, comparisons with N2 are shown) and two-tailed t-test compared with D1 (A, B) were performed; *** = p<0.001 from (Barata, 2022)

We performed an adapted developmental assay in which we assessed the dynamics of dauer recovery of populations arrested in dauer for 1, 10, 20, or 30 days. Worms were assessed every 24 h after recovery induction. We observed no delay in

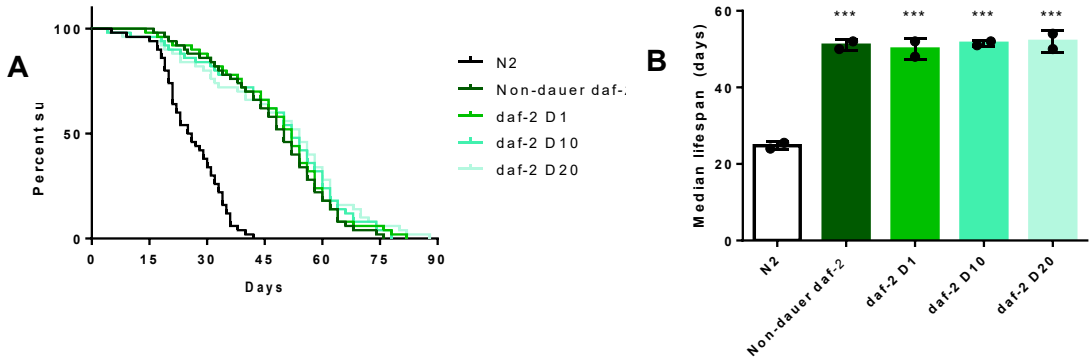

Figure 2.3 Increasing duration of dauer arrest does not affect postdauer lifespan
Lifespan assay of wild-type (N2) and *daf-2* that did not arrest in dauer, and *daf-2* arrested in dauer for 1, 10, or 20 days. One representative experiment is shown in (A) and median ± SD of 2 independent experiments per condition in (B). From (Barata, 2022).

populations arrested in dauer for longer periods, with most worms reaching the young adult stage 96 h after dauer recovery was induced (Fig. 2.2A, B).

Next, we evaluated postdauer lifespan and brood size in wild-type (WT, N2) and *daf-2* worms that were always incubated at 15°C and therefore proceeded with normal development, or were arrested in dauer for 1, 10, or 20 days (Fig. 2.3A,B; Fig.2.4). As reported previously (Kenyon et al., 1993; Tissenbaum & Ruvkun, 1998), *daf-2* worms displayed extended lifespan but reduced brood size compared to WT. More importantly, we saw no difference in either lifespan or brood size between populations arrested in dauer for different periods of time and non-dauer *daf-2* worms.

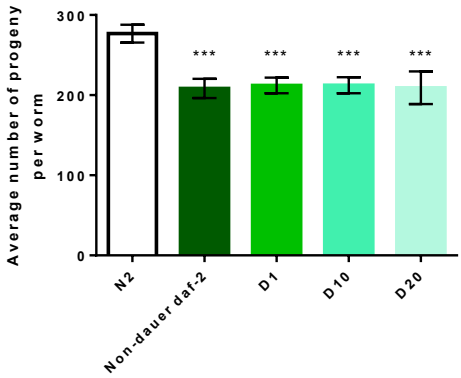

Figure 2.4 Increasing duration of dauer arrest does not affect number of progeny
Brood size assay of wild-type (N2) and *daf-2* that did not arrest in dauer, and *daf-2* arrested in dauer for 1, 10, or 20 days. The summary of 3 independent experiments is shown as mean ± SD; Mantel-Cox Log Rank test (B, comparisons with N2 are shown), one-way ANOVA with Dunnett's multiple comparison test (F, comparisons with N2 are shown) and two-tailed t-test compared with D1 (D, E) were performed; *** = $p<0.001$. The color-coding is the same as in Fig. 2.3. From (Barata, 2022).

Overall, in our experimental settings, there is no observable functional decline in postdauer animals, despite their higher chronological age when compared to worms that never underwent dauer arrest. This suggests that worms arrested in dauer either do not accumulate aging-associated changes to a significant degree or are able to revert them during recovery.

2.2. Predicted biological age of worms during dauer arrest and upon recovery

As mentioned in the introduction, dauer was originally reported as a non-aging developmental stage. Indeed, dauer diapause presents an aging conundrum: after worms undergo dauer arrest and reach adulthood, they are chronologically older than those that developed normally, and yet, postdauer worms are functionally comparable to normally developed worms, based on our results. This suggests that even though postdauer and normally developed worms differ in chronological age, they are of the same or closely comparable biological age, implying that dauers have to somehow compensate for the time they spent in the diapause. This led us to hypothesize that in worms in dauer, or dauers 1) aging proceeds on the same trajectory as before dauer entry followed by an age reversal event, or rejuvenation during dauer recovery, 2) the aging process is halted altogether, circumventing the need for age reversal during

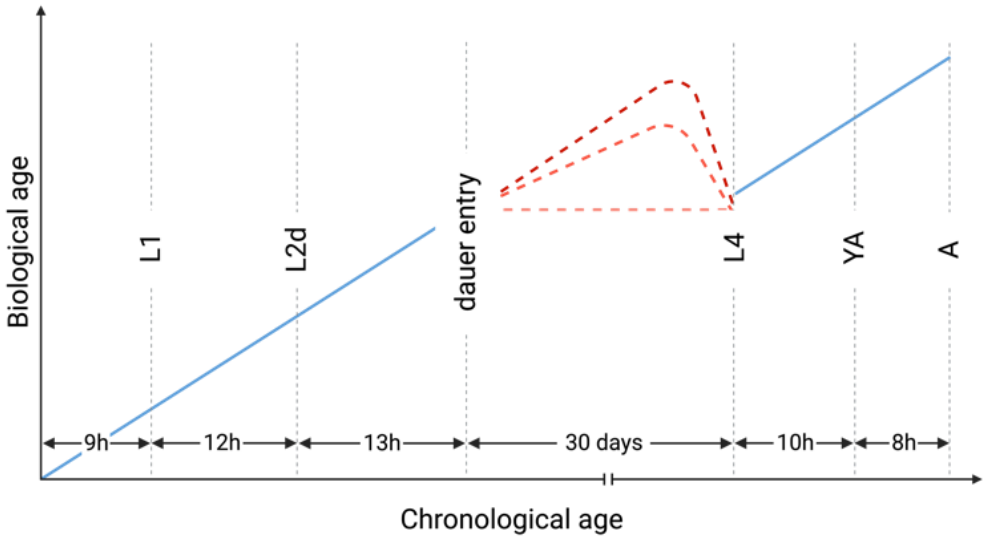

Figure 2.5 A schematic depiction of the research hypotheses and questions
The x-axis is broken for the sake of visualization. The blue line depicts a hypothesized relationship between chronological and biological age during *C. elegans* development and life. The dashed red lines depict three proposed dauer aging trajectories.

dauer exit, or 3) a combination of both strategies transpires by decreasing the aging rate during dauer and reversing the age-related changes that did occur upon dauer exit. (Fig. 2.5).

Since our understanding of aging in development is incomplete, we resort to making a few assumptions that allow us to test these hypotheses. The assumptions are: 1) at the zygote, or at some early developmental stage, an organism has a biological age of zero, 2) the eggs and ensuing larvae experience a linear increase in biological age, as a function of chronological age, continuously throughout development, 3) postdauer worms that recovered and reached the phenotypic L4 stage are the same biological age as L4 worms that developed normally, i.e. they function comparably, appearing to be the same biological age given that their fitness is unaffected by the time spent in dauer (Fig. 2.1.2-4).

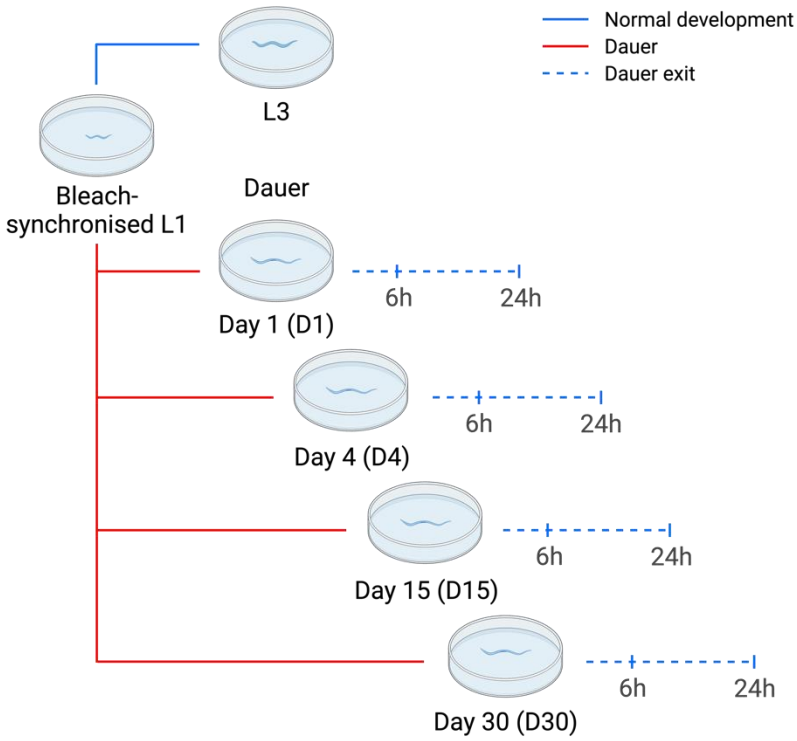

Figure 2.6 A schematic depiction of the experimental setup
Red and blue lines indicate plate incubation at 25°C or 15°C, respectively.

We devised an experimental setup (Fig. 2.6) in which we collected worm populations arrested in dauer for 1, 4, 15, or 30 days and populations undergoing dauer exit (6 h and 24 h post exit induction) and performed bulk RNA-sequencing. Since the worms are still actively recovering from dauer at 6 and 24 h post exit (Fig. 2.2), we hypothesized that the recovery transcription programs would be actively engaged during this time. We additionally included L3 samples to serve as a non-dauer control as dauer is sometimes referred to as an alternative L3 stage.

We first performed principal component analysis (PCA) to explore the relationship between different samples (Fig. 2.7). We observed that samples were separated into dauer and non-dauer (L3) by PC2 which could be considered a developmental axis, while PC1 separated samples that spent a different amount of time in dauer would be a temporal axis. Consistently, dauer exit samples are located in-between dauer and

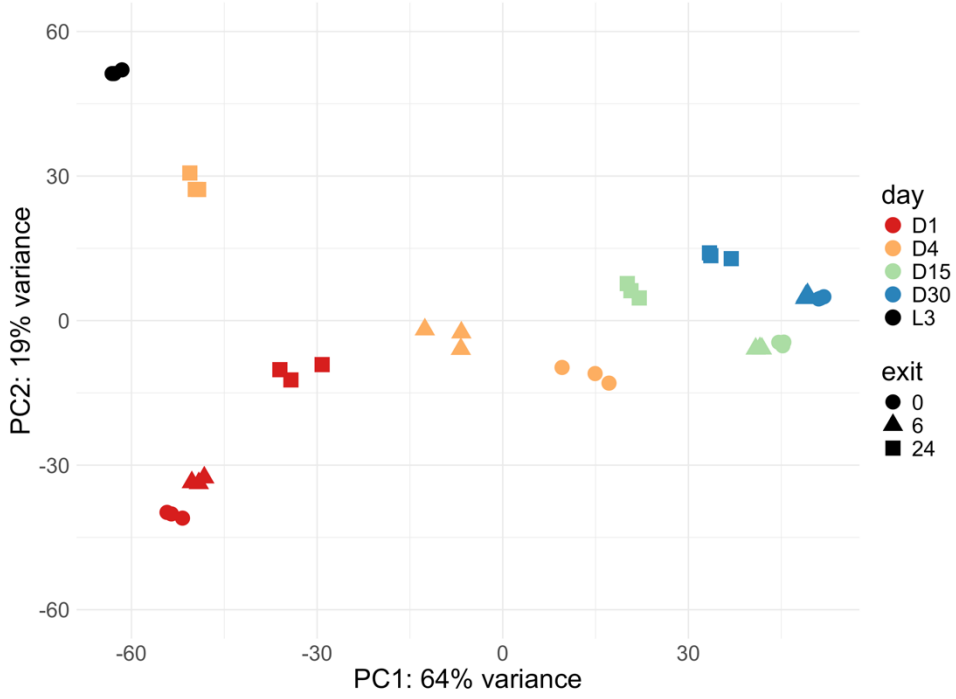

Figure 2.7 Samples cluster by the day of dauer or stage of dauer exit
Principal component analysis (PCA) of bulk RNA-sequencing results grouped by developmental stage (color) and time after exit from dauer was induced (shape). Each dot represents a replicate for the given stage. Variance explained by each principal component (PC) is indicated on the axis label.

L3 samples. Notably, D4.24 samples cluster in closest proximity to L3 out of all 24 h exit time points suggesting a greater similarity and implying a higher rate of recovery from D4 compared to other dauer time points. This result also sets certain expectations for the rest of the analysis: one could expect to see the least variance in gene expression between D15 and D30 exit time points and the most variance along the D4 exit trajectory.

Next, we wanted to estimate the biological age of *C.* elegans larvae during dauer arrest and upon recovery. Since *C. elegans* have extremely low amounts of methylated CpG sites (Hu et al., 2015), it is not feasible to use or develop an epigenetic clock. Thus, we utilized BiT Age transcriptomic clock (Meyer & Schumacher, 2021). Since the

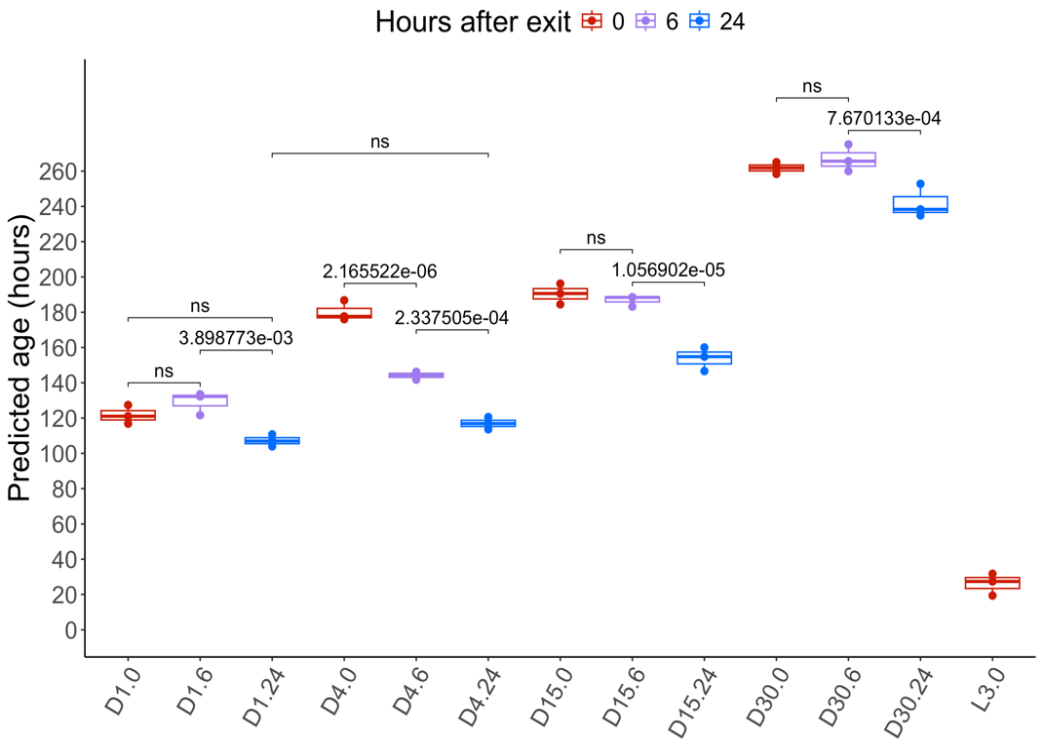

Figure 2.8 Predicted biological age increases during dauer and decreases upon exit
Biological age prediction for dauer and L3 samples using BiT Age transcriptomics clock. Each dot represents a single RNA-seq sample. P-values were calculated using one-way ANOVA with post hoc Tukey test. See Table S1 for results for all comparisons. ns – not significant.

publication of the original clock, BiT Age was updated as briefly described in the Methods section. In general, BiT Age utilizes a binarization approach to transcriptomic data, by setting expression values to 0 or 1 depending on whether the value exceeds the median expression value, as well as temporal scaling to accurately predict biological age.

BiT Age clock was originally developed based on adult *C. elegans* data sets which is important to keep in mind while interpreting the biological age prediction for samples undergoing development, albeit a special case. To date, there are no aging clocks created specifically to be applied to developmental data.

According to BiT Age results (Fig. 2.8), worms in dauer diapause experience aging as the predicted biological age (PBA) increases from D1 through D30 with a plateau around D15. However, the rate of the PBA increase changes during dauer. For example, $PBA_{D1} \approx 122$ h and $PBA_{D4} \approx 180$ h meaning, the rate of biological aging equals 0.8 between these two timepoints (58 h/72 h). Since $PBA_{D30} \approx 246$ h, the rate of biological aging between D4 and D30 is about 0.1 (66 h/624 h). Thus, while the worms continue to age into the diapause, the rate of aging sharply decreases during its later stages.

Further, we observed a significant decrease in PBA upon recovery from D1 at 24 h (D1.24), but not 6 h (D1.6) post recovery. In contrast, predicted age of D4.6 samples is already significantly lower compared to D4 and the difference increases further at D4.24, reaching D1.24 values. Considering that there is no significant difference between D1.24 and D4.24 PBA but there is one between D1 and D4, this suggests

that worms recovering from D4 are able to reduce their biological age faster compared to D1.

Recovery trajectories for D15 and D30 samples differ noticeably from those of D1 and D4 samples while being similar to one another. In both D15 and D30, a significant difference in PBA occurs only 24 h and not 6 h into recovery. Also, D15.24 is predicted to be younger than D30.24, which implies that older dauers take longer to revert changes accumulated during the diapause and are lagging behind worms recovering from D1, D4, and D15. However, based on the results of our developmental assay (Fig 2.2), older dauers do not experience any negative consequences of such a delay during dauer exit, suggesting a decoupling of the processes of age reversal, developmental resumption, and dauer recovery.

Of note is the fact that non-dauer L3 samples are predicted to be much younger than even the younger dauer sample. This result was not predicted but could warrant further investigation.

Taken together, the BiT Age clock results suggest that the biological age progression is increasingly slowed during dauer and reverted during the dauer exit process at a pace dependent on dauer duration.

2.3. Transcriptomic patterns throughout dauer arrest and during recovery

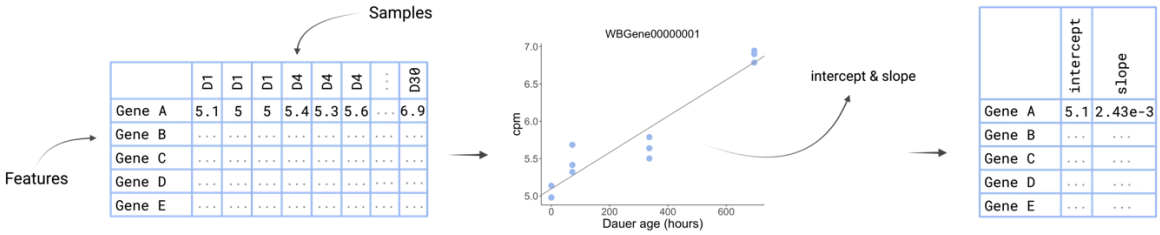

Figure 2.9 A schematic depiction of the linear modelling workflow

We next sought to investigate which transcriptomic patterns could explain our BiT Age results (Fig. 2.8). For this, we developed an approach in which we built a linear model for each gene using expression values from the relevant samples. For the analysis of dauer patterns, we included only D1, D4, D15, and D30 samples. For the analysis of dauer exit trajectories, we split all the samples intro four groups, each containing a dauer sample and two dauer exit samples (6 and 24 h), e.g. D1, D1.6, D1.24. We used the slope derived from the models for downstream analysis (Fig. 2.9)

In order to address the observation of so-called slow aging in the dauer diapause, we examined genes that change in an age-dependent manner during dauer. We performed gene set enrichment analysis (GSEA) using genes ranked by their slope in dauer i.e. if a slope is positive, expression of this gene is increasing during dauer. We used Gene Ontology (GO) (Ashburner et al., 2000; The Gene Ontology Consortium et al., 2023) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa, 2000, 2019; Kanehisa et al., 2023) for the functional enrichment analysis.

During dauer, five groups of pathways are differentially expressed, namely: 1) translation (ribonucleoprotein complex biogenesis, translation, ncRNA processing; ribosome biogenesis in eukaryotes, ribosome, aminoacyl-tRNA biosynthesis, mRNA surveillance pathway), 2) protein processing (protein folding, regulation of dephosphorylation, modification-dependent macromolecule catabolic process, methylation, post-translational protein modification, protein maturation, intracellular protein transport; protein processing in endoplasmic reticulum), 3) sensory perception (cilium organization, sensory perception of chemical stimulus), 4) autophagy (proteasome, autophagy, mitophagy), and 5) defense response (biological process involved in interspecies interaction between organisms, innate (biological process involved in interspecies interaction between organisms, innate immune response), as well as splicing, molting cycle and amide metabolic process pathways (Fig. 2.10A, B).

The change in expression of some of these groups is expected or at least falls in line with what we know about the dauer diapause. For example, underexpression of the defense response genes aligns with the fact that worms in dauer do not feed and do not interact with the external environment. Thus, it is possible that their expression was maintained at a higher level in the beginning of dauer, perhaps, as a preparation for a short diapause, and was later decreased due to the lack of necessity. The same logic could apply to the genes of the molting cycle category.

In a similar vein, an increased expression of genes involved in sensory perception could be explained by the goal of the arrest which is to disperse and find an auspicious environment. Since dauers rely on a finite amount of lipids, the more sensitive the

worm is to exit cues, the more they lower their chances of running out of fat resources, and the sooner they can resume development and reproduce.

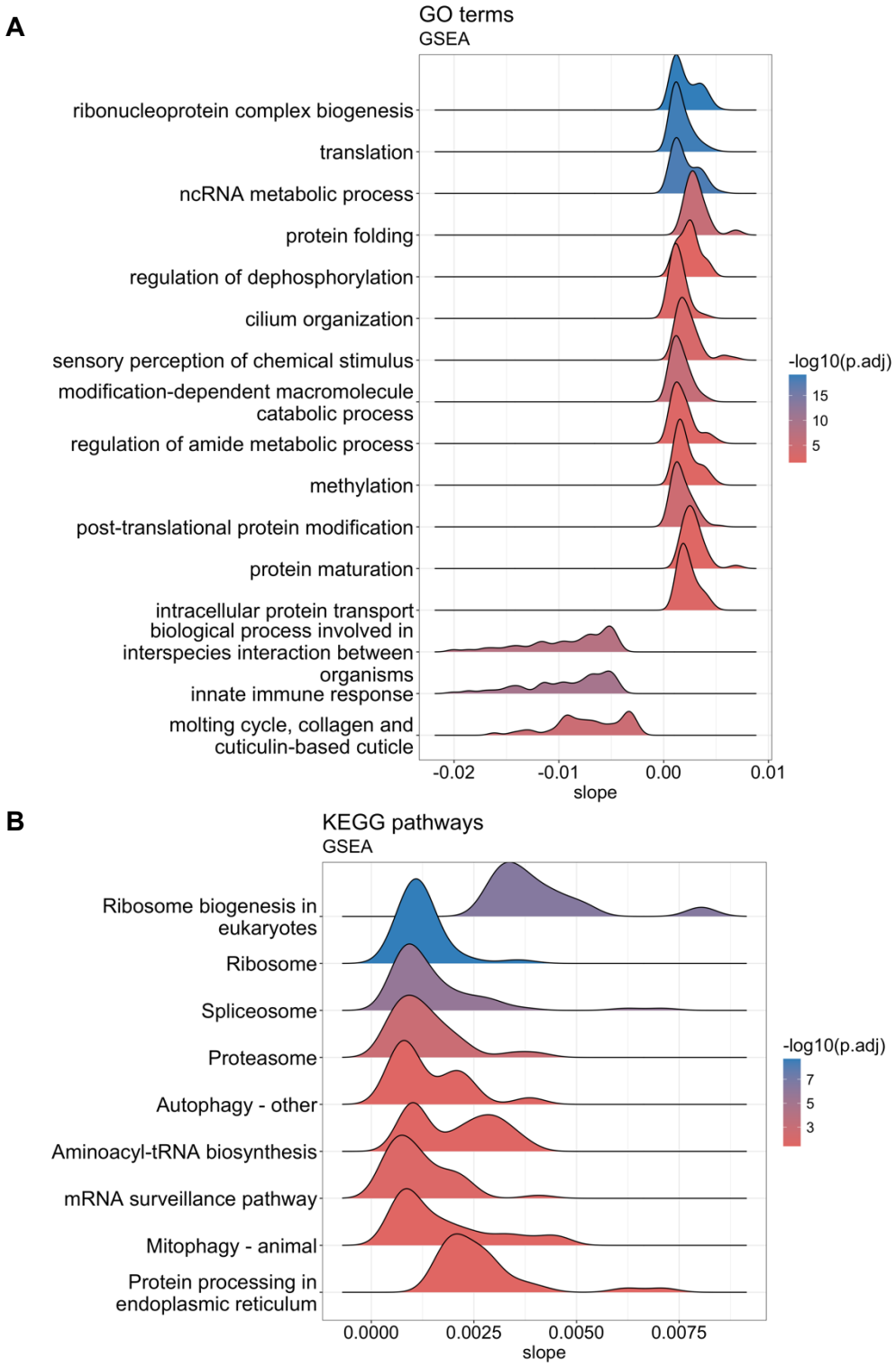

Figure 2.10 Dauer-specific transcriptome patterns

Gene set enrichment analysis (GSEA) of differentially expressed genes throughout dauer using GO terms (A) and KEGG (B) databases. The ridgeplots consist of density plots for genes belonging to each of the indicated pathways; p.adj – adjusted p-value.

The reasons for increased expression of translation, protein processing, and autophagy genes appear less clear at first. If the worms rely on this limited fat storage, why would they activate such energy-demanding processes? However, since both protein production and degradation genes are overexpressed, it could be indicative of improved proteostasis and efficient recycling. Spliceosome genes are also overexpressed throughout dauer which could mean that worms utilize more transcript isoforms during dauer compared to normal development.

Next, we focused on the comparison between D1 and D4 recovery, as both samples reached comparable predicted biological age 24 h after recovery induction (Fig. 2.8). We used the same linear modelling approach as described above, except this time we compared a model for gene A during D1 recovery with a model for gene A during D4 recovery. We then compiled a list of genes that 1) change their expression during recovery (i.e. have a non-zero slope) and 2) differ between D1 and D4 recovery (i.e. slope values are significantly different). Since there is more than one way in which gene expression patterns can differ, we further grouped the genes into 6 categories according to the direction of change and the relationship between expression during exit from D1 and D4 (Fig. 2.11), and used this list to perform an overrepresentation analysis (ORA) using the GO biological process (BP) (Fig. 2.12) and KEGG (Fid. 2.13) databases.

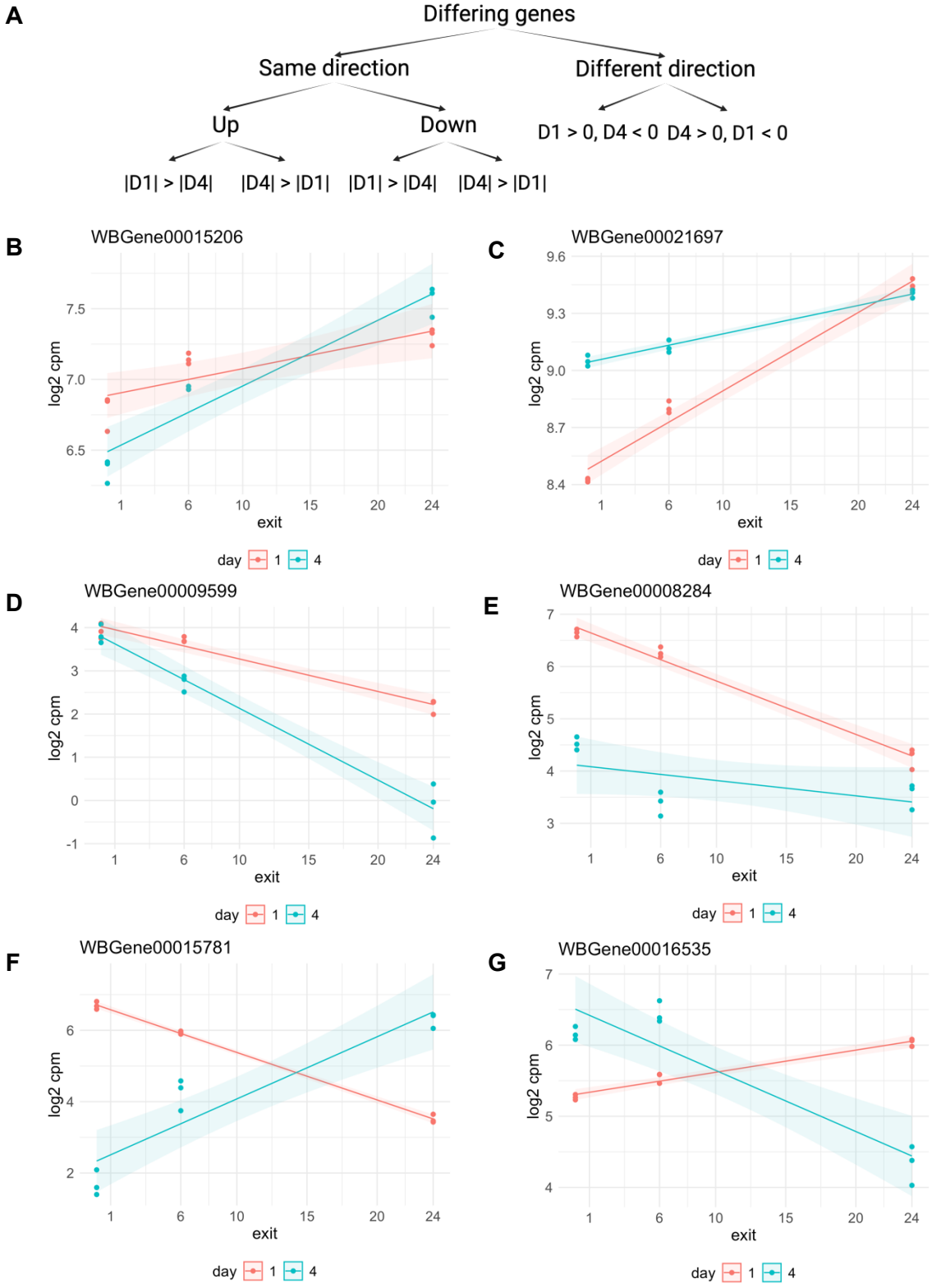

Figure 2.11 Different ways in which gene trajectory can differ during dauer exit
(A) A scheme of six groups of genes. (B-G) Genes that represent each of the groups.

Out of the six groups, one showed no enrichment (same direction, down, |D4 > D1|) (Fig. 2.12, 2.13). Genes whose expression is increasing in both exits but the effect is more pronounced in D1 exit are enriched in ribosome biogenesis, rRNA, and lipid metabolic processes. The |D4| > |D1| scenario will be discussed in detail later. Genes that decrease more drastically during D1 exit are enriched in cell morphogenesis, proliferation and well as neurogenesis, axon regeneration and locomotion pathways. Interestingly, genes increasing in expression during D1 but decreasing during D4 exit are enriched in translation, ncRNA metabolic process as well as ribosome biogenesis, nuclear transport, and spliceosome.

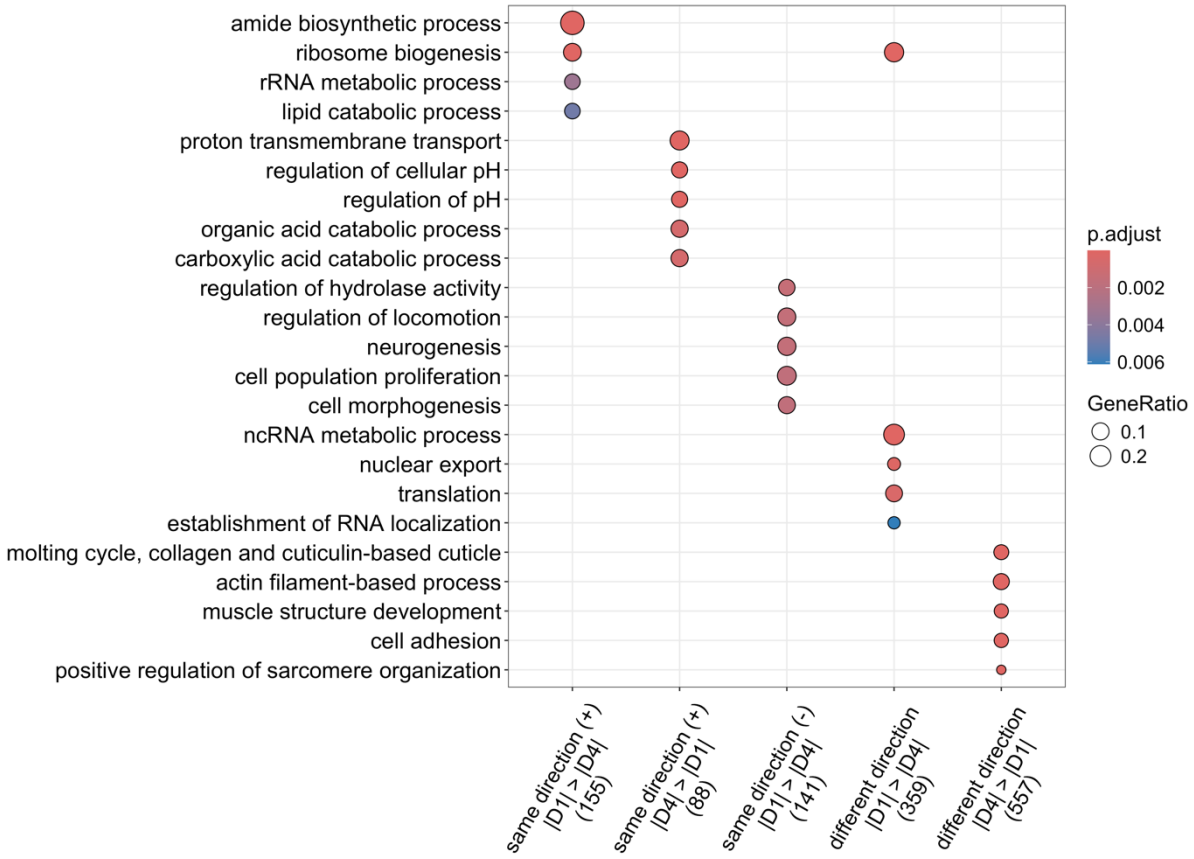

Figure 2.12 ORA using genes with differing expression patterns between D1 and D4 exit (GO BP)

Number of genes found to be enriched in each of the groups is specified in parentheses. See SFig.1 for ORA results including cellular component (CC) and molecular function (MF) ontologies.

These results present many avenues for exploration. For example, the overrepresentation of translation and ribosome biogenesis pathways among overexpressed genes in D1 exit could suggest an ability of worms exiting from D1 to prepare for translation earlier than those exiting from D4.

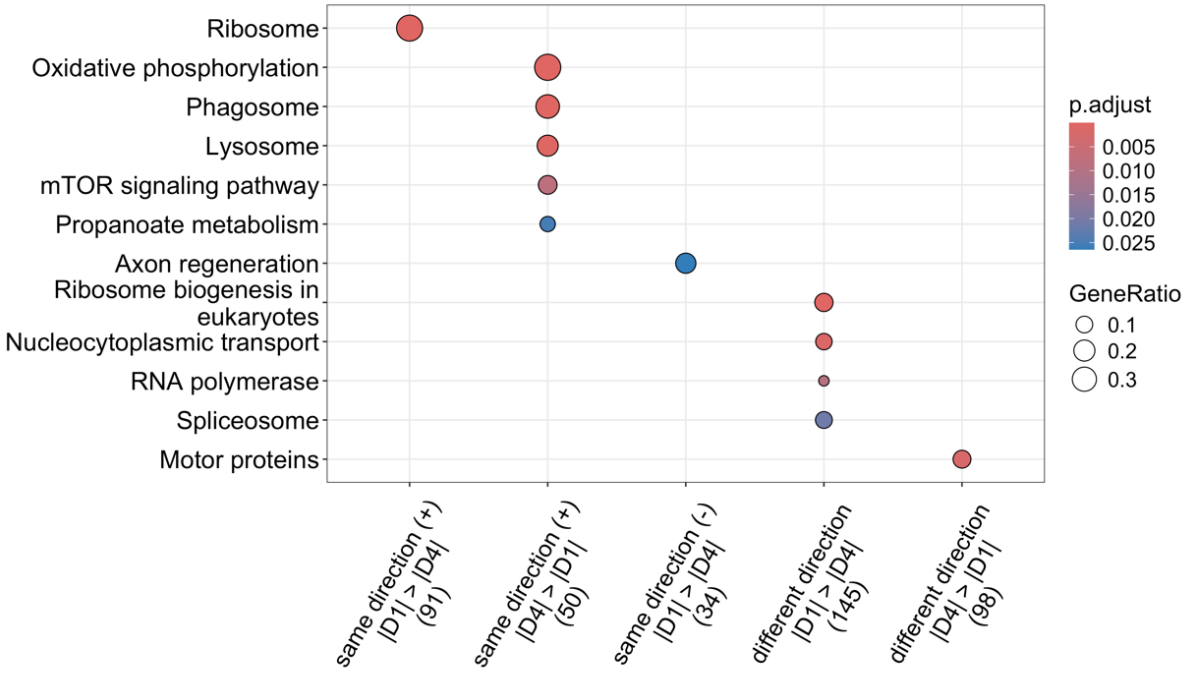

Figure 2.13 ORA using genes with differing expression patterns between D1 and D4 exit (KEGG)
Number of genes found to be enriched in each of the groups is specified in parentheses.

We sought to further elucidate the processes that would capture the essence of the recovery process. We hypothesized that 1) the program responsible for reverting age-associated changes during recovery from dauer is the same between D1 and D4 recovery, and 2) the difference lies in the magnitude of up- or downregulation, i.e. the same pathways are differentially regulated in both cases, but are induced to a greater degree during D4 recovery as these worms accumulated more age-related changes which are reverted in the same amount of time as during D1 recovery.

Thus, we focused on the two groups in which genes change in the same direction during both exits but the change is more pronounced (i.e. the slope is steeper) during D4 exit. We found that such genes are enriched in pH and cation transport related GO terms (Fig. 2.14A) and phagosome, oxphos, mTOR signaling and lysosome KEGG pathways (Fig. 2.14B). Additionally, a group of *vha* genes is represented prominently among those overexpressed. *vha* stands for vacuolar H^+-ATPase (also, V-ATPase) which is a conserved and ubiquitous enzyme responsible for proton transport and acidification of cellular compartment in animals (Futai et al., 2019). All of the *vha* genes from the group encode for a subunit of V-ATPase. As evident from the heat plots (Fig. 2.14), *vha* genes constitute the majority of genes belonging to phagosome, oxphos, mTOR signaling and lysosome pathways (Fig. 2.14B). The rest of the genes include ndus-8, nuo-4, nuo-6, C16A3.5 (predicted to be involved in NADH-related processes, according to WormBase WS292 (Davis et al., 2022)) and metabolic genes (from propanoate and 2-Oxocarboxylic acid metabolism categories).

In order to identify potential transcription factors (TFs) regulating multiple genes throughout dauer or during dauer exit, we performed motif enrichment analysis using HOMER (Heinz et al., 2010) (Table 1).

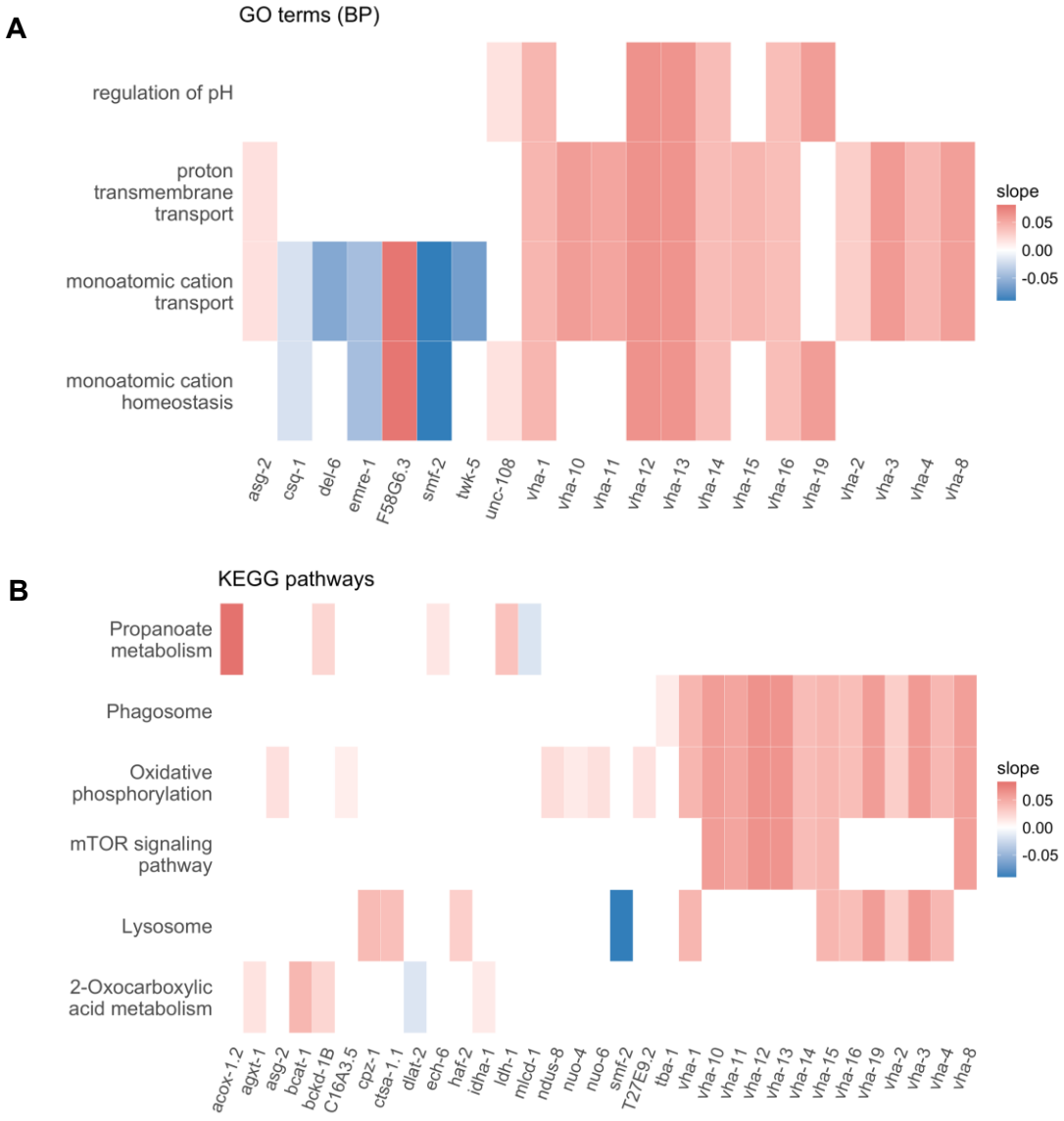

Figure 2.14 ORA using genes whose expression change is induced more in D4 exit
(A) GO (BP) and (B) KEGG databases. The list of enriched terms in (A) was edited for clarity and brevity. For the heatmap with a full list and other ontologies (CC and MF) see SFig. 3. The slope refers to the slope of the gene during exit.

GATA transcription factors are conserved across animals, plants, and fungi and most well-known for playing a key role in embryonic development, cell fate decisions, and tissue morphogenesis (Tremblay et al., 2018). In *C. elegans*, the 11 GATA TFs coordinate development of the gut, epidermis, and vulva and expression of some of them persists into adulthood (Block & Shapira, 2015). Considering that GATA motifs are enriched in the genes overexpressed during D1 and D4 exit and underexpressed

during D1 exit and dauer diapause, a reasonable explanation would be that since GATA TFs orchestrate many developmental processes, they will inevitably appear enriched in any movement along the development axis i.e. continuing (dauer exit) or halting (dauer diapause) development.

Enrichment in TATA box among dauer underexpressed and D4 exit overexpressed genes suggests a global shutdown and reactivation of transcription, respectively.

We also observed that overexpressed D1 exit genes are enriched in a motif, that is targeted by *ces-2* TF. *ces-2* is involved in several processes, including aging and positive regulation of cell death and transcription by RNA polymerase II (Metzstein et al., 1996; Wang et al., 2006). Lastly, overexpressed dauer genes are enriched in *lin-54*-binding motif. LIN-54 has a DNA-binding activity and is a constitutive part of the MuvB subunit of the DREAM complex which regulates cellular quiescence and differentiation (Bujarrabal-Dueso et al., 2023; Müller & Engeland, 2010).

Our findings suggest 1) that an interplay between protein synthesis and overall degradation, including autophagy and mitophagy, could be a part of the dauer longevity program in tandem with alternative splicing variants usage; 2) an existence of a transcriptomic program that rejuvenated worms during dauer recovery, and 3) such rejuvenation process relies on the function of V-ATPases that enable lysosome, oxphos, mTOR and autophagy functioning.

Table 1 Summarised results of motif enrichment analysis using HOMER

% of Targets: percentage of target genes enriched in a motif; % of Bg: percent of background genes enriched in a motif; STD (Bg) STD: standard deviation of position of target (or background) sequences in base pairs; best match/name: manually selected best match based on the HOMER output or a name of the known motif.

Motif	p-value	% of Targets	% of Bg	STD (Bg STD) [bp]	Best match/ name	Enriched in
	1e-13	11.3	4.33	53.8 (65.6)	TATA-Box	D4 exit, up
	1e-12	20.21	10.75	76.4 (99.1)	elt-2 (GATA motif)	D4 exit, up
	1e-20	6.26	0.51	10.8 (49.2)	ces-2	D1 exit, up
	1e-14	5.54	0.63	75.5 (85.1)	elt-3 (GATA motif)	D1 exit, down
	1e-7	29.49	19.14	NA	elt-3 (GATA motif)	D1 exit, up
	1e-16	22.38	8.31	53.5 (79)	elt-3 (GATA motif)	Dauer, down
	1e-12	34.79	19.03	79.6 (93.3)	TATA-box	Dauer, down
	1e-13	5.28	0.8	92.3 (71.9)	lin-54	Dauer, up

2.4. Aging patterns across dauer and non-dauer worms

Considering the image of dauer as a non-aging stage, we wanted to compare the effect that passage of time has on arrested worms and non-dauer worms. For this, we chose to use a publicly available longitudinal transcriptomic dataset (Ham et al., 2022) that includes aging samples of WT (N2) and *daf-2(e1370)* mutant worms from four time points: day 1, 4, 7, and 11.

The PCA of dauer and non-dauer samples (Fig. 2.15) shows that both N2 and *daf-2* adult samples cluster separately from dauer samples and move predominantly but not exclusively along PC1 with age increase. In contrast, dauer samples have a wider

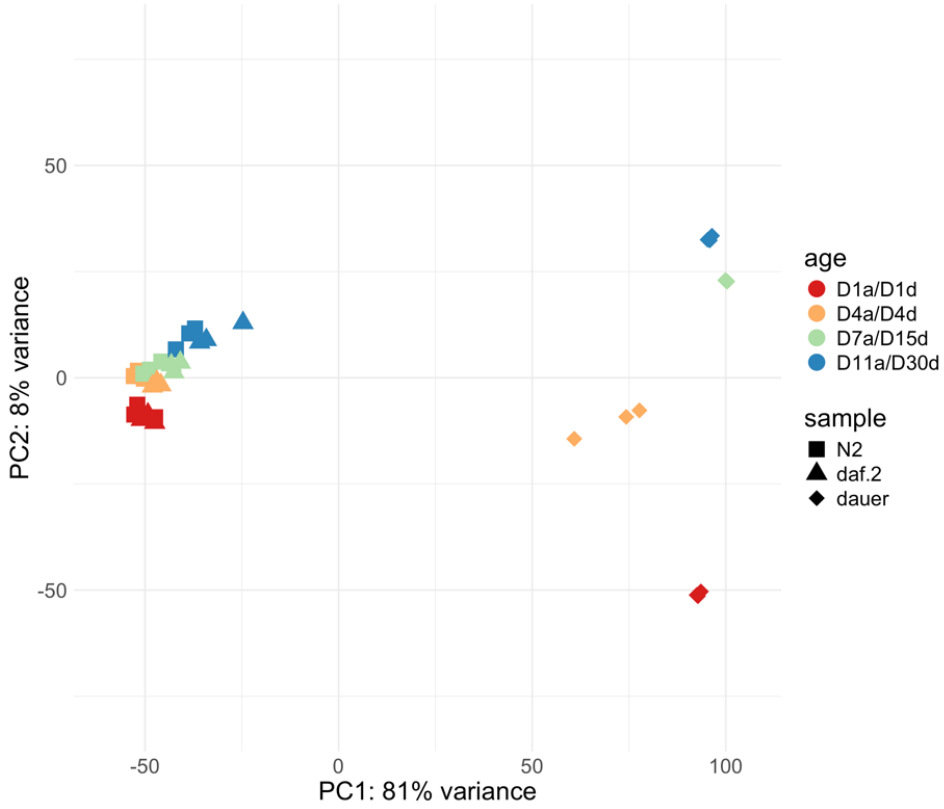

Figure 2.15 Dauer samples cluster separately from adult samples and move mostly along different axes during chronological aging

Principal component analysis (PCA) of RNA-sequencing output for samples of worms in dauer diapause (diamond) and non-dauer wild-type (N2, square) and *daf-2* mutant (*daf-2*, triangle) adult worms. Age legend: a is for adult, d is for dauer. Variance explained by each principal component (PC) is indicated on the axis label.

spread, mostly along the PC2, with a relatively smaller difference in PC1. Such a great variance between dauer samples is surprising as they are expected to age slower than adult non-dauer samples and therefore exhibit fewer differences sample to sample.

First, we wanted to identify transcriptomics patterns that describe the fundamental difference between worms in dauer diapause and adult worms. We separated dauer and adult samples in two groups and compared expression levels between them (Fig. 2.16). Synaptic signaling and related pathways, such as G protein-coupled receptor signaling, regulation of membrane potential and monoatomic ion transmembrane transport, were found to be enriched GO terms in dauer overexpressed genes (Fig. 2.16A). This is mirrored in GSEA KEGG results with neuroactive ligand-receptor interaction, MAPK and calcium signaling and axon regeneration pathways being enriched in genes with elevated expression in dauer (Fig. 2.16B). Aging worms were described to experience a decline in synaptic integrity (Wirak et al., 2022), which could explain the difference in gene expression we observe as worms undergoing dauer must preserve neuronal integrity throughout the diapause as they are critically dependent on it for sensing changes in environmental conditions that would allow for dauer exit and ensuring a successful postdauer life.

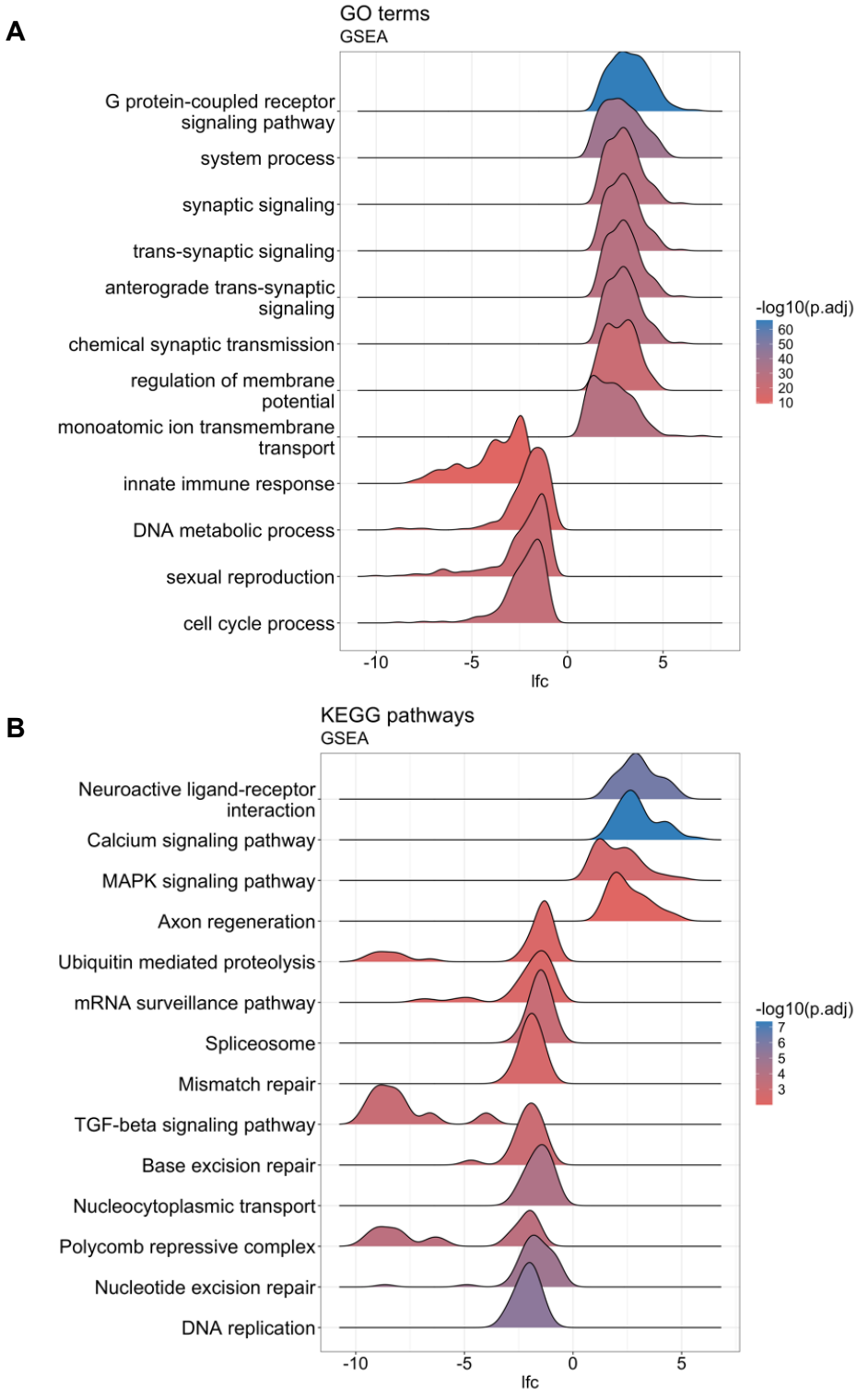

Figure 2.16 Dauer-specific transcriptomic patterns

Gene set enrichment analysis (GSEA) of differentially expressed genes (DEGs) between all dauer and all adult samples using GO terms (A) and KEGG (B) databases. lfc: log fold change, p.adj: adjusted p-value.

At the same time, processes associated with cell cycle (cell cycle process, DNA metabolic process, DNA replication), development (endoderm development, TGF-beta signaling pathways), RNA processing (spliceosome), histone modification (polycomb repressive complex), and DNA repair (BER, NER, mismatch repair) are enriched in underexpressed genes in dauer suggesting developmental and transcriptional quiescence. Interestingly, pathways such as spliceosome and mRNA surveillance are underexpressed in dauer compared to an adult worm while their expression increased during dauer (Fig. 2.10B).

Next, to further address the difference in aging between adult and dauer worms, we focused on the genes whose expression changes during aging differently. To this end, we focused on D1 and D4 dauer samples and d7 and d11 adult samples. The reasoning behind this choice is two-fold: 1) to exclude reproduction-related genes from the analysis as worms have laid most of their eggs by day 5 of adulthood, 2) to include samples that are most comparable to each other in terms of the amount of time between them (3 days between dauer and 4 days between adult samples). Unlike in the dauer exit analysis, we included genes whose slope is not equal zero in at least

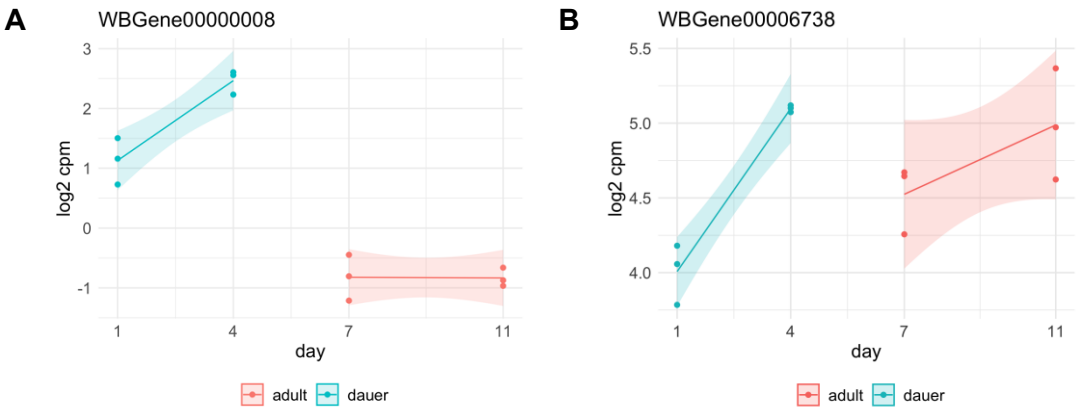

Figure 2.17 Expression trajectory of two example genes during dauer or adulthood
Dauer samples are marked blue, adult samples are marked red. cpm – counts per million.

one of the groups (versus having to be non-zero in both groups) and whose slope differs significantly between the groups (example genes on Fig. 2.17).

Based on this gene list, we once again performed ORA to discover that genes whose trajectory differs in dauer and adulthood are enriched in translation (translation, protein-RNA complex assemble and organization, ribosome) RNA processing (ncRNA processing, spliceosome, RNA polymerase) related processes as well as DNA replication and NER (Fig. 2.18).

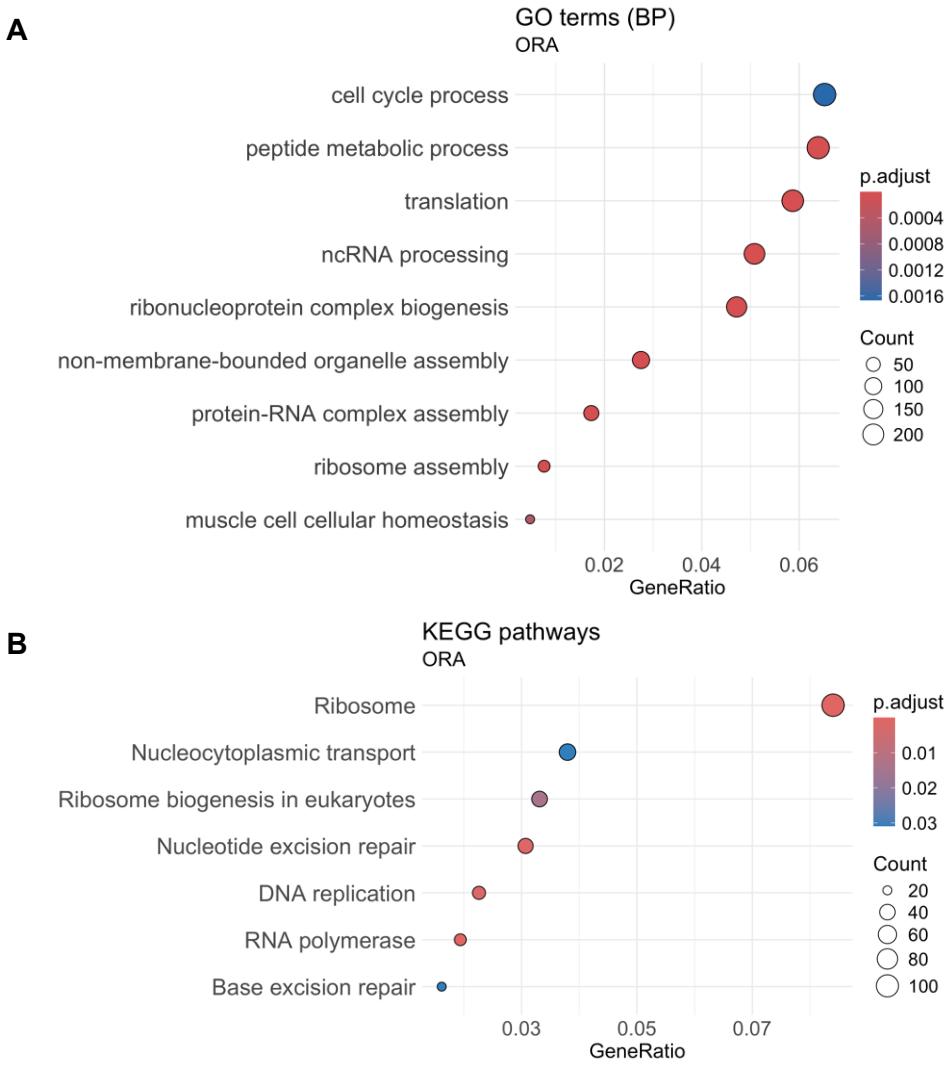

Figure 2.18 Translation and DNA repair genes differ significantly between dauer and non-dauer aging

ORA of genes whose trajectory differs in dauer compared to adult aging.

Altogether, we highlight differences in gene expression patterns that could provide insight on how dauers undergo a successful aging process without phenotypic decline of health.

2.5. Effects of UV on dauer recovery and length-dependent gene expression

Worms in dauer are known to be resistant to various types of stress (Jones et al., 2001). We aimed to know how exposing worms to UV radiation at the beginning of diapause would affect recovery dynamics depending on the time spent in dauer. We chose UVB treatment because it predominantly induces the well-defined cyclobutane pyrimidine dimers (CPDs) that provide a paradigm for helix-distorting lesions. This lesion type interferes with transcription elongation and is thus also highly relevant for non-dividing cell types, such as those comprising the dauer larvae. We irradiated D1 and D4 worms, induced dauer recovery and observed no significant difference between D1 and D4 recovery dynamics (SFig. 3). This means that worms that spent longer in dauer did not experience any disadvantage recovering after UV-treatment despite being older.

To check whether despite the absence of a phenotypic consequence in dauers, applying UV irradiation will have biological consequences and require the repair of UV-induced DNA damage, we continued by testing the consequences of perturbing the NER pathway. NER is the major mechanism required for removing helix-distorting lesions such as UV-induced CPDs. The lesions are sensed by the CSB-1 protein upon stalling of the RNAPII to induce transcription-coupled (TC-) NER or throughout the genome by XPC-1 that induces global-genome (GG-) NER. Both converge on the NER

core pathway by recruiting XPA-1. Upon UV irradiation, mutations in *csb-1* lead to developmental growth delays and premature aging in the worm's postmitotic somatic cells (as they require transcription but not DNA replication), while *xpc-1* mutants are UV sensitive in proliferative germ cells. *xpa-1* mutants are extremely sensitive to UV in all cell types as no NER activity is present (van den Heuvel et al., 2020).

Given the role of NER in DNA damage-driven aging, we then tested the consequences of a perturbed NER pathway for dauer exit by performing a developmental assay on

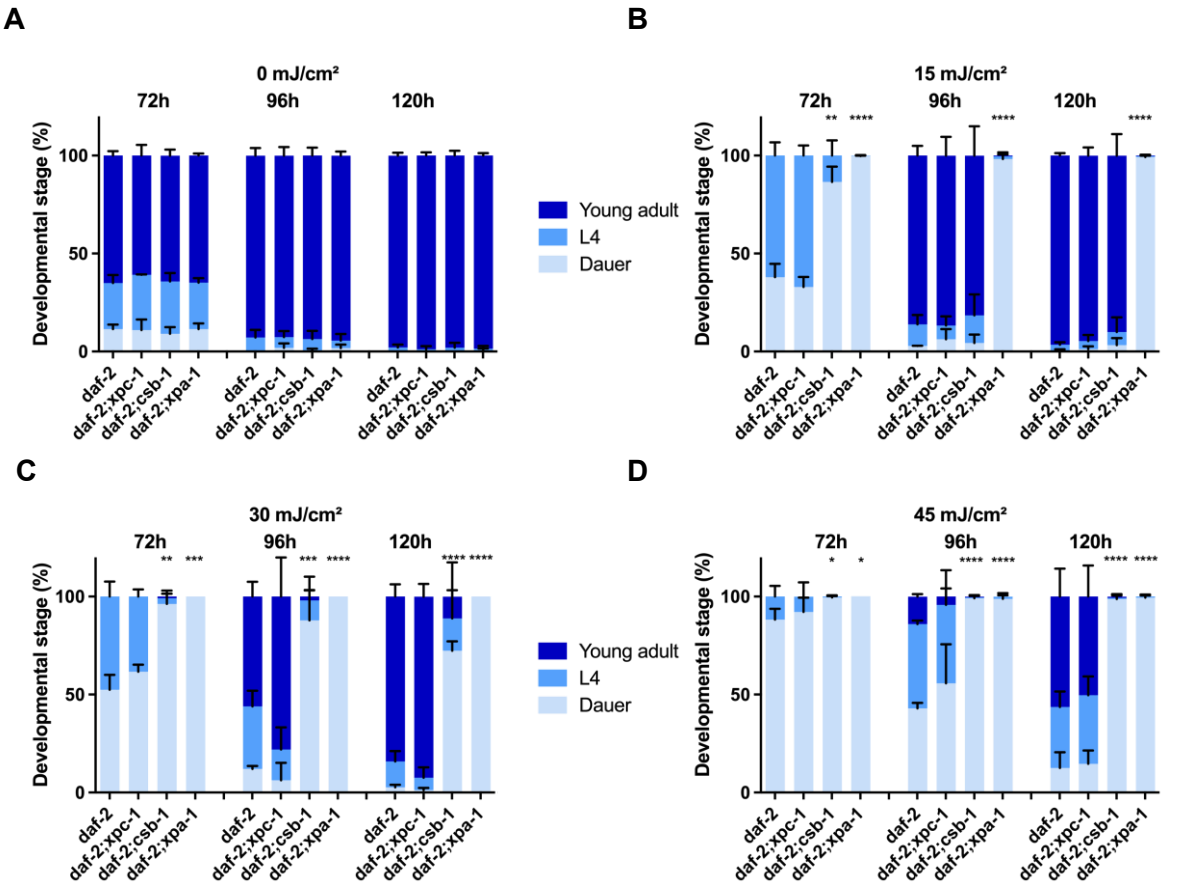

Figure 2.19 NER machinery is required for successful dauer exit which is impaired by UV treatment equally in D1 and D4 worms

daf-2, daf-2;xpa-1, daf-2;xpc-1, and *daf-2;csb-1* animals were UV- or mock-treated at day 1 of dauer. Panels (A) to (D) show, respectively, 0, 15, 30 and 45 mJ/cm2 conditions. Dauer exit was induced immediately after the UV treatment. (average of n=3 independent experiments per strain and dose is shown, error bars represent the standard deviation (SD); ****p <0.0001, Two-tailed t-test compared with *daf-2*). From (Barata, 2022).

NER mutants (Fig. 2.19A-D). All NER mutants had similar recovery dynamics to single

daf-2 mutant in the absence of DNA damage stimulus. However, upon UV exposure we observed a dose- and mutation-dependent delay in resuming development (Fig. 2.19B-D). *daf-2;csb-1* mutants were unable to resume development. *daf-2;xpc-1* mutants exhibited exit dynamics similar to *daf-2* single mutants. *daf-2;xpa-1* mutant worms were unable to resume development even at the lowest dose.

To further address the role of NER pathway during dauer exit, we assessed its activity by quantifying the removal of UV-induced CPDs with a slot blot (Fig. 2.20). The slot blot shows that dauers can remove CPDs. More than 50% of the UV-induced damage is repaired in the first 24 h, with further repair happening slower, similarly to the non-dauer L3 condition and in agreement with previous reports that suggest biphasic repair kinetics (Cipollini et al., 2006).

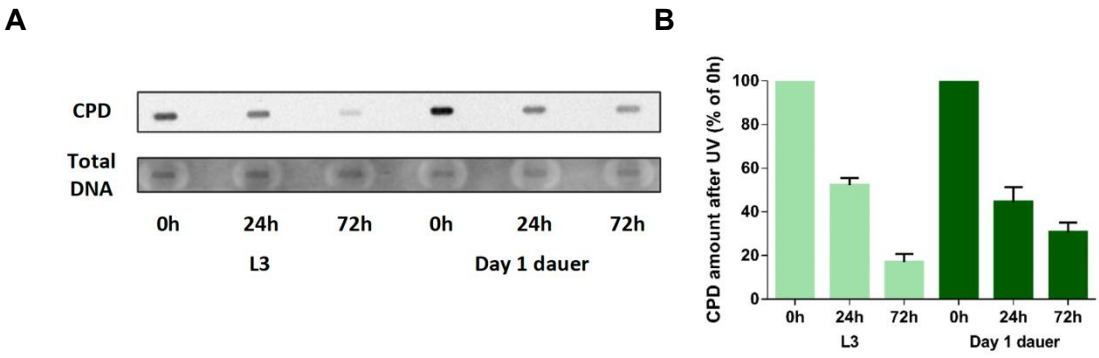

Figure 2.20 Worms in dauer are able of repair UV-induced DNA damage
daf-2 animals were UV-treated (75 mJ/cm2) at the L3 or dauer stage (D1). CPDs were measured via slot blots by antibody staining, 0 h, 24 h, and 72 h after UV treatment, and normalized to the respective 0h time-points (result shown in panel (A) is representative, experiment was repeated 2 times). From (Barata, 2022).

Next, we performed RNA-sequencing on a set of samples that were irradiated with UV at D1, namely, D1.6, D1.24, D4, D4.6, and D4.24 matching their non-irradiated counterparts introduced earlier.

First, we included the UV-treated samples in the PCA shown earlier (Fig. 2.7) and observed little change in clustering of D1.6, D1.24, D4.6, and D4.24 samples, however, D4.UV samples clustered in closer proximity to D4.6 and D1.24 than D4 suggesting a high degree of similarity in transcriptomes (Fig. 2.21). This means that either UV-treatment does not affect transcriptome of recovering worms in a major way or they have a program that allows to at least partially ignore consequences of such treatment.

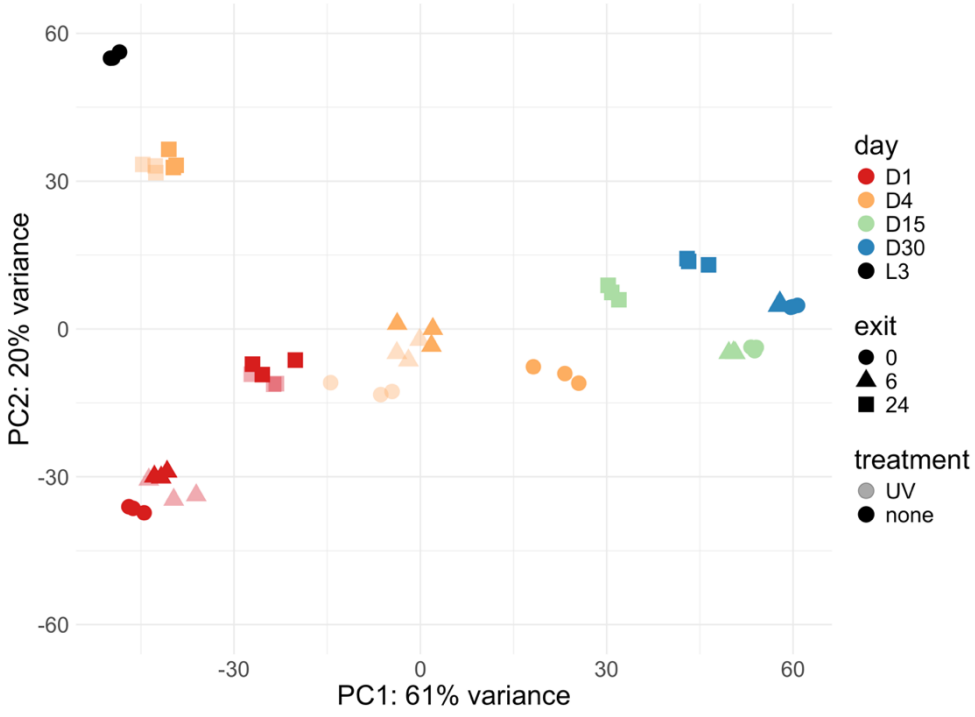

Figure 2.21 Some of the UV-treated samples cluster differently from the untreated counterparts
Principal component analysis (PCA) of bulk RNA-sequencing results grouped by developmental stage (color), time after exit from dauer was induced (shape) and whether a sample is derived from UV treated D1 sample.

Second, we added the UV-treated samples to the BiT Age clock and saw that PBA for UV-treated samples was not significantly different to their untreated counterparts except for D4 samples (Fig. 2.22). D4.UV (D4.0.UV on the figure) is predicted to be significantly younger than D4 which is surprising considering the UV-irradiation. We hypothesize that the UV-triggered DDR could have resulted in an age reversal.

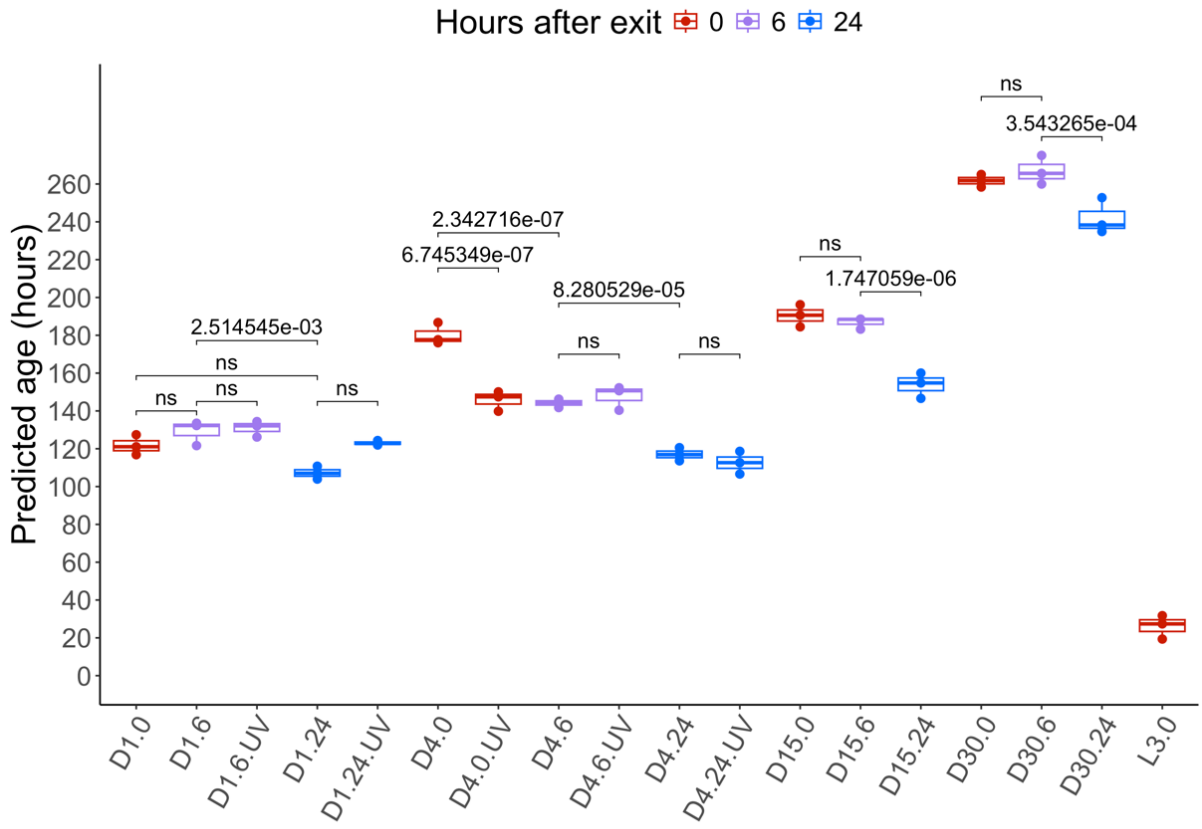

Figure 2.22 Predicted biological age of dauer (untreated and UV-treated) and L3 samples
Each dot represents a single RNA-seq sample. P-values were calculated using one-way ANOVA with post hoc Tukey test. See Table S2 for results for all comparisons. ns – not significant.

To describe transcriptomic changes occurring after and as a response to UV irradiation, we used the same linear modeling approach as earlier, separated untreated and treated samples into groups, and followed up with unsupervised clustering to elucidate which genes behave similarly. We compiled a list of top 1,000

DEGs between non-UV and UV samples and separated them into three clusters (Fig. 2.23).

Cluster 1 (red) consists of genes whose expression decreases during exit (except for D1 UV-treated samples) and it is enriched in genes related to synaptic signaling, GPCR signaling pathway, and neuroactive ligand-receptor interaction (Fig. 2.24). Expression of cluster 2 (green) genes changes depending on the day of exit. In D1 samples, the expression increases in both treated and untreated samples although the starting point is different. At the same time, in D4 samples we see two opposite trajectories: expression decreases in untreated samples while increasing in UV-treated samples. This cluster is enriched in genes involved in cell cycle, DDR and chromosome organization pathways as well as repair pathways (Fig. 2.24). Genes from cluster 3 (blue) demonstrate the opposite behavior during D4 exit: decreased expression in UV-treated samples and increased expression in untreated samples. This is the cluster enriched in defense and immune response genes alongside ABC transporters (Fig. 2.24). Interestingly, during D4 exit all the clusters converge by 24 hours post dauer. The difference in the trajectories between D1 and D4 exit can be a starting point to disentangling the differential UV-response during dauer recovery.

Gene expression patterns both during aging in multiple species and in response to UV-induced DNA damage were recently shown to be characterized by a decrease of the expression of long genes. The gene length-dependent transcription decline (GLTD) is thought to be triggered by the accumulation of transcription blocking lesions

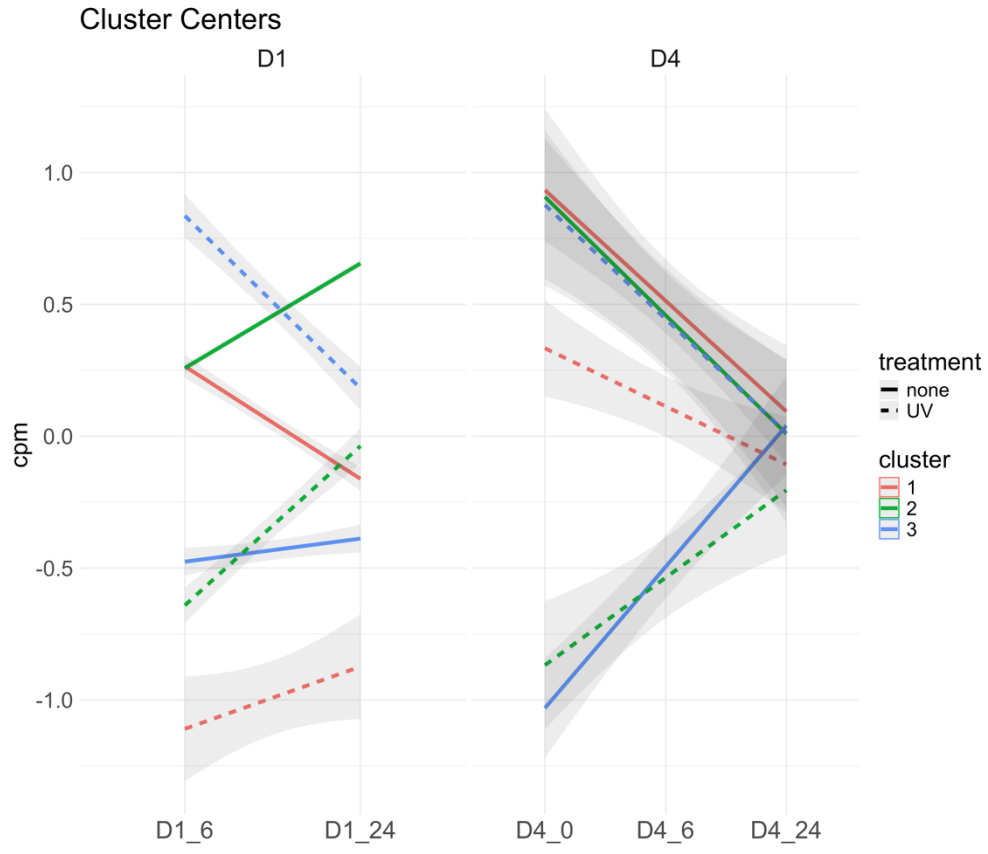

Figure 2.23 Top 1000 DEGs between un- and UV-treated samples split into three clusters
The lines represent cluster centers. Clusters were obtained using non-supervised fuzzy clustering. cpm – counts per million

during the aging process (Gyenis et al., 2023; Ibañez-Solé et al., 2023). Therefore, we inquired whether *C. elegans* dauer larvae exhibit a similar decrease in expression of long genes upon UV irradiation.

We created a list of top 1,000 DEGs between all non-UV and UV samples, as well as five additional top 1,000 lists for each pairwise comparison (D1.6 vs D1.UV.6, etc.). Then, we separated genes into up- and downregulated, and calculated gene length of those groups (Fig.2.25). Consistently, genes that were upregulated following UV-induced DNA damage were significantly shorter, while longer genes were downregulated, consistent with the effect of transcription-blocking lesions. This difference in gene length was most pronounced in the earliest timepoint after UV

(D1.6) and in D4 sample suggesting limited removal of transcription-blocking lesions during the dauer stage. The gene length difference was less pronounced during exit (e.g. D1.24) and completely leveled in D4.6 and D4.24 suggesting highly efficient repair during the dauer exit.

Overall, our data show that transcription-blocking DNA lesions impact pathways that are differentially regulated throughout the recovery and skews transcription patterns

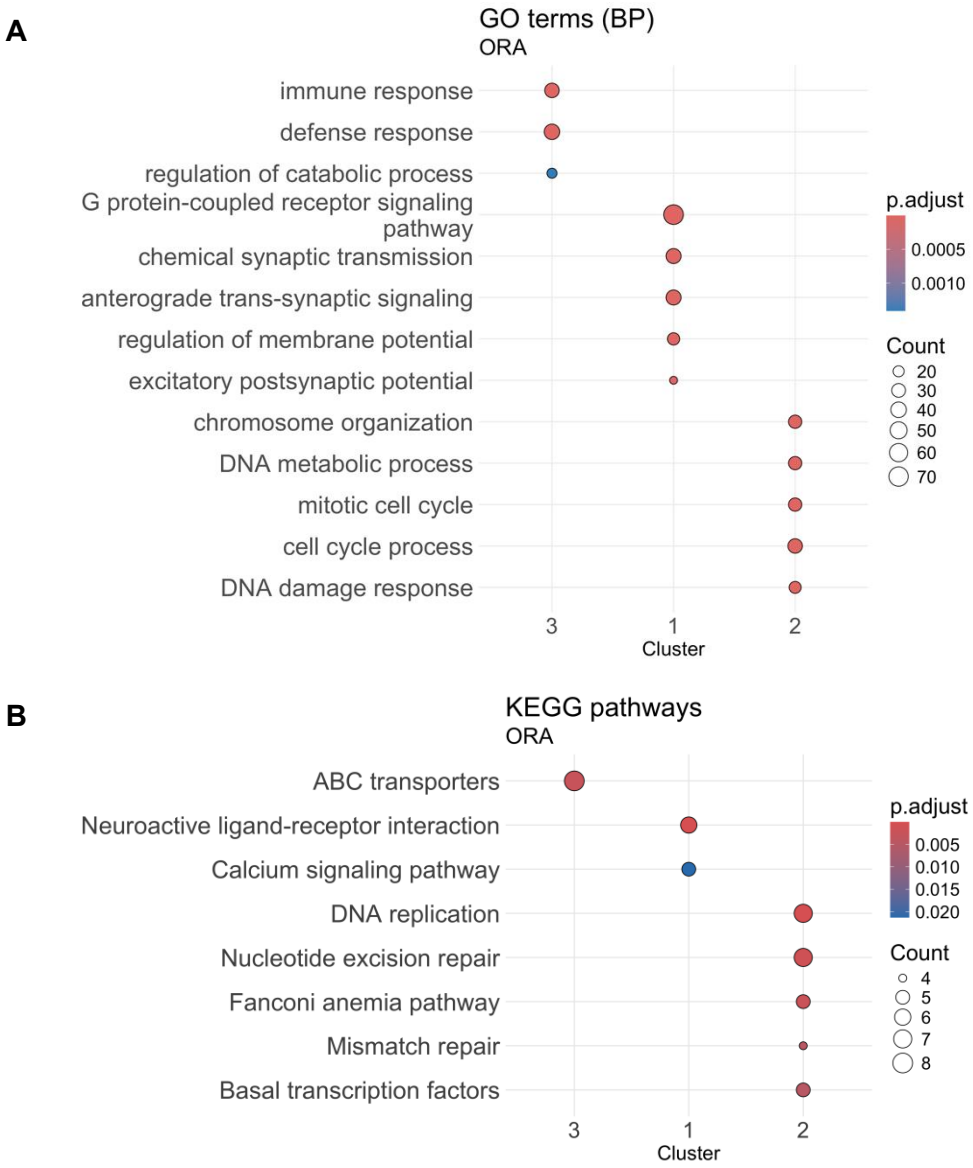

Figure 2.24 Functional enrichment of top DEGs between un- and UV-treated samples
ORA results using GO terms (A) or KEGG (B) databases. Enrichment terms are split by clusters from 2.23

in a gene-length-dependent manner. The combination of our experimental data and transcriptomic analysis suggests that even though worms appear to be capable of DNA repair during dauer, it does not seem to be sufficient to remove all the damage caused by UV-treatment. Gene expression during dauer is affected by transcription-blocking lesions from which the animals only completely recover upon dauer exit.

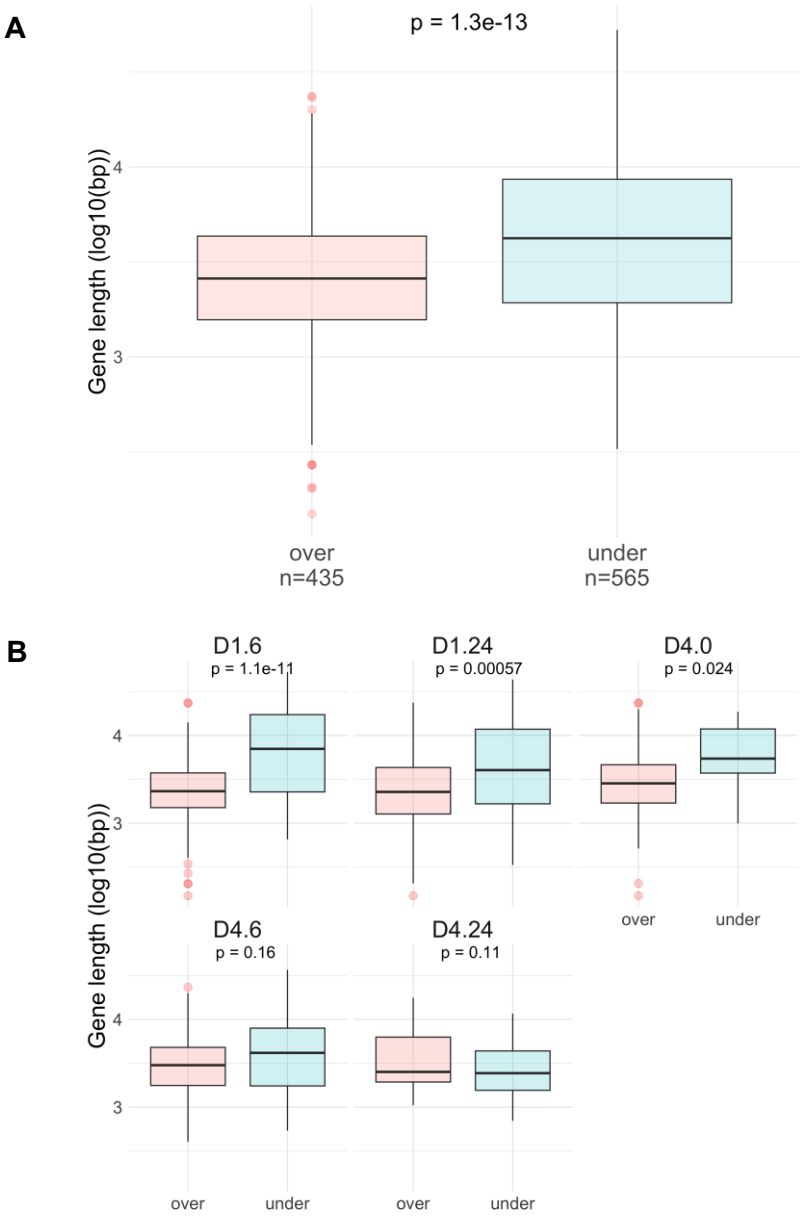

Figure 2.25 Genes underexpressed upon UV-treatment tend to be longer
Gene lengths in $\log_{10}$ of over- and underexpressed DEGs for all treated vs untreated samples (A) or pairwise comparisons (B). p-values were calculated with unpaired Wilcoxon test.

Chapter 3 Discussion

Since *C. elegans* was established as a model organism in the early 70s (Brenner, 1974), because of its small size, ease of handling and cultivation, pre-determined number of cells and developmental trajectory and an array of genetic tools available, among other advantages, it has become a powerful tool in addressing a range of biological questions including matters of development and aging. It possesses certain unique qualities, such as a diapause, that allows to push the envelope of our understanding of aging further. At first glance, a quiescent state in a tiny free-living nematode does not seem to have great implications for biogerontology. And yet, it does. Even considering the variety of reports regarding the postdauer lifespan (S. E. Hall et al., 2010; S. Kim & Paik, 2008; Klass & Hirsh, 1976), none of the reported values are lower compared to the worms that developed normally. According to the classic paper by Klass & Hirsh, this suggests that worms in dauer do not age. But why not? And how do they do this?

3.1 Postdauer fitness and predicted biological age

To fundamentally address these questions, we first wanted to check the impact of dauer diapause on postdauer fitness in our experimental setup. Many of the studies on dauer use WT worms and induce dauer entry by exposing them to the dauer pheromone, which is a mixture of ascarosides that indicates the population density (Ludewig, 2013). In this project, we opted to use a Daf-c mutant *daf-2(e1370)* to achieve high frequency dauer entry after synchronizing worms at L1 stage.

We report that increased time in dauer up to 30 days does not cause a delay in dauer recovery as there are no significant differences between number of worms at different developmental stages and by 96 h after exit induction the majority of worms reached young adult stage (Fig. 2.2). We also checked the postdauer lifespan and brood size and observed, once again, no significant differences between non-dauer *daf-2* and *daf-2* mutants that spent 1, 10, or 20 days in dauer (Fig. 2.3 and Fig. 2.4). All of the *daf-2* mutants were significantly longer lived and produced less progeny than N2 strain as reported previously (Kenyon et al., 1993; Kimura et al., 1997). The discrepancy between our and previously published results could stem from the differences in experimental setups, dauer-induction and scoring protocols.

Transcriptomic data on dauer are very scarce and even those that exist don't include a sufficient number of time points during the diapause, during exit, or both (accession numbers of exemplar datasets: GSE52910 (Hendriks et al., 2014) and GSE214208). Therefore, we devised a setup and performed RNA-sequencing on the samples of worms arrested in dauer for 1, 4, 15, or 30 days and recovering populations at 6 h and 24 h after the recovery was induced (Fig. 2.6).

As a first step, we wanted to assess the biological age of worms undergoing and exiting dauer to establish whether any predictions about their longevity, or "non-aging" and age reversal, or rejuvenation could be made. For this we used an updated version of the transcriptomic clock developed previously in our lab (Meyer & Schumacher, 2021) as *C. elegans* has only very low levels of DNA methylation (Hu et al., 2015) which means that mammalian epigenetic clocks cannot be applied in this case. According to BiT Age results (Fig. 2.8), the PBA increases during dauer arrest and decreases upon exit induction. Specifically, the rate of age increase changes from

0.8 in the first three days to 0.1 from D4 to D30, suggesting an aging plateau is reached after a couple of days of arrest. Additionally, this result demonstrates that 24 h is sufficient for D1 and D4 worms to reach the same decreased biological age, however, worms from D15 and D30 samples are predicted to remain biologically older even after 24 h of recovery. It is also apparent that there is no difference in PBA between D15 and D15.6 just like D30 and D30.6. This suggests that worms that spent more time in dauer while still can decrease their biological age do so later and slower than worms that spent only 1 or 4 days in the arrest. Taken together with developmental resumption results (Fig. 2.2), recovery from dauer and resuming development appear to be decoupled from the age reversal process.

A similar decrease in biological age was shown to occur in early embryonic development (Kerepesi et al., 2021) and reprogramming of human dermal fibroblasts (Olova et al., 2019). Based on the BiT Age results, we propose that exit from *C. elegans* dauer diapause is an example of a naturally occurring rejuvenation.

Applying BiT Age to developmental data as well data from other diapause states such as L1 arrest or ARD would help to gain further insights into the nature of aging during development as well as the underlying principles according to which BiT Age predicts biological age.

3.2 Aging in dauer

To investigate closer the observed phenomenon of slow aging in dauer, we describe how gene expression levels change throughout the diapause. We observe a change in transcript levels in many groups of genes (Fig. 2.10). We will further focus on those that could have the largest implications for dauer longevity.

We observed an increase in expression of sensory perception genes during dauer and think that it can be explained by an increasing urgency to find new, resource-rich environment. Because dauer larvae rely on stored lipids for energy, it is crucial to exit dauer before the storage is exhausted. For example, *C. elegans* lacking LKB1/AMPK signaling are able to enter dauer but consume the energy storage rapidly which leads to organ failure and death, highlighting the importance of rationing (Narbonne & Roy, 2009). Perhaps, there is a feedback loop that induces expression of sensory perception genes as a response to depleting fat storages.

Translation genes and those of related processes (e.g. ribosome, protein folding, etc., see Results for details) also increase their expression the longer dauer persists. Yet, translation was suggested to be inhibited during dauer diapause (Pan et al., 2007). However, since our analysis includes only dauer samples, we do not compare expression levels between dauer and non-dauer worms. Proteomics studies would aid in further dissecting translational dynamics during dauer diapause.

Autophagy, mitophagy, and proteasome pathways' expression increases during dauer as well. Such a combination – protein synthesis and degradation – could suggest an increased rate of protein turnover with dauer age. Impaired protein homeostasis that manifests as protein aggregation is one of the aging hallmarks (López-Otín et al., 2013, 2023) and enhancing proteostasis promotes longevity (X.-Q. Chen et al., 2023). Supporting the idea of this interplay are results demonstrating that autophagy alone is not sufficient to extend lifespan (Hansen et al., 2008) but is required for normal lifespan extension (Meléndez et al., 2003). In later years, more studies have emerged demonstrating that autophagy can have tissue-specific longevity effects and selective types of autophagy, including mitophagy (Palikaras et al., 2015; Schiavi et al., 2015)

whose expression is also increased in dauer, can play a crucial role in promoting and enabling longevity (Hansen et al., 2018). Performing protein aggregation assays on dauer larvae and comparing the results to non-dauer worms would test this hypothesis and help establish the role of proteostasis in dauer maintenance and longevity.

Additionally to its role in protein turnover, the autophagy increase can suggest an exhaustion of dauer's internal lipid storages, forcing them to recycle other macromolecules as an additional energy source (Singh & Cuervo, 2011).

Alternative RNA splicing increases the variety of proteins that can be produced from the same coding sequence. Considering that worms undergo complete remodeling when entering dauer diapause, it is not inconceivable that they might employ alternative splicing to enrich their proteome. At the same time, dysfunctional alternative splicing has been recently associated with aging as age-related changes in splicing factor levels are detected in mice, rats, and humans (Angarola & Anczuków, 2021; Bhadra et al., 2020). Even more intriguing is the finding that some splicing factors are connected to longevity through mTOR and AMPK pathways in *C. elegans* (Angarola & Anczuków, 2021). Long-read sequencing approaches such as PacBio or Nanopore would allow to examine the question of differential transcript usage and, hopefully, illuminate the alternative splicing process in dauer.

3.3 Recovery from dauer and how it is affected by dauer duration

BiT Age results (Fig. 2.8) lead us to focus on the comparison between D1 and D4 recovery trajectories. Since in both cases worms that were recovering for 24 h reach the same biological age (i.e. there is no significant difference), the rejuvenation has to happen faster during D4 exit. We assumed that the recovery and therefore the

rejuvenation program does not change in nature based on dauer duration but does change in its intensity. That is to say that the same genes and pathways are being activated or inhibited but to a different degree depending on the dauer duration.

As such, we created a list of genes whose expression levels change in the same direction during D1 and D4 exit but the change is more pronounced in D4 exit, i.e. if Gene A in overexpressed in D1 exit, it will be even stronger overexpressed in D4 exit. We then performed ORA and discovered a number of pathways enriched in both GO and KEGG databases (Fig. 2.14). GO terms captured regulation of pH and cation or proton transport biological processes (Fig. 2.14A) while KEGG pathways matched the genes to two metabolism pathways (propanoate and 2-Oxocarboxylic acid metabolism) and four pathways whose main components in this case are *vha* genes: phagosome, oxphos, mTOR signaling, and lysosome (Fig. 2.14B).

Propanoate pathway consists of genes that metabolize propionic acid and propionyl-CoA. The latter can be produced via β-oxidation of unsaturated fatty acids which is a major source of energy generation in dauer larvae (M. S. Gill & Olsen, 2017; Wadsworth & Riddle, 1989). Moreover, *ech-6* gene – encodes enoyl-CoA hydratase which catalyzes the second step of β-oxidation – that is overexpressed in D4 exit was shown to have an effect on lifespan in fat diet conditions (Y. J. Liu et al., 2022). While it's the repression of *ech-6* which was shown to have a beneficial effect, the study was conducted on worms with shortened lifespan on a fat diet which is different from the conditions of recovering dauers. Regardless, *ech-6* was identified as a potential metabolic flexibility modulator and it could be modulating metabolism during dauer exit.

Among 2-oxocarboxylic acid metabolism genes is *bcat-1* (branched-chain amino acid transferase-1, homolog of human BCAT2) which metabolizes branched-chain amino acids (BCAAs) and when impaired leads to lifespan extension in an mTOR-dependent manner (Mansfeld et al., 2015). It is required for larval development and was recently shown to improve age-related reproduction in *C. elegans* (Lesnik et al., 2024). Interestingly, both increase and decrease in BCAA were associated with longevity and their intermediates were found to regulate ubiquitin-proteasome system (UPS) (Ravanelli et al., 2022).

Other genes in the pathway include *agxt-1* which is an alanine-glyoxylate aminotransferase that detoxifies peroxisomal glyoxylate and defects in which cause hyperoxaluria type I in humans (Pey et al., 2013).; *bckd-1B* (branched-chain keto acid dehydrogenase) that is involved in positive regulation of fatty acid synthesis and regulation of nematode development according to WormBase (Davis et al., 2022); *dlat-2* (Dihydrolipoamide S-Acetyltransferase), and *idha-1* (isocitrate dehydrogenase alpha-1) that was shown to modulate lifespan and oxidative stress resistance (Lin et al., 2022).

As mentioned previously, *vha* genes make up the majority of genes enriched in phagosome, oxphos, mTOR signaling and lysosome pathways. *vha* genes encode different subunits of V-ATPase which is a proton pump responsible for controlling intra- and extracellular pH and performs many physiological functions (Forgac, 2007; Stevens & Forgac, 1997). Lysosome, phagosome, and oxphos require an acidic environment or a proton gradient (Nguyen & Yates, 2021), while the connection between V-ATPase and mTOR signaling is less obvious at first glance. It is known that mTORC1 is translocated to lysosomal surface by Rag guanosine triphosphatases

(GTPases) activated by amino acids and later V-ATPase was demonstrated to be required for the amino acid mTORC1 activation (Zoncu et al., 2011). At the same time, mTORC1 inhibits lysosomal catabolic activity by regulating V-ATPase assembly (Ratto et al., 2022). V-ATPase works together with mTORC1 to activate mitochondrial unfolded protein response (UPR) and is required for mitochondrial surveillance and stress-induced longevity in *C. elegans* (T. Y. Li et al., 2023).

Lysosome activation was shown to enhance functioning of aged neuronal stem cells (Leeman et al., 2018) and autophagy was demonstrated to play a crucial role in maintaining regenerative capacity of hematopoietic stem cells (Ho et al., 2017). Autophagy is also induced by diets and dietary-restriction mimicking drugs, and during early reprogramming stages (Barzilai et al., 2016; Mahmoudi et al., 2019; Rubinsztein et al., 2011; Y. Wu et al., 2015).

In sum, our results suggest that dauer-duration-dependent transcriptomic signature reflects both developmental processes, as some of the genes and pathways are involved in metabolism control, and age-reversal. We suggest that the age reversal process occurring during dauer recovery preserves crucial features of rejuvenation processes described in other model systems.

Considering that aging does not occur homogenously across all tissues and some processes, such as autophagy, can have tissue-specific longevity effects, it would be interesting to take a closer look at dauer tissues and cells via fluorescence-activated cell sorting or single-cell sequencing.

3.4 Dauer vs non-dauer aging

Having characterized the consequences of chronological aging in dauer, we wondered how they compared to non-dauer, adult aging. For this we used a publicly available dataset (Ham et al., 2022) which included RNA-seq samples of N2 and *daf-2* adult worms from four time points: day 1, 4, 7, and 11 (GSE190068).

First, we compared all dauer to all non-dauer *daf-2* samples to establish a reference point, i.e. what is fundamentally different between adults and dauer larvae. We discovered that expression of synaptic signaling, axon regeneration, calcium and MAPK signaling genes is increased in dauer (Fig 2.16). Aging worms experience a decline in synaptic integrity (Wirak et al., 2022), while worms arrested in dauer diapause are reported to not only preserve neuronal integrity but also to induce axonal regeneration after an insult (Caneo et al., 2019). Thus, a relative increase in synaptic signaling genes supports the idea of dauer's exceptional ability to preserve and maintain neuronal integrity despite chronological aging.

In contrast, repair pathways (mismatch repair, NER, BER), TGF-beta signaling, polycomb repressive complex, spliceosome and ubiquitin mediated proteolysis are all underexpressed in dauer, meaning, they are overexpressed in adult *daf-2*. Since dauer larvae do not feed or interact with their external environment, the repression of repair pathways is not surprising. Importantly, this does not mean that there is no expression of these genes during the dauer diapause, it is simply lower than in adulthood.

Next, we created a list of genes that are affected differently during aging from day 7 to 11 in adult *daf-2* and day 1 to 4 in dauer larvae. We selected these time points to avoid

including germline-related genes from adult samples and have the most chronologically comparable time points. We show that the trajectory of expression of translation and DNA repair genes (NER, BER) is different in the two aging models (Fig. 2.18). Translational decline associated with aging and at the same time reduction of translation increases lifespan (H. S. Kim & Pickering, 2023). This contradiction can be reconciled by viewing translational decline as an adaptive response to the declining autophagy and proteasomal capacity to reduce the proteostatic burden (H. S. Kim & Pickering, 2023). The translation decline could be an explanation for the changes we observe in ribosome and other protein synthesis-related pathways. The difference in NER and BER gene expression could arise from 1) lack of necessity in their activation during dauer, 2) their steady increase in adult aging with a change in dauer as an initial response to dauer diapause entry.

Additionally, we noticed that spliceosome and mRNA surveillance pathways are underexpressed compared to adult worms but do increase during the dauer diapause (Fig. 2.16 and 2.10). Most likely, it is the case that overall expression levels are still lower in dauer larvae but increase due to prolonged arrest.

Further comparisons with later adult time points, for example, 15 and 30 days, would allow to distil further the difference in aging mechanisms between the arrested and adult worms.

3.5 UV-treatment response and GLTD in dauer

Since we were curious when dauer larvae address the age-related changes, including DNA damage, we tested the effect of UV-treatment on NER-deficient strains. We used *daf-2* mutant as a reference and compared its recovery dynamics to

daf-2;xpc-1 (GG-NER), *daf-2;csb-1* (TC-NER), and *daf-2;xpa-1* (all NER) double mutants. We observed that *daf-2;xpa-1* mutants were the most sensitive to UV-treatment and failed to recover ever at the lowest dose (Fig. 2.19). *daf-2;csb-1* worms were the next most sensitive, highlighting the importance of TC-NER in dauer exit. This is also logical as during dauer recovery the transcription increases again leading to the necessity of TC repair.

Additionally, we checked whether dauer larvae are capable of DNA damage repair for which a slot blot assay was performed which showed that indeed, the amount of CPDs decreases in D1 dauer after UV-treatment at a rate comparable to L3 (Fig. 2.20).

Taken together these findings suggest that *C. elegans* is able to address UV-induced DNA damage both during the diapause and during recovery with TC-NER pathways being utilized more, suggesting a two-step process: repair the bulk of damage during the arrest and the rest upon exit.

We also performed RNA-seq on samples that were irradiated at D1 and then either induced to exit dauer (D1.6.UV, D1.24.UV) or stayed in dauer for 72 h and then recovered (D4.UV, D4.6.UV, D4.24.UV). We assessed PCA (Fig. 2.21) and BiT Age (Fig. 2.22) results with the inclusion of UV-treated samples and noted that D4.UV samples appeared to be more similar to D1.24 and D4.6 samples rather than D4, suggesting that the response to UV-treatment has changed their transcriptome to appear more youthful. We think such a rejuvenation-like effect could be an indirect consequence of DDR during dauer. We didn’t see any significant differences in any of the other non-UV:UV pairs.

Based on our clustering and overrepresentation analyses (Fig. 2.23 and 2.24), UV-treated dauer appear to reduce expression of synaptic signaling genes (cluster 1 on both figures). Surprisingly, we noticed that DNA repair and DDR genes appear to be lowly expressed at D4.UV and highly at D4 and their trajectory converge during the recovery (cluster 2). Perhaps, this could be explained in the context of our earlier result (Fig. 2.20) that demonstrates that dauer larvae are capable of DNA repair. As such, if dauers have repaired most of the UV-induced DNA damage by D4, perhaps, they do not maintain high expression levels of DDR genes while untreated dauers keep up a certain level of DDR genes to monitor the state of DNA and repair when necessary.

Lastly, dauers exhibit resilience to UV-induced damage that induces transcription-blocking lesions, which play a particularly important role in organismal aging. The accumulation of transcription-blocking lesions was recently shown to lead to transcription stress that results in the age-related gene length-dependent transcriptional decline (GLTD) (Stoeger et al. 2022, Gyenis et al. 2023). This phenomenon was observed even in *C.elegans* (Gyenis et al., 2023). In light of dauer presenting age reversal characteristics, we wanted to know whether exposure to UV would at all elicit GLTD and whether it will be reversed during the recovery process.

In accordance with previously published results on the effect of genotoxic insults on gene-length-dependent gene expression (Ibañez-Solé et al., 2023), we observed that underexpressed genes tend to be longer when comparing all UV-treated to all the untreated samples (Fig. 2.25A). However, upon unraveling this comparison and taking a closer look at matched sample pairs (e.g. D1.6.UV vs D1.6), we noted that this effect was the most pronounced during recovery from D1 and in D4 samples and disappeared in the D4 exit time points (Fig. 2.25B). Thus, dauer larvae are able to

recover from GLTD during the course of dauer exit. Our results suggest that during the rejuvenation that happens in the dauer recovery phase, DNA repair capacities are highly effective in restoring transcription integrity. In the future studies, a response of adult *C. elegans* to UV-treatment would be interesting to observe as well as tissue-specific GLTD.

Altogether, we present *C. elegans* dauer diapause as a model of longevity and rejuvenation as reflected by functional assay and postdauer fitness, the BiT Age predictions, transcriptomic patterns in and during recovery from dauer, and gene-length-dependent gene expression patterns. Further work comparing dauer mechanisms to other arrested state in *C. elegans* could reveal conserved longevity and age reversal programs.

Chapter 4 Materials and Methods

As mentioned in the beginning of the Results section and Contributions, all the experimental work in the lab was performed by Dr. João C. V. V. Barata. Therefore, for the detailed description of the methods used, please, see Dr. Barata's published doctoral thesis (Barata, 2022).

4.1 RNA-seq and pre-processing

Steps of quality control of the samples prepare for RNA-seq and the sequencing were performed by Cologne Center for Genomics (CCG). The sequencing was performed with ribo depletion using Illumina Hiseq 4000 machine. The sequencing was paired end and produced approximately 15 million reads per sample.

The quality of produced raw sequencing files was assessed using FastQC reports supplied by CCG. The raw files were pre-processed using fastp (S. Chen, 2023) that performs read filtering, adapter trimming, etc. The read quantification was performed using Salmon (Patro et al., 2017) with –gcBias.

4.2 BiT Age

As mentioned in the Contribution, the BiT Age results were produced by David Meyer using his BiT Age tool originally published in 2021 (Meyer & Schumacher, 2021). Since then, it has been updated by including more training data and using all genes for elastic net regression and subsequent biological age prediction. An updated version of BiT Age is on GitHub: https://github.com/Meyer-DH/AgingClock

4.3 Gene expression analysis

Raw count matrix was filtered by expression to exclude lowly expressed genes and further to include only the genes that are expressed in a at 60% of the samples followed by normalization factor calculation using edgeR (Y. Chen et al., 2016, 2024; McCarthy et al., 2012; Robinson et al., 2010).

The following linear modeling analysis for performed using the limma package (Ritchie et al., 2015) using voom, lmFit, and eBeyes functions.

For analysis of gene expression patterns in dauer, only D1, D4, D15, and D30 samples were included in the initial count matrix. After extracting linear function parameters for all the genes (~13000), gene set enrichment analysis (GSEA) was performed using slope values as input with clusterProfiler package (T. Wu et al., 2021; Yu et al., 2012) using gseGO and gseKEGG functions.

For dauer exit analysis, all samples except UV-treated and L3 were included in the count matrix. After building a linear model that accounts for day of dauer and time of exit for each of the genes, the list of linear models was filtered to include only those with $R^2 > 0.7$. Further, emtrends function from emmeans package (https://CRAN.R-project.org/package=emmeans) with parameters ‘revpairwise ~ day, var = "exit"’. The list was filtered to include only the genes whose slope differs between D1 and D4 exit time points and then filtered further to separate genes into the six groups depending on the direction of change and the relationship between D1 and D4 slopes. The obtained lists were using for overrepresentation analysis (ORA) with clusterPofiler using compareCluster and custom wrapper functions whose main goal is to call enrichGO and enrichKEGG functions and used the obtained results and slope values

to create a heat plot. A list of genes that remained after initial filtering was used as a background.

For dauer vs. non-dauer comparisons, a publicly available dataset with the accession number GSE190068 was included in the analysis. Pre-processed data was downloaded and included in the count matrix. The same filtering and linear modeling approach was applied. For global *daf-2* dauer vs. adult *daf-2* comparison, limma functions makeContrasts and contrasts.fit were used. The downstream GSEA was performed identically to dauer analysis. Similarly, for comparison of D1 and D4 against adult d7 and d11, emmeans approach was used as described before with the exception of gene filtering by slope after emtrends step: in this case the genes with slopes around 0 were included in the analysis if the slope in the other samples was non-zero, i.e. a gene's expression can not change between D1 and D4 but then it has to change between d7 and d11. The same wrapper functions were used for ORA.

For analysis of gene patterns after UV treatment the same filtering and processing (voom, etc) was applied, followed by fuzzy clustering of the top 1000 most variable genes using Mfuzz package (Kumar & Futschik, 2007). Based on the resulting clusters, comparative enrichment analysis (ORA) was performed with compareCluster from clusterProfiler.

4.4 Gene length analysis

We used biomaRt package (Durinck et al., 2005, 2009) to retrieve start and end positions of the top 1000 genes as described above. We further split them into over- and underexpressed genes and plotted their length in $\log_{10}$bp. We performed unpaired Wilcoxon test to calculate the p-values.

4.5 Motif enrichment analysis

Lists of all genes, top and bottom 1000 genes were produced from dauer and dauer exit linear model analysis. Software for motif discovery and next-gen sequencing analysis HOMER was used (Heinz et al., 2010) with the parameters '-start -200 -end 100 -len 8,10,12,14,16 -p 4' and using all the genes that remained after initial filtering as a background.

Contributions

Wet lab results for **figures 2.2 – 2.4, 2.19, 2.20 and SFig. 4** were produced and the figures were kindly provided by Dr. João C. V. V. Barata. The BiT Age analysis was performed and data for **figures 2.8 and 2.22** were kindly provided by David Meyer.

All the schematic images were created using BioRender.

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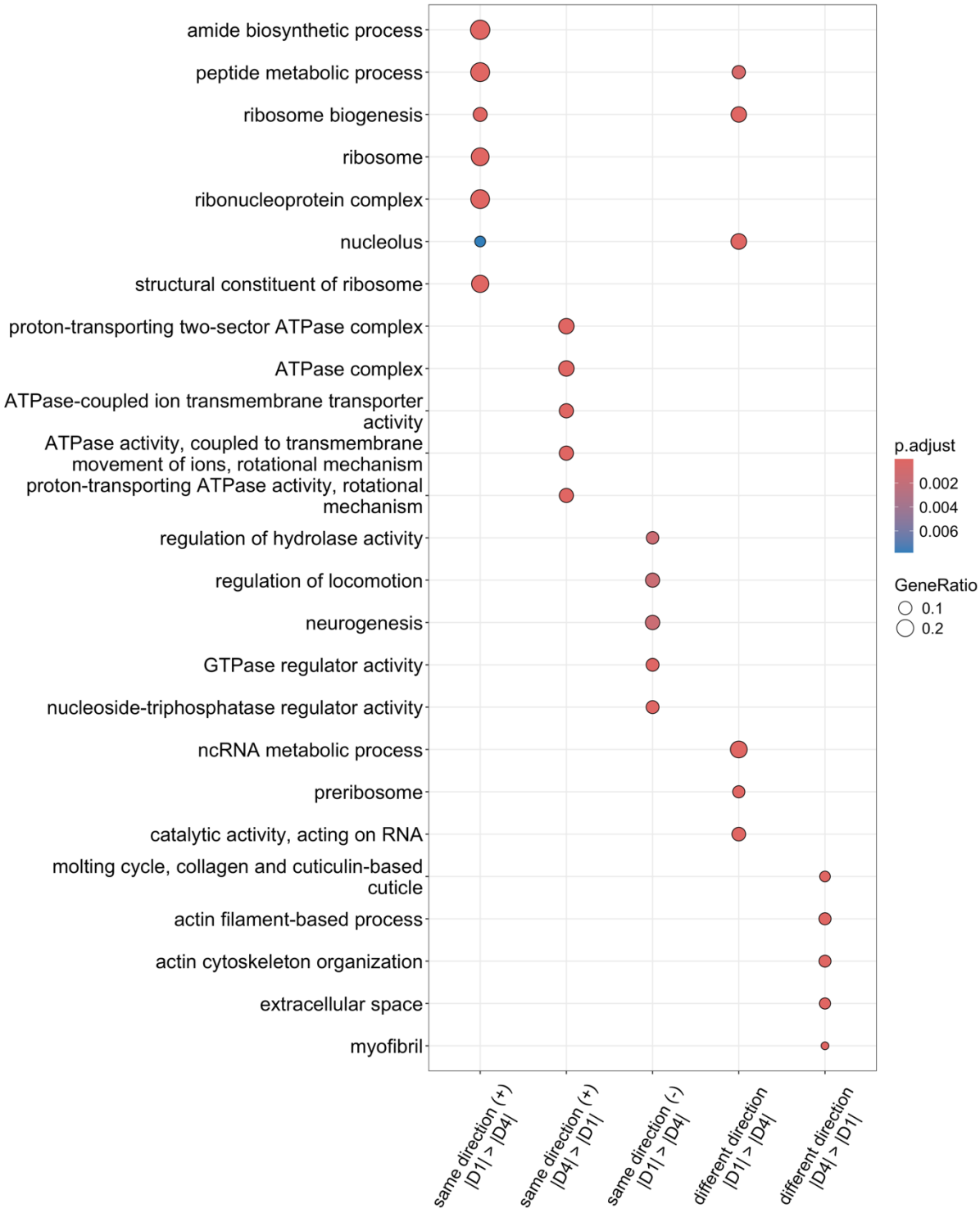
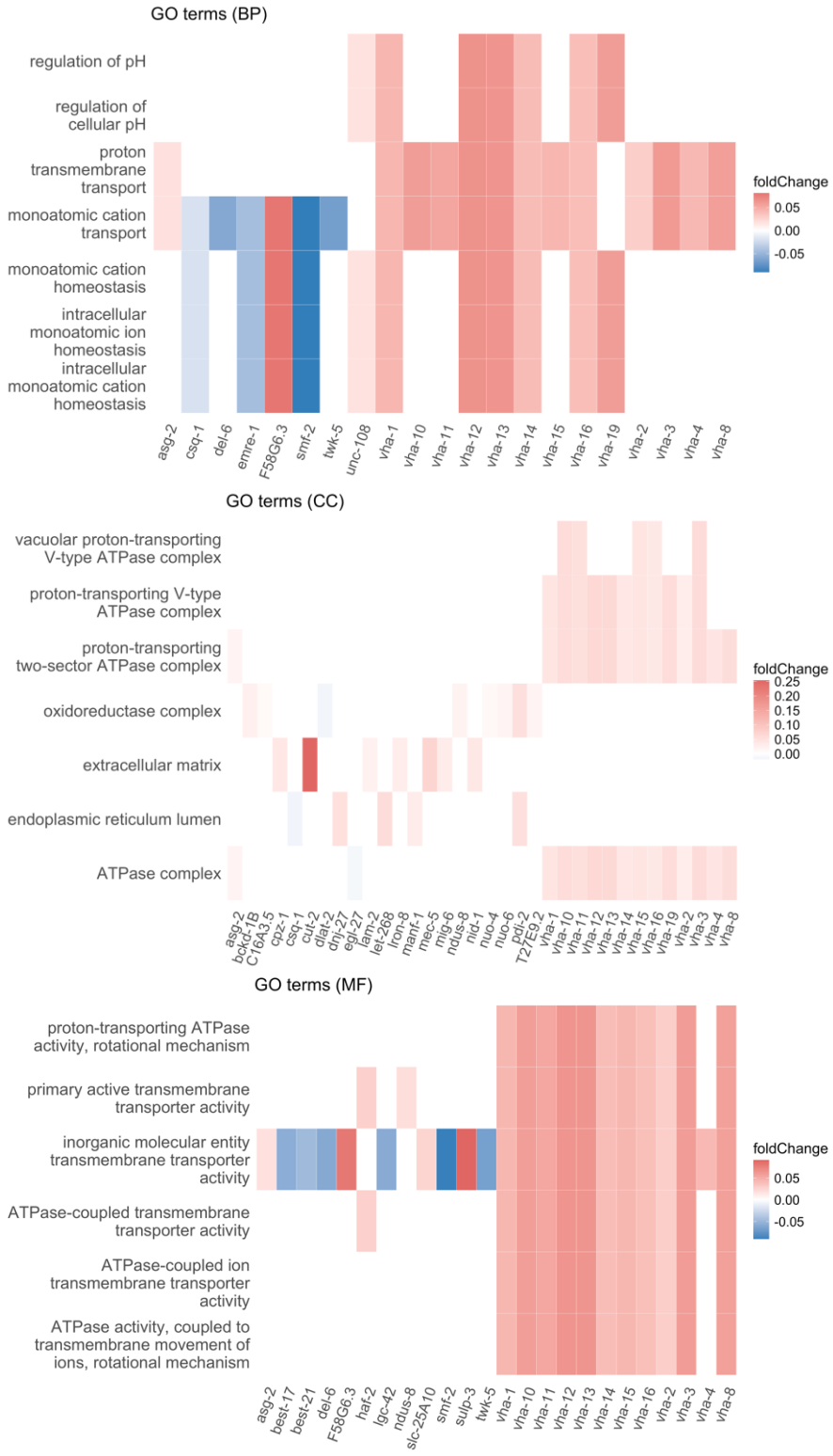
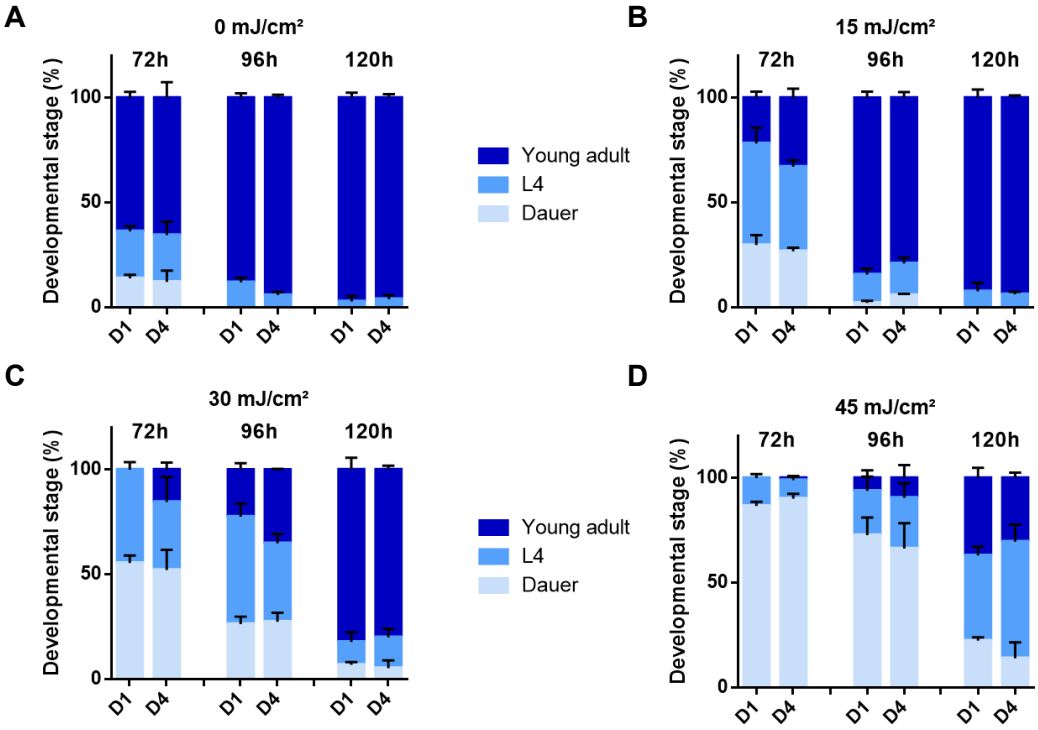
Supplementary Figures and Tables



Supplementary figure 1. Comparative overrepresentation analysis (ORA) for five groups of genes that are differentially expressed between D1 and D4 exit using GO terms including all ontologies (BP, CC, MF) – Figure 2.3.5.



Supplementary Figure 2. Overrepresentation analysis (ORA) of the overlap of genes that are differentially expressed during exits from D1 and D4 stages and the over- or underexpression is more prominent during exit from D4 using GO terms BP, CC, and MF ontologies – Figure 2.3.6.



Supplementary Figure 3. Dauer exit is similar between D1 and D4 UV-treated dauers. daf-2 animals were UV- or mock-treated at day 1 or 4 of dauer. Panels (A) to (D) show, respectively, 0, 15, 30 and 45 mJ/cm2 conditions. Dauer exit was induced immediately after UV treatment. (average of n=3 independent experiments per dose is shown, error bars represent the standard deviation (SD). Two-tailed t-test. No significant differences detected between D1 and D4). From (Barata, 2022)

	COMPARISON	DIFF	LWR	UPR	P ADJ
1	D1.6-D1.0	7.37357748704846	-9.52175301970797	24.2689079938049	0.918926188027956
2	D1.24-D1.0	-14.5212474977608	-31.4165780045172	2.37408300899563	0.144709853274969
3	D4.0-D1.0	58.3843892324515	41.4890587256951	75.279719739208	1.09567466211047e-10
4	D4.6-D1.0	22.3714996961354	5.47616918937897	39.2668302028918	0.00302161822908931
5	D4.24-D1.0	-4.73397174225096	-21.6293022490074	12.1613587645055	0.997392164202428
6	D15.0-D1.0	68.6528670068389	51.7575365000825	85.5481975135954	2.66686672745209e-12
7	D15.6-D1.0	64.9639700191878	48.0686395124314	81.8593005259442	9.62740998033951e-12
8	D15.24-D1.0	32.040991215367	15.1456607086106	48.9363217221234	1.67607914064449e-05
9	D30.0-D1.0	139.972488309751	123.077157802995	156.867818816508	2.99760216648792e-14
10	D30.6-D1.0	145.126013837106	128.23068333035	162.021344343863	2.99760216648792e-14
11	D30.24-D1.0	120.213962978516	103.31863247176	137.109293485272	2.99760216648792e-14
12	L3.0-D1.0	-95.6165209000368	-112.511851406793	-78.7211903932804	3.07531777821168e-14
13	D1.24-D1.6	-21.8948249848093	-38.7901554915657	-4.99949447805283	0.00389877327238974
14	D4.0-D1.6	51.0108117454031	34.1154812386466	67.9061422521595	2.103803686814 84e-09
15	D4.6-D1.6	14.9979222090869	-1.89740829766949	31.8932527158434	0.11829815891409
16	D4.24-D1.6	-12.1075492292994	-29.0028797360559	4.78778127745701	0.355656426568956
17	D15.0-D1.6	61.2792895197905	44.383959013034	78.1746200265469	3.68161057195948e-11
18	D15.6-D1.6	57.5903925321393	40.6950620253829	74.4857230388958	1.48675849409585e-10
19	D15.24-D1.6	24.6674137283185	7.77208322156211	41.562744235075	0.000875728882883009
20	D30.0-D1.6	132.598910822703	115.703580315946	149.494241329459	2.99760216648792e-14
21	D30.6-D1.6	137.752436350058	120.857105843301	154.647766856814	2.99760216648792e-14
22	D30.24-D1.6	112.840385491467	95.945054984711	129.735715998224	2.99760216648792e-14
23	L3.0-D1.6	-102.990098387085	-119.885428893842	-86.0947678803289	2.99760216648792e-14
24	D4.0-D1.24	72.9056367302123	56.0103062234559	89.8009672369687	6.751266212745 58e-13
25	D4.6-D1.24	36.8927471938962	19.9974166871398	53.7880777006526	1.39161950618583e-06
26	D4.24-D1.24	9.78727575550984	-7.10805475124659	26.6826062622663	0.659615692941356
27	D15.0-D1.24	83.1741145045997	66.2787839978433	100.069445011356	6.08402217494586e-14
28	D15.6-D1.24	79.4852175169486	62.5898870101922	96.380548023705	1.17905685215192e-13
29	D15.24-D1.24	46.5622387131278	29.6669082063714	63.4575692198842	1.44157644710319e-08
30	D30.0-D1.24	154.493735807512	137.598405300755	171.389066314268	2.99760216648792e-14
31	D30.6-D1.24	159.647261334867	142.75193082811	176.542591841623	2.99760216648792e-14
32	D30.24-D1.24	134.735210476277	117.83987996952	151.630540983033	2.99760216648792e-14
33	L3.0-D1.24	-81.095273402276	-97.9906039090325	-64.1999428955196	8.5487172896137e-14
34	D4.6-D4.0	-36.0128895363161	-52.9082200430726	-19.1175590295597	2.16552203247122e-06
35	D4.24-D4.0	-63.1183609747025	-80.0136914814589	-46.223030467946	1.87313498045683e-11
36	D15.0-D4.0	10.2684777743874	-6.62685273236902	27.1638082811438	0.593996104652396
37	D15.6-D4.0	6.57958078673627	-10.3157497200202	23.4749112934927	0.962031119405137
38	D15.24-D4.0	-26.3433980170845	-43.238728523841	-9.4480675103281	0.000353091493059177
39	D30.0-D4.0	81.5880990772996	64.6927685705431	98.483429584056	7.82707232360735e-14
40	D30.6-D4.0	86.7416246046546	69.8462940978981	103.636955111411	4.10782519111308e-14
41	D30.24-D4.0	61.8295737460644	44.934243239308	78.7249042528209	3.00336422398573e-11
42	L3.0-D4.0	-154.000910132488	-170.896240639245	-137.105579625732	2.99760216648792e-14
43	D4.24-D4.6	-27.1054714383864	-44.0008019451428	-10.2101409316299	0.000233750540145183
44	D15.0-D4.6	46.2813673107035	29.3860368039471	63.17669781746	1.63388162999212e-08
45	D15.6-D4.6	42.5924703230524	25.697139816296	59.4878008298088	8.82040435135067e-08
46	D15.24-D4.6	9.6694915192316	-7.22583898752483	26.564822025988	0.675420289949809
47	D30.0-D4.6	117.600988613616	100.705658106859	134.496319120372	2.99760216648792e-14
48	D30.6-D4.6	122.754514140971	105.859183634214	139.649844647727	2.99760216648792e-14
49	D30.24-D4.6	97.8424632823805	80.9471327756241	114.737793789137	3.04201108747293e-14
50	L3.0-D4.6	-117.988020596172	-134.883351102929	-101.092690089416	2.99760216648792e-14
51	D15.0-D4.24	73.3868387490899	56.4915082423335	90.2821692558463	5.8386628865037e-13
52	D15.6-D4.24	69.6979417614388	52.8026112546823	86.5932722681952	1.87860837996823e-12
53	D15.24-D4.24	36.774962957618	19.8796324508615	53.6702934643744	1.47612000578334e-06
54	D30.0-D4.24	144.706460052002	127.811129545246	161.601790558758	2.99760216648792e-14
55	D30.6-D4.24	149.859985579357	132.964655072601	166.755316086113	2.99760216648792e-14
56	D30.24-D4.24	124.947934720767	108.05260421401	141.843265227523	2.99760216648792e-14
57	L3.0-D4.24	-90.8825491577859	-107.777879664542	-73.9872186510295	3.33066907387547e-14
58	D15.6-D15.0	-3.68889698765113	-20.5842274944076	13.2064335191053	0.999763046980192
59	D15.24-D15.0	-36.6118757914719	-53.5072062982284	-19.7165452847155	1.60186007525098e-06
60	D30.0-D15.0	71.3196213029122	54.4242907961557	88.2149518096686	1.10733644476113e-12
61	D30.6-D15.0	76.4731468302672	59.5778163235107	93.3684773370236	2.44249065417534e-13
62	D30.24-D15.0	51.561095971677	34.6657654649206	68.4564264784335	1.67080860258295e-09
63	L3.0-D15.0	-164.269387906876	-181.164718413632	-147.374057400119	2.99760216648792e-14
64	D15.24-D15.6	-32.9229788038208	-49.8183093105772	-16.0276482970644	1.05690166986028e-05
65	D30.0-D15.6	75.0085182905633	58.1131877838069	91.9038487973197	3.64375196681976e-13
66	D30.6-D15.6	80.1620438179183	63.2667133111619	97.0573743246747	1.02473585172902e-13
67	D30.24-D15.6	55.2499929593282	38.3546624525717	72.1453234660846	3.71927377784687e-10
68	L3.0-D15.6	-160.580490919225	-177.475821425981	-143.685160412468	2.99760216648792e-14
69	D30.0-D15.24	107.931497094384	91.0361665876277	124.826827601141	2.99760216648792e-14
70	D30.6-D15.24	113.085022621739	96.1896921149826	129.980353128496	2.99760216648792e-14
71	D30.24-D15.24	88.172971763149	71.2776412563925	105.068302269905	3.73034936274053e-14
72	L3.0-D15.24	-127.657512115404	-144.55284262216	-110.762181608647	2.99760216648792e-14
73	D30.6-D30.0	5.15352552735499	-11.7418049794014	22.0488560341114	0.994470223493295

74	D30.24-D30.0	-19.7585253312351	-36.6538558379916	-2.86319482447872	0.0119986529670449
75	L3.0-D30.0	-235.589009209788	-252.484339716544	-218.693678703032	2.99760216648792e-14
76	D30.24-D30.6	-24.9120508585901	-41.8073813653466	-8.0167203518337	0.000767013263399341
77	L3.0-D30.6	-240.742534737143	-257.637865243899	-223.847204230386	2.99760216648792e-14
78	L3.0-D30.24	-215.830483878553	-232.725814385309	-198.935153371796	2.99760216648792e-14

Supplementary Table 1. One-way ANOVA with post hoc Tukey test – Figure 2.8

	COMPARISON	DIFF	LWR	UPR	P ADJ
1	D1.6-D1.0	7.37357748704868	-9.50795791743536	24.2551128915327	0.966568354302219
2	D1.6.UV-D1.0	9.11570180228792	-7.76583360219612	25.997237206772	0.83822875360142
3	D1.24-D1.0	-14.5212474977606	-31.4027829022446	2.36028790672345	0.162764185007292
4	D1.24.UV-D1.0	1.25772055725747	-15.6238148472266	18.1392559617415	1
5	D4.0-D1.0	58.3843892324517	41.5028538279676	75.2659246369357	3.27959881474271e-13
6	D4.0.UV-D1.0	24.0057497677257	7.1242143632417	40.8872851722098	0.000642591547341809
7	D4.6-D1.0	22.3714996961356	5.48996429165155	39.2530351006196	0.00185414312875842
8	D4.6.UV-D1.0	25.9800346009751	9.09849919649108	42.8615700054592	0.000174876084321984
9	D4.24-D1.0	-4.73397174225077	-21.6155071467348	12.1475636622333	0.999727286573201
10	D4.24.UV-D1.0	-9.13488556553925	-26.0164209700233	7.74664983894479	0.836119779786965
11	D15.0-D1.0	68.6528670068391	51.771331602355	85.5344024113231	0
12	D15.6-D1.0	64.963970019188	48.0824346147039	81.845505423672	0
13	D15.24-D1.0	32.0409912153672	15.1594558108831	48.9225266198512	3.12164151716754e-06
14	D30.0-D1.0	139.972488309751	123.090952905267	156.854023714235	0
15	D30.6-D1.0	145.126013837106	128.244478432622	162.00754924159	0
16	D30.24-D1.0	120.213962978516	103.332427574032	137.095498383	0
17	L3.0-D1.0	-95.6165209000366	-112.498056304521	-78.7349854955526	0
18	D1.6.UV-D1.6	1.74212431523924	-15.1394110892448	18.6236597197233	0.999999999916934
19	D1.24-D1.6	-21.8948249848093	-38.7763603892933	-5.01328958032522	0.00251454464084866
20	D1.24.UV-D1.6	-6.11585692979121	-22.9973923342752	10.7656784746928	0.994507165849312
21	D4.0-D1.6	51.010811745403	34.129276340919	67.892347149887	2.88526980085635e-11
22	D4.0.UV-D1.6	16.6321722806771	-0.249363123806983	33.5137076851611	0.0571597222658976
23	D4.6-D1.6	14.9979222090869	-1.88361319539713	31.8794576135709	0.130449420171082
24	D4.6.UV-D1.6	18.6064571139264	1.7249217094424	35.4879925184105	0.0189113157663809
25	D4.24-D1.6	-12.1075492292995	-28.9890846337835	4.77398617518459	0.421359164281811
26	D4.24.UV-D1.6	-16.5084630525879	-33.389998457072	0.373072351896109	0.0610399444865989
27	D15.0-D1.6	61.2792895197904	44.3977541153063	78.1608249242744	0
28	D15.6-D1.6	57.5903925321393	40.7088571276552	74.4719279366233	6.23501250629488e-13
29	D15.24-D1.6	24.6674137283185	7.78587832383444	41.5489491328025	0.000416265082062051
30	D30.0-D1.6	132.598910822703	115.717375418219	149.480446227187	0
31	D30.6-D1.6	137.752436350058	120.870900945574	154.633971754542	0
32	D30.24-D1.6	112.840385491467	95.9588500869832	129.721920895951	0
33	L3.0-D1.6	-102.990098387085	-119.871633791569	-86.1085629826012	0
34	D1.24-D1.6.UV	-23.6369493000485	-40.5184847045325	-6.75541389556446	0.000817613470340373
35	D1.24.UV-D1.6.UV	-7.85798124503044	-24.7395166495145	9.02355415945359	0.943305749691033
36	D4.0-D1.6.UV	49.2686874301638	32.3871520256797	66.1502228346478	7.67008678792536e-11
37	D4.0.UV-D1.6.UV	14.8900479654378	-1.99148743904622	31.7715833699219	0.137264492280236
38	D4.6-D1.6.UV	13.2557978938477	-3.62573751063637	30.1373332983317	0.278453772949739
39	D4.6.UV-D1.6.UV	16.8643327986872	-0.017202605796836	33.7458682031712	0.0504667933563028
40	D4.24-D1.6.UV	-13.8496735445387	-30.7312089490227	3.03186185994535	0.218534695121738
41	D4.24.UV-D1.6.UV	-18.2505873678272	-35.1321227723112	-1.36905196334313	0.0232517490043408
42	D15.0-D1.6.UV	59.5371652045511	42.6556298000671	76.4187006090352	7.061018436616e-14
43	D15.6-D1.6.UV	55.8482682169	38.966732812416	72.7298036213841	1.90314430881244e-12
44	D15.24-D1.6.UV	22.9252894130792	6.0437540085952	39.8068248175633	0.00129787485693367
45	D30.0-D1.6.UV	130.856786507463	113.975251102979	147.738321911947	0
46	D30.6-D1.6.UV	136.010312034818	119.128776630334	152.891847439302	0
47	D30.24-D1.6.UV	111.098261176228	94.216725771744	127.979796580712	0
48	L3.0-D1.6.UV	-104.732222702325	-121.613758106809	-87.8506872978405	0
49	D1.24.UV-D1.24	15.7789680550181	-1.10256734946598	32.6605034595021	0.0889954795148094
50	D4.0-D1.24	72.9056367302123	56.0241013257282	89.7871721346963	0
51	D4.0.UV-D1.24	38.5269972654863	21.6454618610023	55.4085326699704	4.7252884050053e-08
52	D4.6-D1.24	36.8927471938962	20.0112117894121	53.7742825983802	1.33278062031117e-07
53	D4.6.UV-D1.24	40.5012820987357	23.6197466942517	57.3828175032197	1.37720748139003e-08
54	D4.24-D1.24	9.78727575550981	-7.09425964897423	26.6688111599938	0.756644053162763
55	D4.24.UV-D1.24	5.38636193222133	-11.4951734722627	22.2678973367054	0.998673373389733
56	D15.0-D1.24	83.1741145045996	66.2925791001156	100.055649909084	0
57	D15.6-D1.24	79.4852175169485	62.6036821124645	96.3667529214326	0
58	D15.24-D1.24	46.5622387131277	29.6807033086437	63.4437741176118	3.62061713943262e-10
59	D30.0-D1.24	154.493735807512	137.612200403028	171.375271211996	0
60	D30.6-D1.24	159.647261334867	142.765725930383	176.528796739351	0
61	D30.24-D1.24	134.735210476277	117.853675071793	151.616745880761	0
62	L3.0-D1.24	-81.095273402276	-97.9768088067601	-64.213737997792	0
63	D4.0-D1.24.UV	57.1266686751942	40.2451332707102	74.0082040796783	8.57758308825396e-13
64	D4.0.UV-D1.24.UV	22.7480292104683	5.86649380598422	39.6295646149523	0.00145528030253006
65	D4.6-D1.24.UV	21.1137791388781	4.23224373439408	37.9953145433622	0.00411994947454986

66	D4.6.UV-D1.24.UV	24.7223140437176	7.84077863923361	41.6038494482017	0.000401494096984978
67	D4.24-D1.24.UV	-5.99169229950824	-22.8732277039923	10.8898431049758	0.995589708809761
68	D4.24.UV-D1.24.UV	-10.3926061227967	-27.2741415272808	6.48892928168732	0.672353576011997
69	D15.0-D1.24.UV	67.3951464495816	50.5136110450975	84.2766818540656	0
70	D15.6-D1.24.UV	63.7062494619305	46.8247140574464	80.5877848664145	0
71	D15.24-D1.24.UV	30.7832706581097	13.9017352536256	47.6648060625937	7.17144411821469e-06
72	D30.0-D1.24.UV	138.714767752494	121.83323234801	155.596303156978	0
73	D30.6-D1.24.UV	143.868293279849	126.986757875365	160.749828684333	0
74	D30.24-D1.24.UV	118.956242421258	102.074707016774	135.837777825743	0
75	L3.0-D1.24.UV	-96.8742414572941	-113.755776861778	-79.99270605281	0
76	D4.0.UV-D4.0	-34.378639464726	-51.26017486921	-17.4971040602419	6.74534930689141e-07
77	D4.6-D4.0	-36.0128895363161	-52.8944249408001	-19.1313541318321	2.34271620658255e-07
78	D4.6.UV-D4.0	-32.4043546314766	-49.2858900359606	-15.5228192269925	2.45694518374862e-06
79	D4.24-D4.0	-63.1183609747025	-79.9998963791865	-46.2368255702184	0
80	D4.24.UV-D4.0	-67.5192747979909	-84.400810202475	-50.6377393935069	0
81	D15.0-D4.0	10.2684777743874	-6.61305763009666	27.1500131788714	0.690231510139167
82	D15.6-D4.0	6.57958078673627	-10.3019546177478	23.4611161912203	0.988384779435241
83	D15.24-D4.0	-26.3433980170845	-43.2249334215686	-9.46186261260049	0.000137414501116706
84	D30.0-D4.0	81.5880990772997	64.7065636728156	98.4696344817837	0
85	D30.6-D4.0	86.7416246046546	69.8600892001705	103.623160009139	0
86	D30.24-D4.0	61.8295737460643	44.9480383415802	78.7111091505483	0
87	L3.0-D4.0	-154.000910132488	-170.882445536972	-137.119374728004	0
88	D4.6-D4.0.UV	-1.63425007159015	-18.5157854760742	15.2472853328939	0.999999999970886
89	D4.6.UV-D4.0.UV	1.97428483324938	-14.9072505712347	18.8558202377334	0.999999999366257
90	D4.24-D4.0.UV	-28.7397215099765	-45.6212569144605	-11.8581861054925	2.78939171814985e-05
91	D4.24.UV-D4.0.UV	-33.140635333265	-50.022170737749	-16.2590999287809	1.51454226171577e-06
92	D15.0-D4.0.UV	44.6471172391133	27.7655818346293	61.5286526435974	1.11538933644795e-09
93	D15.6-D4.0.UV	40.9582202514622	24.0766848469782	57.8397556559463	1.03866544254316e-08
94	D15.24-D4.0.UV	8.03524144764143	-8.84629395684261	24.9167768521255	0.932518797477107
95	D30.0-D4.0.UV	115.966738542026	99.0852031375416	132.84827394651	0
96	D30.6-D4.0.UV	121.120264069381	104.238728664896	138.001799473865	0
97	D30.24-D4.0.UV	96.2082132107902	79.3266778063062	113.089748615274	0
98	L3.0-D4.0.UV	-119.622270667762	-136.503806072246	-102.740735263278	0
99	D4.6.UV-D4.6	3.60853490483953	-13.2730004996445	20.4900703093236	0.999993183549649
100	D4.24-D4.6	-27.1054714383864	-43.9870068428704	-10.2239360339023	8.28052936613366e-05
101	D4.24.UV-D4.6	-31.5063852616748	-48.3879206661589	-14.6248498571908	4.44320528114606e-06
102	D15.0-D4.6	46.2813673107035	29.3998319062194	63.1629027151875	4.26402801956272e-10
103	D15.6-D4.6	42.5924703230524	25.7109349185683	59.4740057275364	3.82566456291755e-09
104	D15.24-D4.6	9.66949151923157	-7.21204388525247	26.5510269237156	0.772013551712697
105	D30.0-D4.6	117.600988613616	100.719453209132	134.4825240181	0
106	D30.6-D4.6	122.754514140971	105.872978736487	139.636049545455	0
107	D30.24-D4.6	97.8424632823804	80.9609278778963	114.723998686864	0
108	L3.0-D4.6	-117.988020596172	-134.869556000656	-101.106485191688	0
109	D4.24-D4.6.UV	-30.7140063432259	-47.5955417477099	-13.8324709387419	7.50845028330005e-06
110	D4.24.UV-D4.6.UV	-35.1149201665144	-51.9964555709984	-18.2333847620303	4.18235419186175e-07
111	D15.0-D4.6.UV	42.6728324058639	25.7912970013799	59.554367810348	3.643835344568 91e-09
112	D15.6-D4.6.UV	38.9839354182128	22.1024000137288	55.8654708226969	3.545264792848e-08
113	D15.24-D4.6.UV	6.06095661439204	-10.820578790092	22.9424920188761	0.995010004865889
114	D30.0-D4.6.UV	113.992453708776	97.1109183042922	130.87398911326	0
115	D30.6-D4.6.UV	119.145979236131	102.264443831647	136.027514640615	0
116	D30.24-D4.6.UV	94.2339283775408	77.3523929730568	111.115463782025	0
117	L3.0-D4.6.UV	-121.596555501012	-138.478090905496	-104.715020096528	0
118	D4.24.UV-D4.24	-4.40091382328848	-21.2824492277725	12.4806215811956	0.999894269403822
119	D15.0-D4.24	73.3868387490898	56.5053033446058	90.2683741535739	0
120	D15.6-D4.24	69.6979417614387	52.8164063569547	86.5794771659228	0
121	D15.24-D4.24	36.7749629576179	19.8934275531339	53.656498362102	1.4369847844975e-07
122	D30.0-D4.24	144.706460052002	127.824924647518	161.587995456486	0
123	D30.6-D4.24	149.859985579357	132.978450174873	166.741520983841	0
124	D30.24-D4.24	124.947934720767	108.066399316283	141.829470125251	0
125	L3.0-D4.24	-90.8825491577858	-107.76408456227	-74.0010137533018	0
126	D15.0-D4.24.UV	77.7877525723783	60.9062171678943	94.6692879768623	0
127	D15.6-D4.24.UV	74.0988555847272	57.2173201802432	90.9803909892112	0
128	D15.24-D4.24.UV	41.1758767809064	24.2943413764224	58.0574121853904	9.08452635339785e-09
129	D30.0-D4.24.UV	149.107373875291	132.225838470807	165.988909279775	0
130	D30.6-D4.24.UV	154.260899402645	137.379363998161	171.14243480713	0
131	D30.24-D4.24.UV	129.348848544055	112.467313139571	146.230383948539	0
132	L3.0-D4.24.UV	-86.4816353344974	-103.363170738981	-69.6000999300133	0
133	D15.6-D15.0	-3.68889698765111	-20.5704323921351	13.1926384168329	0.999990663525525
134	D15.24-D15.0	-36.6118757914719	-53.4934111959559	-19.7303403869879	1.59503950936113e-07
135	D30.0-D15.0	71.3196213029123	54.4380858984283	88.2011567073964	0
136	D30.6-D15.0	76.4731468302672	59.5916114257831	93.3546822347512	0
137	D30.24-D15.0	51.5610959716769	34.6795605671929	68.442631376161	2.12390105502891e-11
138	L3.0-D15.0	-164.269387906876	-181.15092331136	-147.387852502392	0
139	D15.24-D15.6	-32.9229788038208	-49.8045142083048	-16.0414433993368	1.74705882582948e-06
140	D30.0-D15.6	75.0085182905634	58.1269828860794	91.8900536950475	0
141	D30.6-D15.6	80.1620438179183	63.2805084134342	97.0435792224023	0
142	D30.24-D15.6	55.249992959328	38.368457554844	72.131528363812	2.70095057430808e-12
143	L3.0-D15.6	-160.580490919225	-177.462026323709	-143.698955514741	0

144	D30.0-D15.24	107.931497094384	91.0499616899002	124.813032498868	0
145	D30.6-D15.24	113.085022621739	96.203487217255	129.966558026223	0
146	D30.24-D15.24	88.1729717631488	71.2914363586648	105.054507167633	0
147	L3.0-D15.24	-127.657512115404	-144.539047519888	-110.77597671092	0
148	D30.6-D30.0	5.15352552735487	-11.7280098771292	22.0350609318389	0.999216693353295
149	D30.24-D30.0	-19.7585253312354	-36.6400607357194	-2.87698992675137	0.00951551110659121
150	L3.0-D30.0	-235.589009209788	-252.470544614272	-218.707473805304	0
151	D30.24-D30.6	-24.9120508585903	-41.7935862630743	-8.03051545410624	0.000354326496405433
152	L3.0-D30.6	-240.742534737143	-257.624070141627	-223.860999332659	0
153	L3.0-D30.24	-215.830483878553	-232.712019283037	-198.948948474069	0

Supplementary Table 2. One-way ANOVA with post hoc Tukey test – Figure 2.22.