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MASTER'S THESIS:

Decoding redox signaling in host microbe interactions

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"Molecular – Cellular Biology and Biotechnology"

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Abstract

Interactions between microbes (symbiotic or pathogenic) and their hosts are central for both health and disease. Current interventions show efficacy but lack mechanistic insight into the molecular pathways affected. ROS produced at the host–microbe interface are signaling regulators in host physiology. Previous studies have shown that mild oxidation (oxidative eustress) during early development increases stress resistance and lifespan in *C. elegans* populations by targeting directly an epigenetic modifier, i.e., the redox-sensitive COMPASS complex. In this thesis, I employ *C. elegans*, as an established host model system for the study of human opportunistic bacteria and examine whether an early-in-life exposure to oxidizing conditions can protect against *Pseudomonas aeruginosa* infection later in life. Similar to mammalian phagocytes and in response to microbial infection, *C. elegans* produces ROS via a single NADPH oxidase (Ce-Duox1/BLI-3) whose function I found is indispensable for worm survival against *Pseudomonas*. I also found that application of mild concentrations of oxidants either directly to worms or via dietary bacteria during early-in-life stages extends *C. elegans* survival after exposure to *P. aeruginosa*, independently of the COMPASS complex. My future goal is to explore temporal and spatial dynamics of ROS at the host-microbe interface during infection and identify redox-sensitive pathways that protect against it.

Περίληψη

Οι αλληλεπιδράσεις μεταξύ μικροβίων (συμβιωτικών ή παθογόνων) και των ξενιστών τους είναι κρίσιμες τόσο για την υγεία όσο και σε ασθένειες. Οι τρέχουσες επεμβάσεις δείχνουν αποτελεσματικές, αλλά στερούνται μηχανιστικής γνώσης για τα μοριακά μονοπάτια που επηρεάζονται. Τα ROS που παράγονται κατά τις αλληλεπιδράσεις ξενιστή-μικροβίου είναι ρυθμιστές της σηματοδότησης στη φυσιολογία του ξενιστή. Προηγούμενες έρευνες έδειξαν ότι η ήπια οξείδωση (οξειδωτικό eustress) κατά την πρώιμη ανάπτυξη αυξάνει την αντοχή στο στρες και τη διάρκεια ζωής σε πληθυσμούς του *C. elegans* στοχεύοντας απευθείας έναν επιγενετικό τροποποιητή, το οξειδοαναγωγικά-ευαίσθητο σύμπλεγμα COMPASS. Σε αυτή τη διατριβή, χρησιμοποιώ το *C. elegans*, ως ένα καθιερωμένο σύστημα μοντέλου ξενιστή για τη μελέτη ανθρώπινων, ευκαιριακά παθογόνων βακτηρίων και εξετάζω εάν μια πρώιμη έκθεση σε οξειδωτικές συνθήκες μπορεί να προστατεύσει από τη μόλυνση από *P. aeruginosa* αργότερα στη ζωή. Παρόμοια με τα φαγοκύτταρα των θηλαστικών και ως απόκριση σε μικροβιακή μόλυνση, ο *C. elegans* παράγει ROS μέσω μιας μοναδικής οξειδάσης NADPH (Ce-Duox1/BLI-3) της οποίας η λειτουργία βρήκα ότι είναι απαραίτητη για την επιβίωση των σκουληκιών έναντι της μόλυνσης με *Pseudomonas*. Βρήκα επίσης ότι η εφαρμογή ήπιων συγκεντρώσεων οξειδωτικών είτε απευθείας σε σκουλήκια είτε μέσω βακτηρίων που καταναλώνουν ως τροφή κατά τη διάρκεια των πρώιμων σταδίων της ζωής τους επεκτείνει την επιβίωση του *C. elegans* μετά την έκθεση στη *P. aeruginosa*, ανεξάρτητα από το σύμπλεγμα COMPASS. Ο μελλοντικός μου στόχος είναι να διερευνήσω τη χρονική και χωρική δυναμική των ROS στις αλληλεπιδράσεις ξενιστή-μικροβίου κατά τη διάρκεια της μόλυνσης και να εντοπίσω μονοπάτια που αποκρίνονται σε ROS και προστατεύουν από αυτήν.

1. Introduction

1.1 *C. elegans* developmental stages and reproduction

C. elegans are hermaphrodites and self-fertile. *C. elegans* life cycle includes 4 larval stages (L1-L4, ~ lasting 3 days) and adulthood (~ lasting 3 weeks) (**Fig. 1**) Developmental stages are controlled from the expression of multiple genes, specifically, from interactions between miRNA-transcription factors and genes expressed at developmental stage (Resnick et al., 2010). Unfavorable conditions such as high temperature and limited food supply promote the development of C. elegans into the dauer stage, a stage characterized by reduced mobility, smaller body and organ size, and a specialized cuticle. Dauer is an alternative to the L3 stage, but the decision to enter this state is made in the L1 stage. Worms in dauer stage can survive for several weeks and have high stress resistance (Ewald, Castillo-Quan, et al., 2017). At late L4 stage worms accumulate fertilized eggs in the uterus; a young adult hermaphrodite will generally have 10-15 eggs in its uterus at any given time (Schafer, 2006).

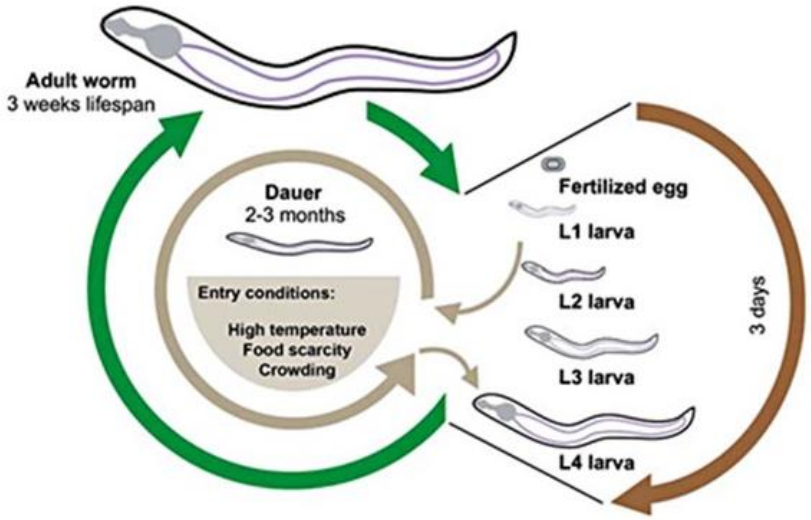


Figure 1: *C. elegans* life cycle. Worms' life cycle incudes L1, L2,L3,L4 larval stages and adulthood. The dauer stage is characterized by unfavorable conditions (high temperature and limited food supply) (Ewald, Castillo-Quan, et al., 2017).

1.2 Microbial pathogenesis and defense mechanisms in *C. elegans*

C. elegans is a free-living soil nematode that feeds on bacteria and is constantly exposed to potential pathogens, including Gram-positive bacteria like *E. faecalis, S. aureus, and S. pneumoniae*, as well as Gram-negative bacteria such as S. enterica and *P. aeruginosa*. Like other invertebrates, *C. elegans* lacks an adaptive immune system and does not appear to have specialized immune cells. To eliminate pathogens, worms employ three immune defense mechanisms: (1) behavioral responses which serve as the first line of defense by avoiding pathogens based on previous exposure (Hasshoff et al., 2007), (2) the epidermis which blocks, as the physical barrier, the entrance of microbes but also activates local innate immunity pathways (Taffoni & Pujol, 2015), (3) the muscular pharynx which, with its structure and muscular contractions helps crushing and grinding ingested food particles, including potential pathogens (Labrousse et al., 2000), and (3) the intestine which protects against pathogens by offering nutrients, producing antimicrobial molecules, and metabolites that affect the virulence of pathogens (Roeder et al., 2010). (4) Gut-residing symbiotic bacteria can also produce antimicrobial compounds and induce immune responses for host protection, (Pees et al., 2024)

1.3 Pathogen recognition in *C. elegans*

Like other invertebrates, *C. elegans* possesses an innate immune system to eliminate pathogens. The initial phase of an inducible defense involves the pathogen recognition. Animals use a set of pattern recognition receptors (PRRs), that function as both cell surface and cytoplasmic pathogen surveillance proteins and sense microbial replication by directly recognizing pathogen-associated molecular patterns (PAMPs). The major PRRs are categorized into four subfamilies: TLRs, nucleotide-binding domain leucine-rich repeat/NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and C-type lectin receptors (CLRs) (Li et al., 2023). Nematodes have lost these classical mechanisms of pattern recognition for the detection of pathogens during evolution and genes encoding proteins of these families are absent. However, recent studies have revealed the detection of a pathogen-derived metabolite by a nuclear hormone receptor in *C. elegans* (Non canonical pattern recognition). Specifically, phenazine-1-carboxamide (PCN), a major phenazine metabolite produced from *P. aeruginosa* binds to the *C. elegans* nuclear hormone receptor NHR-86 (mammalian homolog is HNF4). Activation of NHR-86/HNF4 results in the recruitment of genes involved in host protection from bacterial killing (Peterson et al., 2023).

1.4 Conserved pathways of immune signaling in *C. elegans*

Several signaling cascades are regulated during infection and contribute to immune defenses against pathogens. One of these, includes the DAF-2/Insulin Like Signaling (ILS) pathway, through the generation of PIP3 and activation of PDK-1. Activated PDK-1 leads to the phosphorylation and regulation of DAF-16 by AKT-1, AKT-2, and SGK-1. AKT-1 and AKT-2 contribute to the regulation of pathogen resistance and lifespan, whereas SKG-1 regulates lifespan but not pathogen resistance, rendering this pathway pivotal in controlling key aspects of organismal health (Evans et al., 2008).

The Mitogen Activated Protein Kinase (MAPK) pathway is considered the most ancient signal transduction cascade in immunity. In *C. elegans*, the NSY-1-SEK-1-PMK-1 MAPK pathway is orthologous to the mammalian ASK1-MKK3-p38 MAPK pathway and is crucial for resistance to the Pseudomonas aeruginosa strain PA14 (Kim et al., 2002).

The TGFβ signaling pathway is also conserved. The Sma/Mab pathway is initiated when the ligand, DBL-1, binds to the DAF-4, and the SMA-6 receptors, forming a ligand-receptor complex. SMA-6 receptor phosphorylates the R-Smad proteins, SMA-2 and SMA-3, which form a complex with SMA-4. These activated Smad complexes translocate into the nucleus, where they regulate transcription of target genes (Yamamoto & Savage-Dunn, 2023).

Another important immune mechanism found in both *C. elegans* and mammals involves the production of ROS via the action of NADPH oxidases (NOX). In *C. elegans* increased ROS levels trigger a protective stress response in the host, leading to p38 MAPK signaling activation. This results in the phosphorylation and nuclear localization of SKN-1, a transcription factor crucial for detoxification and stress resistance, encoding proteins involved in antioxidant defense to eliminate ROS and prevent oxidative damage (van der Hoeven et al., 2011). The regulation of NADPH oxidases (NOX) remains an open question.

1.5 Reactive Oxygen Species (ROS)

ROS are oxygen-derived small molecules, including oxygen radicals [superoxide (O_2•−), hydroxyl (•OH), peroxyl (RO2•), and alkoxyl (RO·)] and certain nonradicals that are either oxidizing agents and/or are easily converted into radicals, such as hypochlorous acid (HOCl), ozone (O_3), singlet oxygen (O_2), and hydrogen peroxide (H_2O_2) (Phaniendra et al., 2015). Such oxygen-containing radicals are highly unstable and reactive. Excessive amounts of ROS can cause irreversible molecular damage (a state known as “oxidative distress”) and contribute to the pathophysiology of many diseases (Forman & Zhang, 2021). On the other hand, tightly regulated ROS production is indispensable for cellular homeostasis due to the signaling properties of these molecules. This ability of ROS allows the modulation of redox-sensitive processes including growth, metabolism and cellular adaptation to a changing environment (Sies & Jones, 2020). Notably, ROS at supraphysiological, but still tolerant levels, can provide additional benefits, a condition known as “oxidative eustress” (Sies, 2017). For example, a

moderately elevated and localized ROS production has shown to increase health span and lifespan via an adaptive response that leads to improved defense mechanisms and increased stress resistance (Ristow & Schmeisser, 2011).

(ROS exert their signaling function via an array of oxidative post-translational modifications (OxiPTMs) (**Fig. 2**). The most prominent mode of OxiPTMs involves the reaction of ROS with nucleophilic thiol groups (–SH, reduced form) of specific cysteines, which are initially oxidized into sulfenic acid intermediates (–SOH) and subsequently converted into more stable forms, including disulfides (–SS–, oxidized form) (Paulsen & Carroll, 2013). Disulfides are reversible OxiPTMs and can rapidly alter the function of regulatory proteins (Finkel, 2011; Hourihan et al., 2016; Kramer-Drauberg et al., 2020; Putker et al., 2013). Redox-mediated signaling is typically activated by ROS generated in response to external stimuli such as growth factors, cytokines, and microbes (Dröge, 2002). Hydrogen peroxide (H_2O_2) and superoxide ($O_2-\bullet$) are considered the primary ROS species contributing to signaling events.

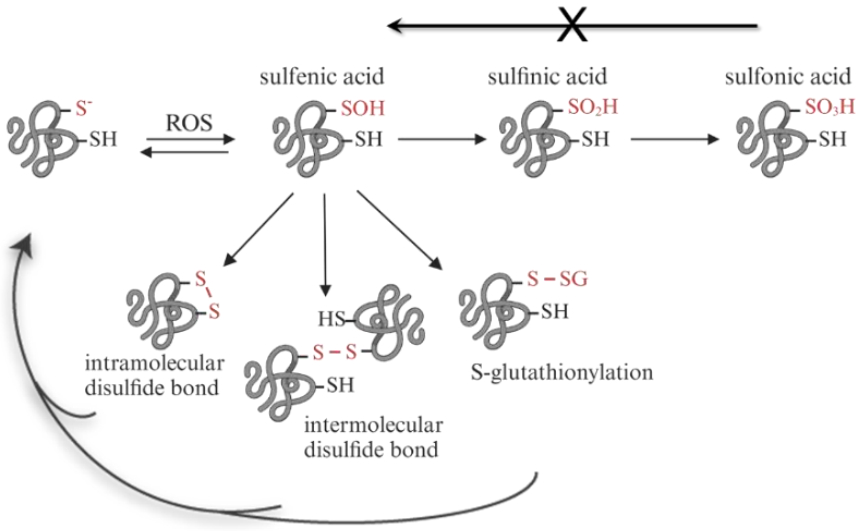


Figure 2: Upon oxidation of protein targets, redox-sensitive thiol groups in cysteines (-SH) rapidly form sulfenic acids (-SOH). These sulfenates are highly reactive and tend to quickly react to nearby cysteine thiols to form reversible inter- or intramolecular disulfide bonds (-S-S-).

1.6 The role of ROS in host microbe interactions

ROS play important roles in the complex interactions between hosts, microorganisms and their environment. ROS, primarily known for their bactericidal activity, are produced by NADPH oxidases (NOX) in host phagocytes (Bedard & Krause, 2007). Members of the NOX family, are also present in many non-phagocytic cell types, including skin cells and epithelia of the oral cavity and respiratory and gastrointestinal tracts of humans (Lambeth et al., 2007). ROS production by host intestinal epithelia can initiate an immediate immune response against invading pathogens or under dysbiosis (Ha et al., 2009). At that point, redox signaling becomes crucial for host defense i.e., for inhibiting the microbial killing and adverse effects in host physiology (Spooner & Yilmaz, 2011). This occurs through the transcriptional activity of SKN-1 and ATF-7 in PMK-1-dependent manner, together with BLI-3 (NOX) activity. Specifically, pathogens can also secrete redox-active metabolites that cause damage to epithelial tissues (Rada & Leto, 2013). To counteract this oxidative burst comes from pathogen, host cells activate SKN-1, an ortholog of the mammalian Nrf transcription factor which is a major oxidative stress response regulator (Blackwell et al., 2015) that is required for pathogen resistance. In mammals, the production of H_2O_2 is essential for pathogen killing, and is carried out by phagocytic cells. This H_2O_2 production results in HOCl and chloride ions catalyzed by myeloperoxidase (MPO)in the phagolysosome. HOCl contributes to microbicidal killing by producing chloramines such as monochloramine (NH_2Cl), a potent microbicidal agent that is highly lipophilic and can cross cell membranes (Beilke et al., 1989). The peroxidase gene skpo-1 (ShkT-containing peroxidase) in *C. elegans* is also crucial in immune defenses since its absence results in increased vulnerability to *E. faecalis* infection (Tiller & Garsin, 2014).

Another important role of ROS is to regulate the physiological processes under non-pathological conditions. Contact with symbiotic or commensal bacteria generates ROS that secure gut homeostasis, maintain the gut barrier protection and promote cell proliferation and differentiation in self-renewing epithelia (Hajjar et al., 2017). Besides the host, a recently discovered class of NOX-like enzymes in bacteria may also catalyze ROS formation. In addition, certain commensal and pathogenic bacteria excrete millimolar levels of hydrogen peroxide generated by specialized oxidases (Echlin et al., 2016).

Overall, ROS at the host–microbe interface are particularly well positioned to modulate signaling pathways that regulate host physiology. To maintain these physiological processes, it is crucial to balance ROS production and detoxification (Papp et al., 2012; van der Hoeven et al., 2011).

1.7 NADPH Oxidase (NOX)/ Dual Oxidase (DUOX) family

Mammals have seven NADPH oxidase family members, which are found in almost every tissue and are localized at cellular membranes and within intracellular compartments, such as endosomes and the endoplasmic reticulum (ER) (Bedard & Krause, 2007). In mammals six homologs of the cytochrome subunit of the phagocyte NADPH oxidase are found: NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2. These enzymes transport electrons across the plasma membrane and generate superoxide and other downstream ROS. Together with the phagocyte NADPH oxidase (NOX2/gp91^{phox}), these homologs form the NOX family of NADPH oxidases. All NOX enzymes have a cytosolic hydrogenase (DH) domain and a 6n-transmembrane (TM) coordinating domain. The DH domain includes an N-terminal lobe for FAD co-factor binding (FAD Binding Lobe, FBL) and a C-terminal lobe for NADPH binding (NBL). The calcium-dependent NOX5 and DUOX1/2 enzymes possess four and two calcium-binding EF-hand (EF) domains, respectively, located N-terminal to the TM domain. The activity of most NOX enzymes is triggered by binding of specific proteins such as p22phox and Rac, whereas NOX4 is regulated by ATP binding (Ogboo et al., 2022).

The nematode *C. elegans* encodes 2 Duox genes (*bli-3* and *duox-2*) that have 94% similarity amino acid sequence and 30% to human Duox 1 and 2 (**Fig. 3**). In *C. elegans* Duoxs consist of a 7 transmembrane domain and an extracellular peroxidase domain. . Intracellularly, they contain an intracellular flavin adenine dinucleotide (FAD) binding and NADPH oxidase domains and 2 EF hand calcium- binding domains (Ewald, 2018).

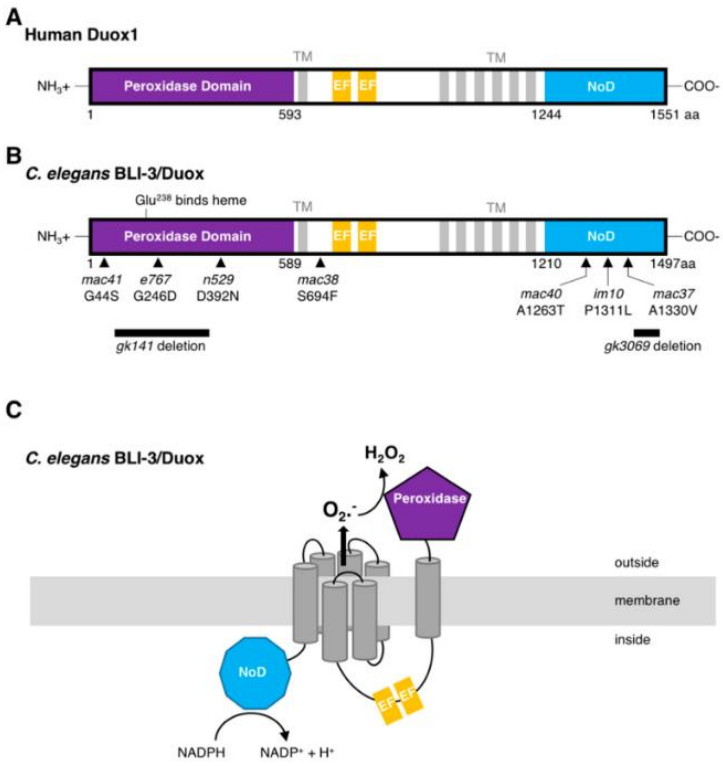


Figure 3: Schematic representation of human Duox1 and *C. elegans* BLI-3/Duox. **a)** Human Duox1. **b)** *C. elegans* BLI-3/Duox. **c)** Proposed topology model of *C. elegans* BLI-3/Duox. TM = transmembrane region, EF = EF-hand, NoD = NADPH oxidase domain (Ewald et al., 2018).

1.8 Early-life ROS in stress resistance and lifespan

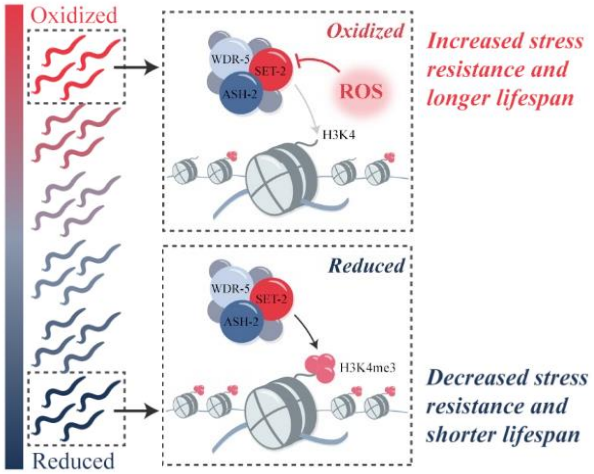
We previously reported that the 2nd larval stage (L2) is a critical time window for ROS to impact *C. elegans* physiology (Bazopoulou et al., 2019; Oleson et al., 2021). We used the redox-sensing protein GRX-1::roGFP2, which faithfully responds to the cellular ratio of oxidized and reduced glutathione (GSSG:GSH) (Gutscher et al., 2008) to monitor and sort worms based on their endogenous redox environment. Analysis of animals sorted according to their redox states at the L2 larval stage, showed that the most oxidized individuals during development are significantly more stress resistant and develop into more reduced and longer-lived adults compared to their oxidized counterparts. In agreement with this, L2-staged populations treated with low concentrations of oxidants (i.e., paraquat) exhibited increased stress resistance and lifespan compared to populations treated with reductants (i.e., N-acetylcysteine, NAC).

These results suggest that naturally occurring early-in-life events exist which can significantly improve health and lifespan. Similar beneficial early-in-life events have also been reported for mammals, where food reduction in pre-weaning mice or rapamycin treatment for the first 4 weeks after birth were sufficient to extend lifespan up to 15% (Shindyapina et al., 2022; Sun et al., 2009). Mechanistic follow-up studies in *C. elegans* revealed that the highly conserved methyltransferase SET-2, the central component of the H3K4me3-depositing COMPASS complex (Shilatifard, 2012), is redox sensitive, and its reversible inactivation is responsible for a substantial reduction in global H3K4me3 levels in the oxidized subpopulation of developing *C. elegans* (Bazopoulou et al., 2019) (**Fig. 4**). The COMPASS complex (COMplex of Proteins ASsociated with Set1), includes the MLL/SET methyltransferase along with ASH-2, WD repeat-containing protein 5 (WDR-5), DPY-30, and RBPP-5, known as WARD components (Sze & Shilatifard, 2016). The MLL/SET family enzymes responsible for depositing the H3K4me3 chromatin mark include two members in *C. elegans* (SET-2 and SET-16) and six members in mammals (SET1A, SET1B, and MLL1–4) (Oleson et al., 2021).

Genetic downregulation of members of the COMPASS complex, i.e., SET-2 and ASH-2, increased stress resistance and substantially extended lifespan, essentially phenocopying the oxidized subpopulation (Bazopoulou et al., 2019; Greer et al., 2010). Notably, we observed very similar phenotypical consequences and augmented HSF1-dependent stress responses when we knocked down components of the H3K4me3 complex in mammalian cells (Bazopoulou et al., 2019). The heat shock factor HSF-1 is a master transcriptional regulator of proteostasis and one of the best studied longevity factors in biology (Hsu et al., 2003; Morley & Morimoto, 2004; Richter et al., 2010). We concluded that H3K4me3 plays a hitherto unknown role in HSF-1-mediated stress gene

expression, protein homeostasis and stress resistance. Overall, our results suggest that developmental oxidative stress leads to the transient inactivation of the COMPASS complex, which alters the histone epigenetic landscape and triggers persistent gene expression changes to improve proteostasis throughout adulthood and extend lifespan.

a



b

C. elegans	Mammalian
SET-2	SET1A
	SET1B
SET-16	MLL1
	MLL2
	MLL3
	MLL4
	MLL5
WDR-5.1	WDR5
ASH-2	ASH2L
RbBP-5	RbBP5
DPY-30	DPY30

Figure 4: a) Redox environment during development varies widely among individuals. The COMPASS complex, including SET-2, WDR-5.1 and ASH-2, is targeted under oxidizing conditions and global H3K4me3 levels are reduced. As a result, stress responses and lifespan are increased. Modified from (Oleson et al., 2021). **b)** H3K4me3 methyltransferases and WARD proteins in *C. elegans* and mammals.

1.9 *C. elegans-P. aeruginosa* interactions

C. elegans lives in the presence of microbiota both in nature and in the laboratory and is an established model for studies of host-microbe interactions (Zhang et al., 2017). Due to its genetic tractability and inexpensive husbandry, *C. elegans* studies allow direct causality to be assigned to the presence or absence of well-defined microbiota, which initially consumed as food (dietary bacteria), can colonize and propagate in the gut. Moreover, since the first infection model involving a *Pseudomonas* species (Kirienko et al., 2014), a plethora of studies report the susceptibility of the nematode to numerous bacterial pathogens, many of them infecting humans (Burkhardt, 2023; Garsin et al., 2001; Rangan et al., 2016; Sifri et al., 2003). Similar to mammalian phagocytes and in response to microbial infection, *C. elegans* produces ROS via the single functional NADPH oxidase (Ce-Duox1/BLI-3) whose function is indispensable for worm survival (Ewald, Hourihan, et al., 2017).

Comparative analysis of *C. elegans* transcriptional responses to microbial pathogens (i.e., *E. faecalis, P. luminescens, S. marcescens*) that we and others previously performed reveal a common, pathogen-shared signature that involves the upregulation of survival promoting mechanisms including antimicrobial and stress response genes (Engelmann et al., 2011; Wong et al., 2007). Notably, when we compare these datasets to our transcriptomics profiling of upregulated genes in subpopulations of developing, naturally oxidized worms-OX(UP) (Bazopoulou et al., 2019) we find a significant overlap (53 out of 191 genes) (**Fig. 5a**). These naturally oxidized worms at the L2 stage have reduced H3K4me3 levels and display enhanced fitness and longer lifespan. When we look at individual pathogens, we find that more 50% of the upregulated genes in naturally oxidized worms are common with genes upregulated upon *Pseudomonas aeruginosa* infection (Engelmann et al., 2011) (**Fig. 5b**). *Pseudomonas aeruginosa*, a Gram-negative human opportunistic, is one of the leading causes of hospital-acquired infections as it can target a plethora of tissue types including skin and soft tissues, contributing to both acute and chronic pathologies (Markou & Apidianakis, 2014; Sadikot et al., 2005). Burn victims as well as immunocompromised, mechanically ventilated, and cystic fibrosis patients are particularly susceptible to *P. aeruginosa* infection, (Engelmann et al., 2011). *P. aeruginosa* is also a common colonizer of the human intestine upon hospitalization, antibiotic treatment, surgery, severe trauma (Alverdy et al., 2000; Andremont et al., 1989; Stoodley & Thom, 1970). *P. aeruginosa* can also infect a wide range of model organisms, including *C. elegans* and has an immediate effect on host health. PA14, a highly virulent, clinical strain isolate of *P. aeruginosa* can kill *C. elegans* over a few days in an infection-like process that requires live bacteria (“slow killing”) (Tan et al., 1999). This is to be distinguished from rapid PA14 killing of *C. elegans* (‘‘fast killing’’) that is most likely mediated by exported toxins, occurs on high osmolarity and does not require the presence of live bacteria. Upon exposure to live *P. aeruginosa*, worms show a quick avoidance behavior, starting at 30 min of exposure, which gradually increases in the first 6 hours of exposure (Saito et al., 2019). Studies have shown that this aversive response is initiated by a gut-to-neurons communication promoted by oxidative cues such as bacterially released, reactive oxygen species, nitic oxide (Hao et al., 2018) and extracellular GSSG (Lalsiamthara & Aballay, 2022).

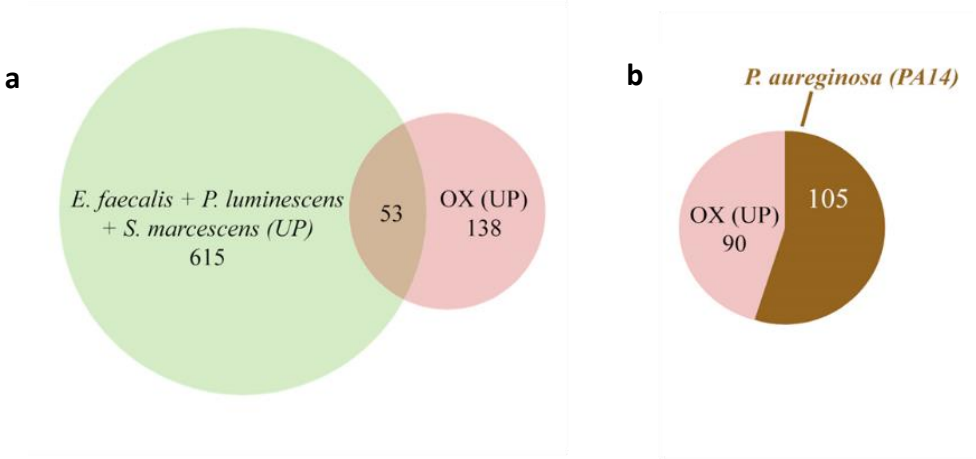


Figure 5: Upon exposure to pathogenic bacteria *C. elegans* activates transcriptional responses that lead to expression of antimicrobial, stress response and immune defense genes. Venn diagrams showing the overlap between upregulated genes in naturally oxidized *C. elegans* (191 genes) and (a) upregulated genes as part to the transcriptional responses to infection by three bacteria (*Serratia marcescens*, *Enterococcus faecalis*, *Photorhabdus luminescens*) (Wong et al., 2007) or (b) *Pseudomonas aeruginosa* (Engelmann et al., 2011).

1.10 Aim of the study

According to the preliminary transcriptomics data *C. elegans* genes that are being upregulated during *P. aeruginosa* infection have a significant overlap with genes being upregulated during oxidative eustress, suggesting that the redox environment may influence the host's susceptibility to infection. This suggests that redox signaling may be critical for the response to pathogens. The aim of this MSc thesis is to understand certain aspects of ROS during host-microbe interactions. Specifically, I will:

1. Investigate whether early-life oxidative stress can be protective for host defense during *P. aeruginosa* infection.
2. Determine if a highly oxidized environment in host cells can initiate redox signaling, which may be part of the host defense mechanism against *P. aeruginosa.*
3. Discover if the COMPASS complex plays role in host's innate immunity.

2. Experimental Procedures

2.1 *C. elegans* strains, maintenance and lifespan assays

The following *C. elegans* strains were used in this study: *N2 (*wild-type Bristol isolate), PB020: *N2jrIs2*[P*rpl-17*::Grx-1-roGFP2], AY101: *acIs101*[F35E12.5p::GFP + *rol-6* (su1006)]. Worms were cultured at 20°C, unless noted otherwise. Synchronization was performed as described below. Survival assays were performed at 25 °C in the presence of fluorodeoxyuridine (FuDR). Survival was scored every day or every 2 days and worms were scored as dead when no movement was observed upon gentle prodding, whereas worms which had crawled off the plate or hatched inside, were scored as censored. Survival plots were generated using GraphPad Prism. Lifespan data were analyzed for statistical significance with log-rank (Mantel–Cox) or Gehan–Breslow–Wilcoxon test.

2.2 Worm synchronization by egg-laying

Adult *C. elegans* were picked and transferred to NGM (3g NaCl, 2.5g of BactoPeptone, 17g BactoAgar, 1M $CaCl_2$, 1M $MgSO_4$, 1M $KHPO_4$, 5 mg/ml cholesterol, 10 mg/ml nystatin and 100 mg/ml from appropriate antibiotic (streptomycin or ampicillin) plates seeded with *E. coli* OP50 (the standard dietary bacteria for C. elegans laboratory growth and maintenance) for two hours to lay eggs. After 2 hours, adults were removed from the plates, leaving synchronized eggs behind.

2.3 Worm synchronization by bleaching adult worms and egg plating

Adult hermaphrodites were transferred to fresh NGM plates seeded with OP50 bacteria. Worms were washed with distilled water (dH_2O) and placed into a 15 ml canonical tube. Bleach solution (1 ml; consisting of 2 ml 5N NaOH, 2.5 ml Chlorox, and 5.5 ml distilled water) was added until 50% of the worm population was dissolved. The worm population was then washed three times with M9 buffer. Eggs were placed in a flask for hatching at 20°C on a shaker. After hatching, L1 larvae were transferred to NGM plates seeded with food by pipetting.

2.4 Preparation of agarose pads

To immobilize worms for microscopy, a 2% (w/v) agarose pad was used. A drop of 2% molten agarose was placed in the center of a slide and covered with a cover glass, forming an agarose sandwich. After 2 minutes, the cover glass was removed.

2.5 Ratiometric imaging

For redox state measurements, experiments were performed using the strain *N2*[*Prpl-17*::Grx-1::roGFP2]. The fluorescence of the redox-sensing protein Grx1–roGFP2 was measured using excitation filters for 405 nm (a proxy for oxidized glutathione GSSG) and 488 nm (a proxy for reduced glutathione GSH). The redox state was quantified from the GSSG:GSH ratio (405/488). Worms were collected from plates seeded with the bacterial strains *E. coli* MG1655 or PA14, transferred into tubes, and washed twice with M9 buffer. Synchronized *C. elegans* (20-30 worms) were placed on the agarose pad, and 25 mM of levamisole was added for anesthesia. Redox state measurements were performed using an Olympus IX73 inverted microscope with a CoolSNAP HQ2 camera and a 10x optical lens. Image analysis was performed with ImageJ software, with an intensity threshold chosen by the user, and ROS measurements defined from the worms' nose to tail.

2.6 Molecular cloning for *bli-3* knockdown

The cloning strategy for the *bli-3* RNAi construct involves amplifying the *bli-3* gene from nematode cDNA through PCR. cDNA was synthesized using the PrimeScript Reverse Transferase Kit (Takara). The 834 bp-PCR fragment was then inserted into the PCR-II TOPO vector (Invitrogen) and transformed into DH5a *E. coli* strain. The *bli-3* fragment was then digested from the PCR-II TOPO vector with KpnI/Xbal enzymes, inserted into the pL4440 (RNAi feeding vector) and transformed into the *E. coli* HT115 strain (RNAi feeding strain). The above experimental steps are described in detail below.

2.6.1 RNA isolation

For RNA isolation, 15 adult worms were picked and transferred into 5 µl of worm lysis buffer (5 mM Tris, 0.5 % Triton, 0.5% Tween,0.25 mM EDTA, 1 mg/ml Proteinase K) on the lid of a PCR tube. The tubes were placed in liquid nitrogen for 3 freeze/thaw cycles. Samples were incubated at 65°C for 10 minutes, followed by incubation at 85°C for 1 minute to inactivate proteinase K, and then cooled on ice.

2.6.2 cDNA synthesis protocol

The Takara cDNA synthesis kit was used according to the manufacturer's instructions. Initially the following components were mixed:

Components	Reaction
Random 6 mers (50 µM)	1 µl
dNTP Mixture (10 mM each)	1 µl
Template RNA	total RNA: < 5 µg
Sterile water	Up to 10 µl

The above mixture was then incubated at 65 °C for 5 minutes and then placed on ice where the following components were added:

Components	Reaction
Reaction mixture (A1)	10 µl
5X PrimeScript Buffer	4 µl
RNase Inhibitor (40 U/µl)	0.5 µl
PrimeScript RTase	0.5 µl
Sterile water	Up to 20 µl

The mixture was incubated at 30°C for 10 minutes, then at 42°C for 60 minutes. For enzyme inactivation, the mixture was incubated at 95°C for 5 minutes and then stored at 20°C.

2.6.3 Polymerase chain reaction

PCR was used to amplify 834bp of the *bli-3* cDNA using Taq DNA polymerase from Enzyquest, following the manufacturer's instructions. The reaction prepared was the following:

Components	Concentration	Reaction 25 µl
Enzyquest Taq pol buffer (10x)	1x	2.5 µl
$MgCl_2$	25mM	1.5 µl
dNTP mix (10mM)	0.2 mM	0.5 µl
Forward primer (10µM)	0.5 mM	0.5 µl
Reverse primer (10µM)	0.5 mM	0.5 µl
Template DNA	1-500 ng	3 µl
Enzyquest Taq DNA pol (5u/µl)	1 unit	0.25 µl
Sterile ultrapure water		Up to 25 µl

The primers for amplification were the following:

Forward primer: 5'- CAAAGTTGTTGGATTCTCCCA-3'
Reverse primer: 5'- TGGATTGCTCCTTATGTTCACTC-3'

For the polymerase chain reaction, the following thermal stages were used:

Stage	**Temperature °C**	**Time**
Initial denaturation	95	5 minutes
Denaturation	95	30 seconds
Annealing	59	20 seconds
Extending	72	50 seconds
Final extending	72	5 minutes
Hold	8	∞

The above stages were repeated for 30 cycles.

2.6.4 TOPO TA cloning

The amplified PCR product (*bli-3)* was inserted into the TOPO vector following the manufacturer's instructions. The following components were mixed:

Reagent	**Volume**
PCR product	3 µl
Salt solution	1 µl
TOPO vector	1 µl
Sterile water	up to 6 µl

The mixture was incubated at room temperature for 5 minutes, and then transformed into competent DH5α cells.

2.6.5 Restriction digest of plasmid DNA

For cleaving DNA at specific sequences, KpnI and XbaI restriction enzymes from Enzyquest were used. Both the TOPO construct and the pL4440 vector were digested with these enzymes, in the following reaction which was incubated for 1 hour at 37°C to allow the digest.

Reagent	Volume
10x EQ Buffer	2 µl
Plasmid DNA	1 µg
Enzyme KpnI	1 µl
Enzyme XbaI	1 µl
Sterile water	up to 20 µl

2.6.6 Ligation protocol

The digested *bli-3* insert from TOPO was inserted into the digested pL4440 vector using the quick ligation protocol from NEB (New England Biolabs). A 1:3 molar ratio was used (1 molar vector and 3 molars insert DNA).

The following components were mixed:

Component	Volume
Quick ligase reaction buffer (2x)	10 µl
Vector DNA	50 ng
Insert DNA	37.5 ng
Sterile water	up to 20 µl
Quick ligase	1 µl

The mixture was incubated for 5 minutes at room temperature and then 10 µl of the reaction was used to transform competent HT115 cells (RNAi feeding *E. coli* strain).

2.6.7 Plasmid DNA extraction

For DNA plasmid extraction, 6 ml cultures of *E. coli* in LB containing an appropriate antibiotic at 37°C for 16-18 hours. Cells were collected by centrifugation at 11.000 g for 1 minute at 4°C, then lysed by adding 100 µl of Solution I (50 mM Glucose, 25 mM Tris-Cl EDTA, 10 mM). 200 µl of Solution II (NaOH 0.2 N, SDS 1% w/v) was then added, and the lysate was neutralized by adding 150 µl of Solution III (Potassium Acetate 5M: 60 ml, Glacial acetic acid: 11.5 ml, H2O: 28.5 ml). The lysate was centrifuged at 11.000 g for 5 minutes. The supernatant was collected, 900 µl of 100% ethanol was added, and the mixture was centrifuged at 11.000 g for 20 minutes. The DNA pellet was washed with 70% (v/v) ethanol, dried, and then resuspended in sterile water.

2.6.8 Transformation and selection

To insert plasmid DNA into competent cells, the following transformation technique was employed: Initially, 10 µl from the ligation reaction was added to competent cells and incubated on ice for 30 minutes. Subsequently, a heat shock at 42°C for 1 minute was performed, followed by immediate placement on ice for 1 minute. Next, 900 µl of LB medium was added, and the tube was incubated at 37°C for 1 hour to allow recovery of the cells. After recovery, the tube was centrifuged at 3.000 rpm for 3 minutes and the supernatant was discarded, leaving approximately 100 µl of LB medium with the pellet. The pellet was then resuspended, spread onto selection LB-agar medium, and incubated overnight at 37°C. The following day, colonies were selected and inoculated into LB liquid medium with the appropriate antibiotic. After plasmid extraction, digests were conducted to confirm the presence of the insert.

2.6.9 RNAi Interference by feeding

RNAi was performed by feeding worms *E. coli* HT115 (DE3) bacteria carrying the plasmid expressing the 834bp cDNA fragment of *bli-3* flanked by two antiparallel T7 promoters (pL4440). HT115 bacteria carry a defective RNase III and an isopropylthiogalactoside (IPTG)-inducible T7 polymerase gene. Addition of 1 mM IPTG will generate the dsRNA of interest (*bli-3*) through T7 polymerase-inducible transcription.

2.7 *E. coli* pretreatment with N-acetylcysteine (NAC) and Paraquat (PQ)

For pretreatment of *E. coli* OP50 with antioxidant (NAC) or oxidant (PQ), an overnight *E. coli* liquid culture was diluted 1/100 using LB in a Falcon tube. 5 mM of NAC or 2 mM PQ were added in the liquid culture, which was then placed in a shaking incubator at 37°C and 250 rpm for 8 hours. The OD at 600 nm was measured using a spectrometer to achieve an optimal range of 0.6-0.9. The culture was then centrifuged at 2000 rcf for 10 minutes, the supernatant discarded, and the pellet washed with LB.

C. elegans at the L2 stage were washed with M9 from NGM OP50 plates and transferred into a Falcon tube containing the pretreated bacterial pellet. The tube was filled with 3ml M9, and the worms were incubated at 20°C and 80 rpm for 16 hours. *C. elegans* were then washed three times with M9 and transferred onto NGM plates seeded with OP50. The day after, worms were transferred to PA14 or *E. coli* MG1655 plates.

2.8 Slow Killing of *C. elegans* by *P. aeruginosa*

For Slow Killing (SK) infection, 8 µl of saturated liquid culture of PA14 and *E. coli* MG1655 were spread onto 3.5 cm SK agar plates. The inoculated plates were first incubated at 37°C for 24 hours, then transferred to 25°C and further incubated for 24 hours. Prior to transferring worms to SK agar plates, 45 µl of 100× FUDR was added to each plate to inhibit worm reproduction and plates were allowed to dry for 40 minutes. Synchronized L4-staged worms were then transferred to the Slow Killing plates and incubated at 25°C. Worms were assessed for survival every 24 hours.

2.9 *P. aeruginosa* and *E. coli* treatment with Paraformaldehyde (PFA)

For bacterial treatment with Paraformaldehyde (PFA), a single colony of bacteria was inoculated in LB liquid and grown for 16-18 hours. The following day, PFA solution was added to the LB liquid culture to achieve a 5% concentration. Bacterial cultures containing PFA were incubated at 37°C for 2 hours, followed by centrifugation at 2000 g for 20 minutes and washed three times with LB. The pellet was resuspended to 1/10 of the original volume using LB, and 200 µl were plated on Slow Killing agar plates.

2.10 Worm treatments with NAC and PQ

For exposure of *C. elegans* to PQ (oxidant) or NAC (antioxidant), L2-staged worms were placed in a 15 ml Falcon tube containing 5 ml M9, 200 µl OP50, 5 µl streptomycin, and the specified concentrations of PQ (2 and 3 mM) or NAC (8 mM) for 15 hours. The following day (L4 larval stage), worms were transferred to NGM OP50 plates for 24 hours, and then transferred to PA14 or *E. coli* MG1655 plates.

2.11 Statistical analysis

Statistical analysis was conducted using Prism software (GraphPad Software 8). Detailed information on statistical tests, *p*-values, and sample sizes can be found in the corresponding methods and figures.

3. Results

3.1 Worm survival after Slow Killing infection with *P. aeruginosa*

The Slow Killing assay (SK) was used to assess the impact of *Pseudomonas aeruginosa* (PA14) on the survival of *C. elegans*. This assay involves an active infection where live PA14 bacteria colonize the intestinal lumen of the worms. Synchronized *C. elegans* at the L4 developmental stage, grown on NGM plates seeded with OP50 *E. coli*, were transferred to plates containing either PA14 or MG1655 (a non-pathogenic *E. coli* strain used as a control). I observed that worms infected with PA14 died gradually over a period of 10 days, whereas those exposed to MG1655 remained alive throughout the observation period (**Fig. 6a**).

To confirm that this observation is indeed due to live bacteria, I employed the lab's recently developed 0.5% PFA regiment tested on metabolic and proliferation assays (Beydoun et al., 2021), which kills bacteria but still allows them to serve as a preferred

dietary source for worms as opposed to standard bacteria-killing methods such as heat, UV or antibiotics which make bacteria less edible, with confounding effects to worm growth and metabolism (Gems & Riddle, 2000; Qi et al., 2017). As expected, worms exposed to PFA-treated PA14 exhibited significantly extended lifespan compared to those infected with live PA14, and comparable to worms exposed to PFA-treated MG1655, suggesting that only live PA14 bacteria are pathogenic under the conditions of the SK assay (**Fig. 6b**).

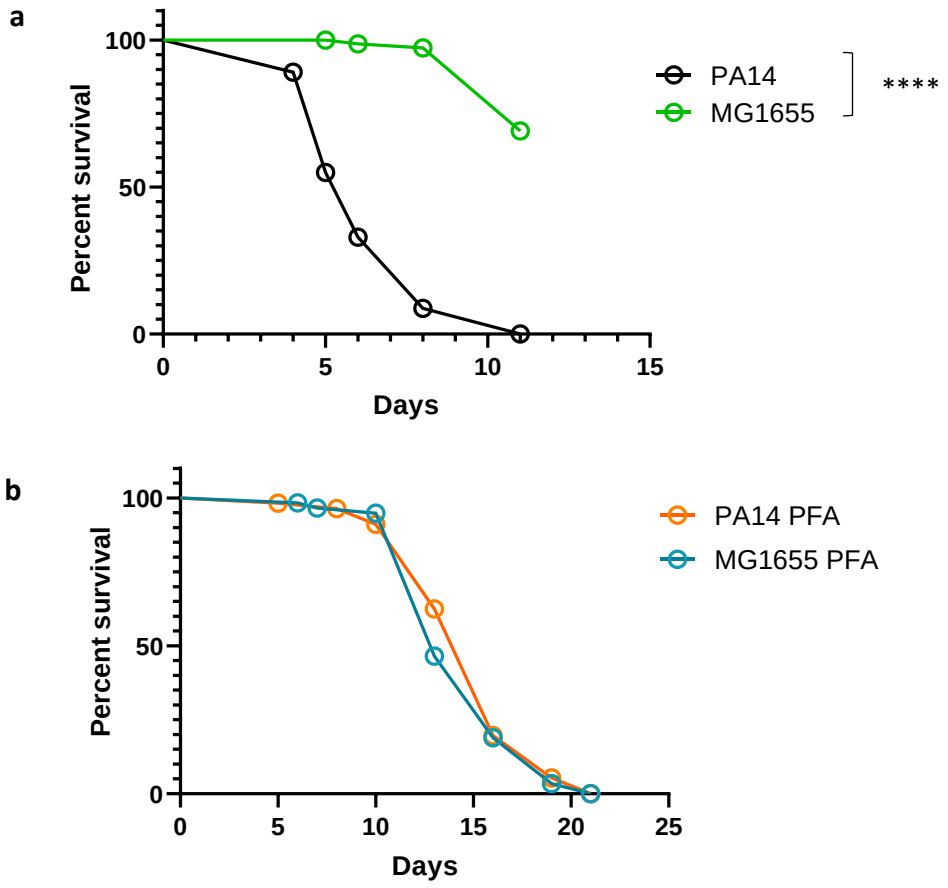
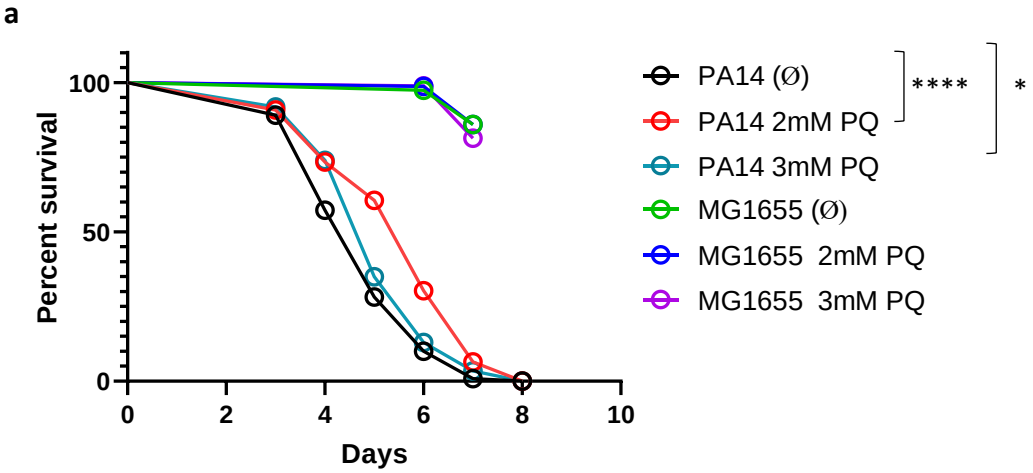


Figure 6: Worm survival after exposure to PA14. a) Survival curves of worms maintained on SK plates seeded with MG1655 or PA14. **b)** Survival curves of worms maintained on SK plates seeded with PFA-treated MG1655 or PFA-treated PA14. Experiments were performed with the wild-type *C. elegans* strain, *N2*. Curves represent data from populations of *n*:20-100 worms. Statistical analysis was performed with the Log-rank (Mantel-Cox) test. ****: p <0.0001.

3.2 An oxidizing environment during *C. elegans* development extends survival during infection with *P. aeruginosa*

According to recent studies a direct application of mild concentrations of paraquat, to L2-staged worms extended their lifespan and increased their stress resistance (Bazopoulou et al., 2019). Similarly, here, I hypothesized that treatments with oxidants, will extend its survival in the presence of PA14. To investigate this, I exposed L2-staged worms to 2 mM or 3 mM paraquat (PQ) for 15 hours. Following this treatment, at the L4-stage, worms were tested for survival in the presence pf PA14 (Slow Killing assay). I observed that worms pre-treated with PQ had an extended survival upon PA14 infection, compared to untreated worms, suggesting that an oxidizing environment during the developmental L2 stage confers protection against a subsequent exposure to live PA14 (**Fig. 7a**).

To confirm the positive effect of oxidation on worm survival, I also treated L2-staged worms with 8 mM N-acetylcysteine (NAC), an antioxidant, for 15 hours. As it was anticipated, NAC pre-treatment reduced the survival of worms during PA14 infection, strengthening my initial hypothesis (**Fig. 7b**).



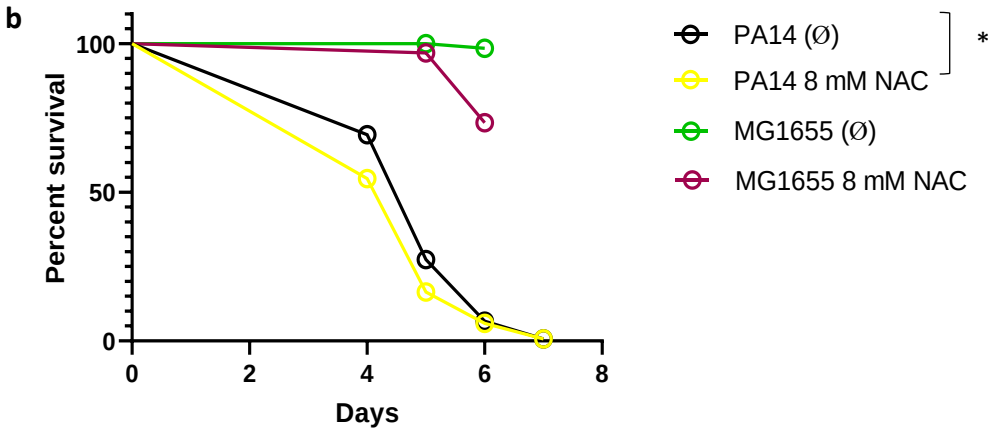


Figure 7: Survival of worms pretreated with oxidants or antioxidants, after exposure to PA14. a) Survival curves of worms treated with 2 mM or 3 mM PQ, and then transferred to SK plates seeded with MG1655 or PA14. **b)** Survival curves of worms treated with 8 mM NAC and then transferred to SK plates seeded with MG1655 or PA14. Experiments were performed with wild-type *C. elegans* strain, *N2*. Curves represent data from populations of *n*: 20-100 worms. Statistical analysis was performed with the Log-rank (Mantel-Cox) test. ****: p <0.0001, *: p <0.05.

Our lab recently found that the redox state of dietary bacteria affects *C. elegans* lifespan under normal conditions (no infection) (Falalakis, *Bachelor Thesis*, 2023). Specifically, it was observed that worms exposed during the L2 stage to OP50 bacteria that were treated with NAC had reduced lifespan. Based on these observations, I decided to also explore whether similar conditions, i.e. exposure to reduced or even oxidized dietary bacteria, have an effect of worm survival against pathogenic bacteria. For this purpose, I exposed L2-staged worms to OP50 *E. coli* pre-treated with 1 mM PQ or 5 mM NAC. These concentrations were previously shown to effectively oxidize or reduce bacteria, respectively (Falalakis, *Bachelor Thesis*, 2023). Following this exposure, worms were transferred to SK plates with PA14. While worms exposed to PQ pre-treated OP50 (oxidized dietary bacteria) showed no significant difference in survival compared to those fed with untreated OP50, worms fed NAC pre-treated OP50 (reduced dietary bacteria) exhibited decreased survival during PA14 infection and also during exposure to MG1655 bacteria (**Fig. 8**). Overall, these findings suggest that dietary bacteria can affect host survival against subsequent bacterial infection and that antioxidants can diminish any positive effects of these bacteria even in normal conditions.

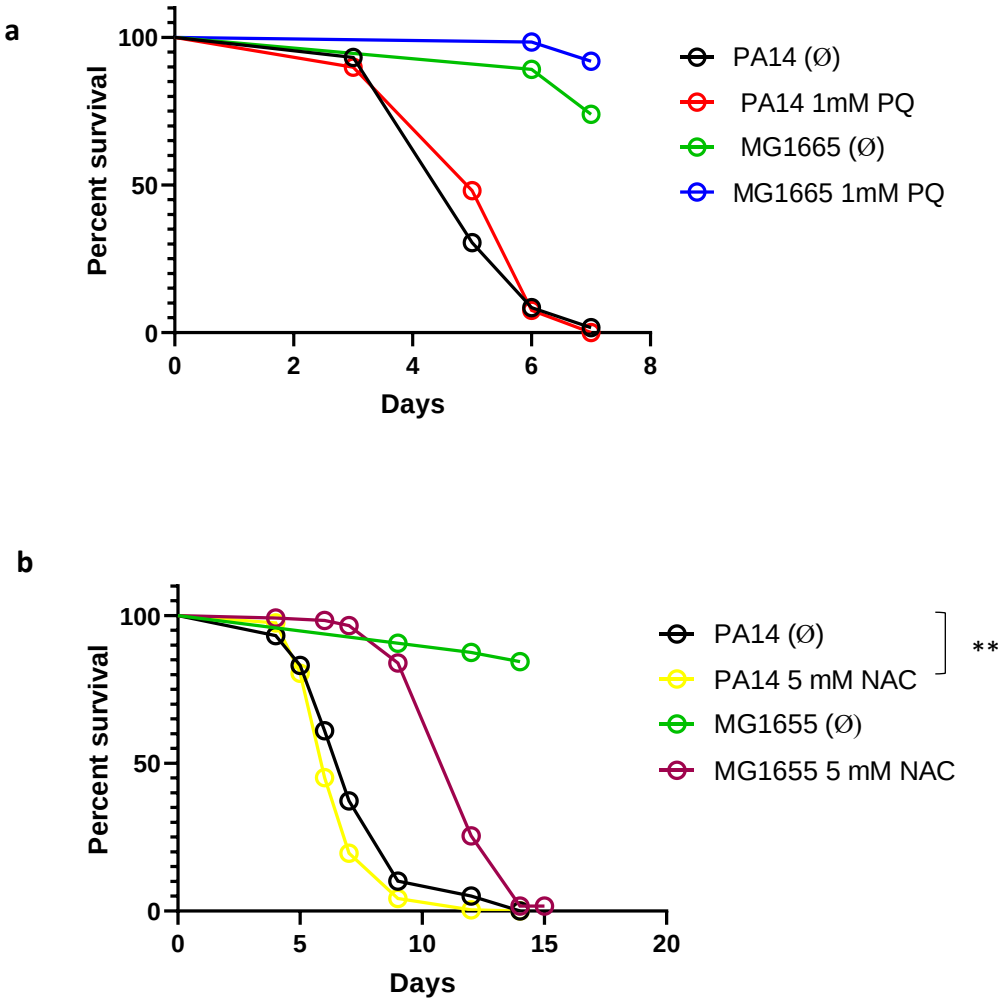


Figure 8: Survival of worms pretreated with oxidized or reduced dietary bacteria, after exposure to PA14. a) Survival curves of worms after exposure to OP50 bacteria treated with 1 mM PQ and then maintained on SK plates seeded with MG1655 or PA14. **b)** Survival curves of worms after exposure to OP50 bacteria treated with 5 mM NAC and then maintained on SK plates with MG1655 or PA14. Experiments were performed with wild-type *C. elegans* strain, *N2*. Curves represent data from populations of *n*: 20-100 worms. Statistical analysis was performed with the Log-rank (Mantel-Cox) test. **: $p < 0.01$.

3.3 The *C. elegans* redox state during infection correlates with *P. aeruginosa* infection outcome

In *Caenorhabditis elegans*, a burst of reactive oxygen species (ROS) is observed during infection, mediated by the dual oxidase BLI-3, which produces H_2O_2 suggesting a significant role in the immune response (van der Hoeven et al., 2011). However, there is still no report on the host's redox state under these conditions and how this redox state may affect worm survival. To monitor this, I employed wild-type *N2* worms expressing the integrated redox-sensing protein Grx1–roGFP2, which faithfully responds to the cellular ratio of oxidized (GSSG) to reduced glutathione (GSH) (Gutscher et al., 2008). Following the same SK protocol as before, I initially found that L4-staged worms exposed to PA14 for 5 hours were significantly reduced compared to worms transferred to SK plates seeded with MG1655 (**Fig. 9**). Although this may seem counterintuitive due to the reported increase in BLI-3/DUOX activity, we believe that at this stage of infection the intracellular redox environment has already received significant impact from antioxidant defenses. This is supported by i) studies that show the timeline of SKN-1 antioxidant function (van der Hoeven et al., 2011) and ii) by previous data from our lab (not shown) that show a peak in intracellular oxidation at 1-2 hours' time mark, post PA14 exposure (Fast Killing assay, *data not shown*).

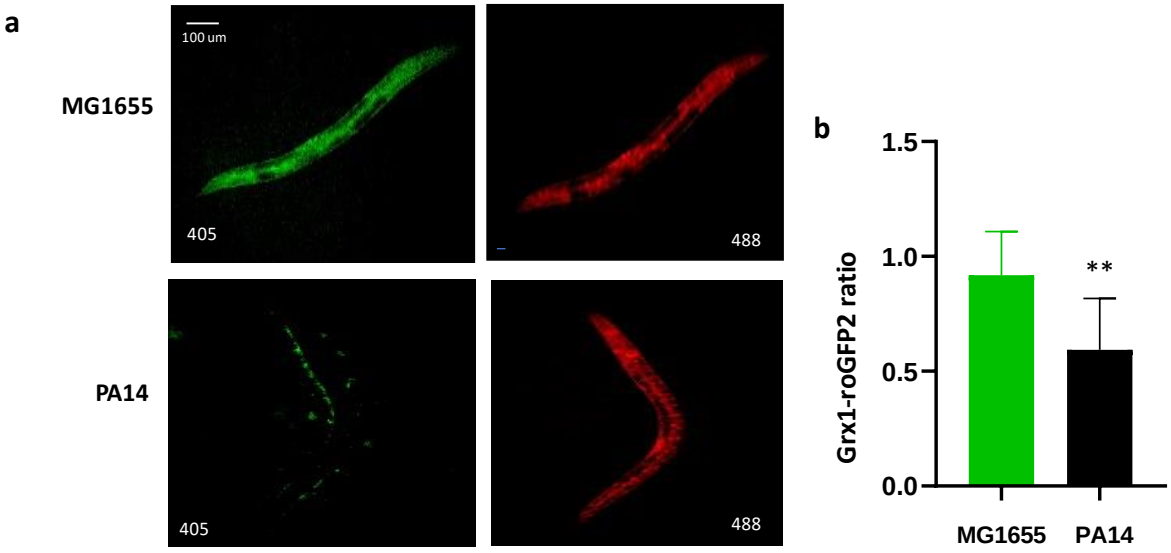
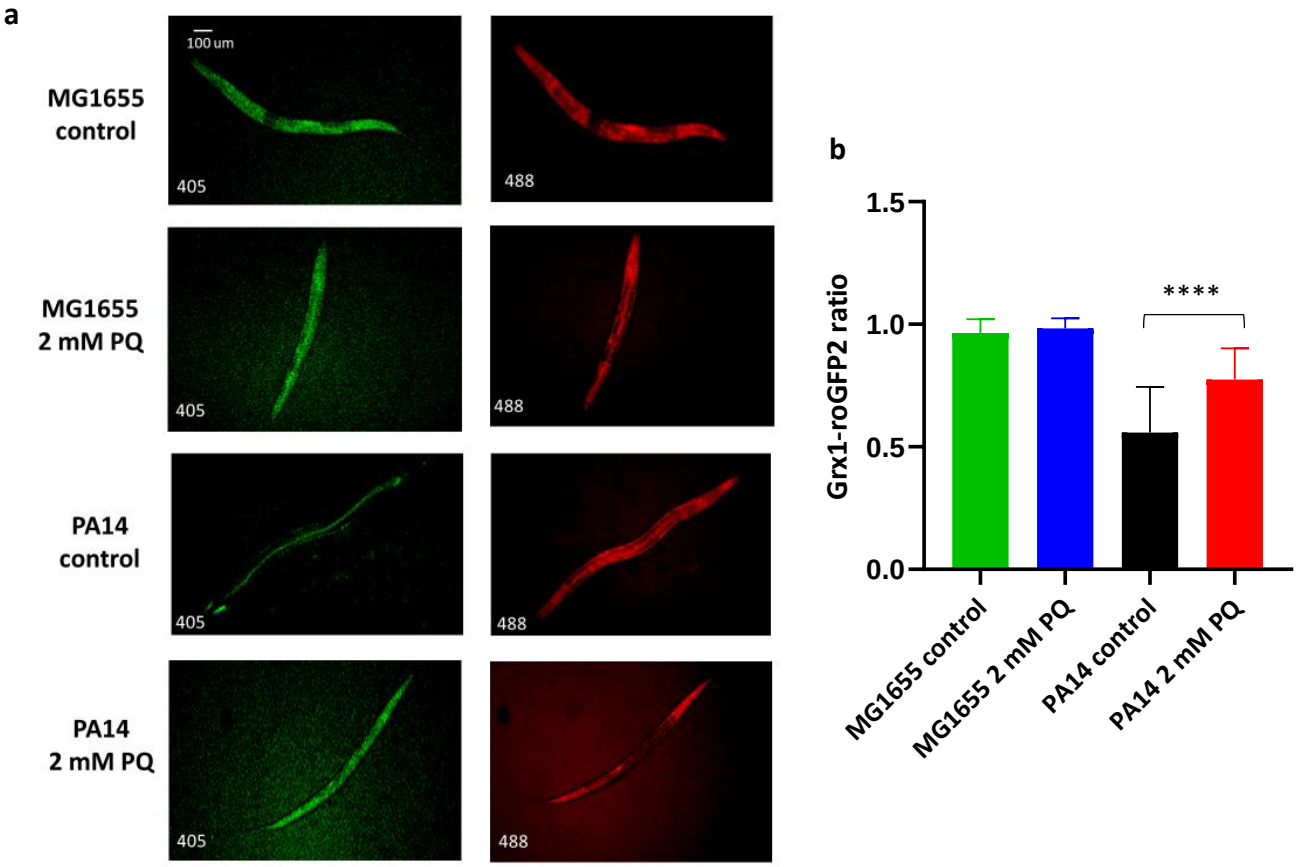


Figure 9: Intracellular redox state after exposure to PA14. a) Representative images of worms expressing the redox-sensing protein Grx1-roGFP2 maintained on SK plates seeded with MG1655 or PA14 for 5 hours and **b)** analysis of Grx-1-roGFP ratio (405/488) from populations of at least 20 worms per condition. Experiments were performed with the PB020 *N2*jrIs2[P*rpl-17*::Grx-1-roGFP2] strain. Statistical analysis was performed with the unpaired *t* test. **: $p < 0.01$.

To further elaborate, I tested if the host's redox is subject to changes during infection, as a result of pre-treatments, and whether any of these changes are consistent with our survival results. Indeed, I found that the 2 mM PQ treatment of L2-staged worms, which increases survival during their subsequent exposure to PA14 (**Fig.10a**), significantly increased the oxidation levels of *N2* worms expressing the integrated redox-sensing protein Grx1–roGFP2, 5 hours post infection with *P. aeruginosa* (**Fig.10 a&b**). However, the host's redox state has no significant difference between worms treated with PQ and untreated worms exposed to MG1655. One explanation for this observation could be that the Grx1–roGFP2 has reached its maximum oxidation potential under these conditions. On the other hand, pretreatment with an antioxidant (8 mM NAC) which decreases survival during infection (**Fig.10b**) did not reduce the intracellular host environment (**Fig.10 c&d**). The host's redox state remains also unchanged on MG1655 bacteria between treated and untreated worms) the same with untreated worms MG1655.



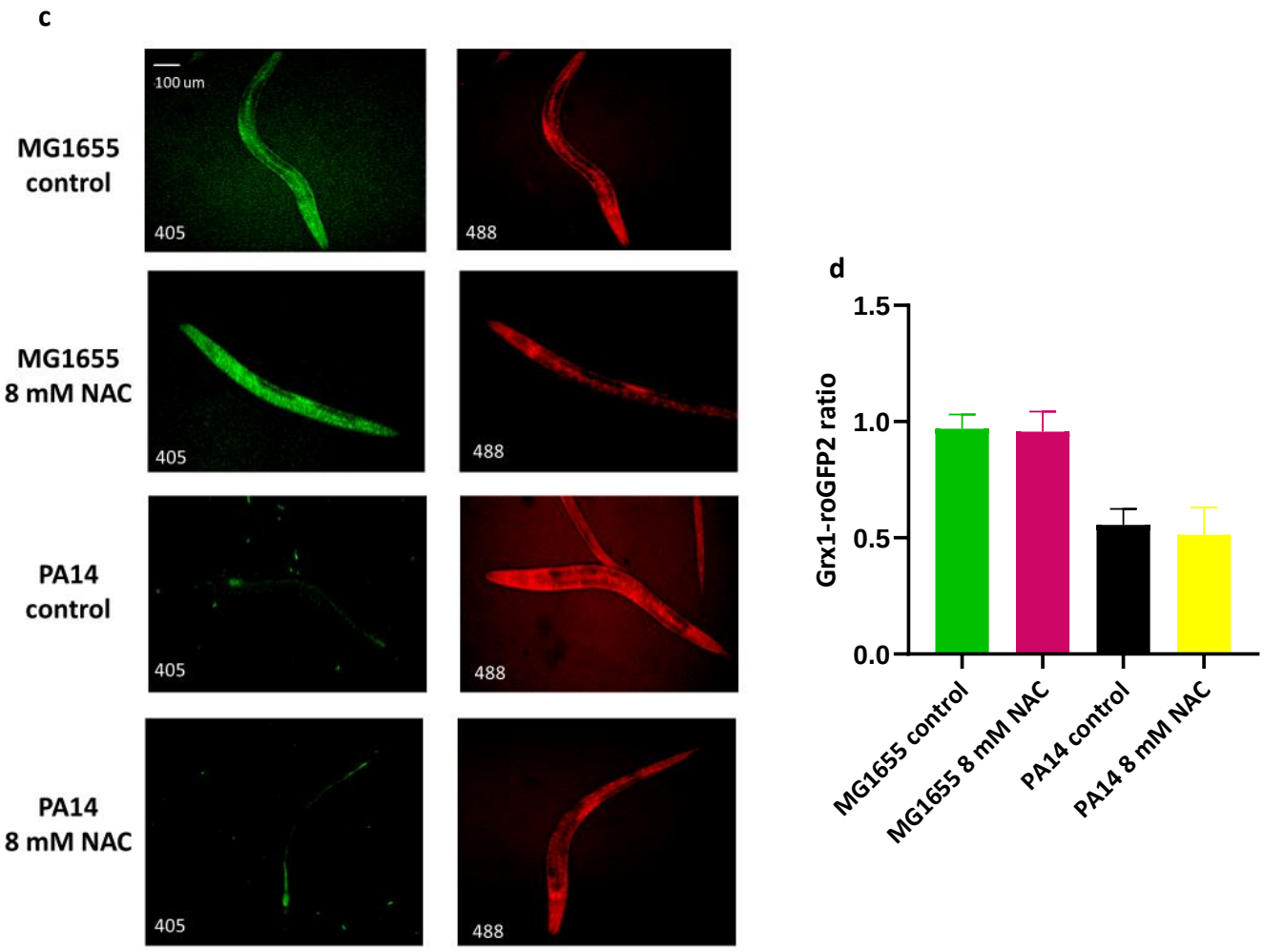


Figure 10: Intracellular redox state of worms pretreated with paraquat or NAC and after exposure to PA14. **a) & c)** Representative images of *N2* worms expressing the redox-sensing protein Grx1-roGFP2, pretreated (or not) with paraquat or NAC respectively and then maintained on SK plates seeded with MG1655 or PA14 for 5 hours and **b) & d)** respective analysis of Grx-1-roGFP ratio (405/488) from populations of at least 20 worms per condition. Experiments were performed with the PB020 *N2*jrIs2[P*rpl-17*::Grx-1-roGFP2] strain. Statistical analysis was performed with the unpaired *t* test. ****: $p < 0.0001$.

However, the treatment of L2-staged worms with 8 mM NAC increases the expression on the infection response gene *irg-5*, when worms were placed in SK plated seeded with PA14. Expression of *irg-5* is controlled by the p38 mitogen-activated protein (MAPK) kinase PMK-1 pathway, a central regulator of anti-pathogen defenses in *C. elegans* (van der Hoeven et al., 2011) and serves as a readout of innate immune activation (the higher it is expressed, the more sever the infection is). Notably, the same NAC pre-treatment increased *irg-5* expression in worms placed in SK plates seeded with the control MG1655 bacteria, a condition that led to decreased lifespan compared to untreated worms exposed to MG1655 (**Fig. 11**). Overall, these data suggest that manipulations that lead to an increased oxidation of the host during initial stages of infection correlate with increased survival, whereas conditions that reduce the worms have a negative effect on survival under pathogenic (and non-pathogenic bacteria).

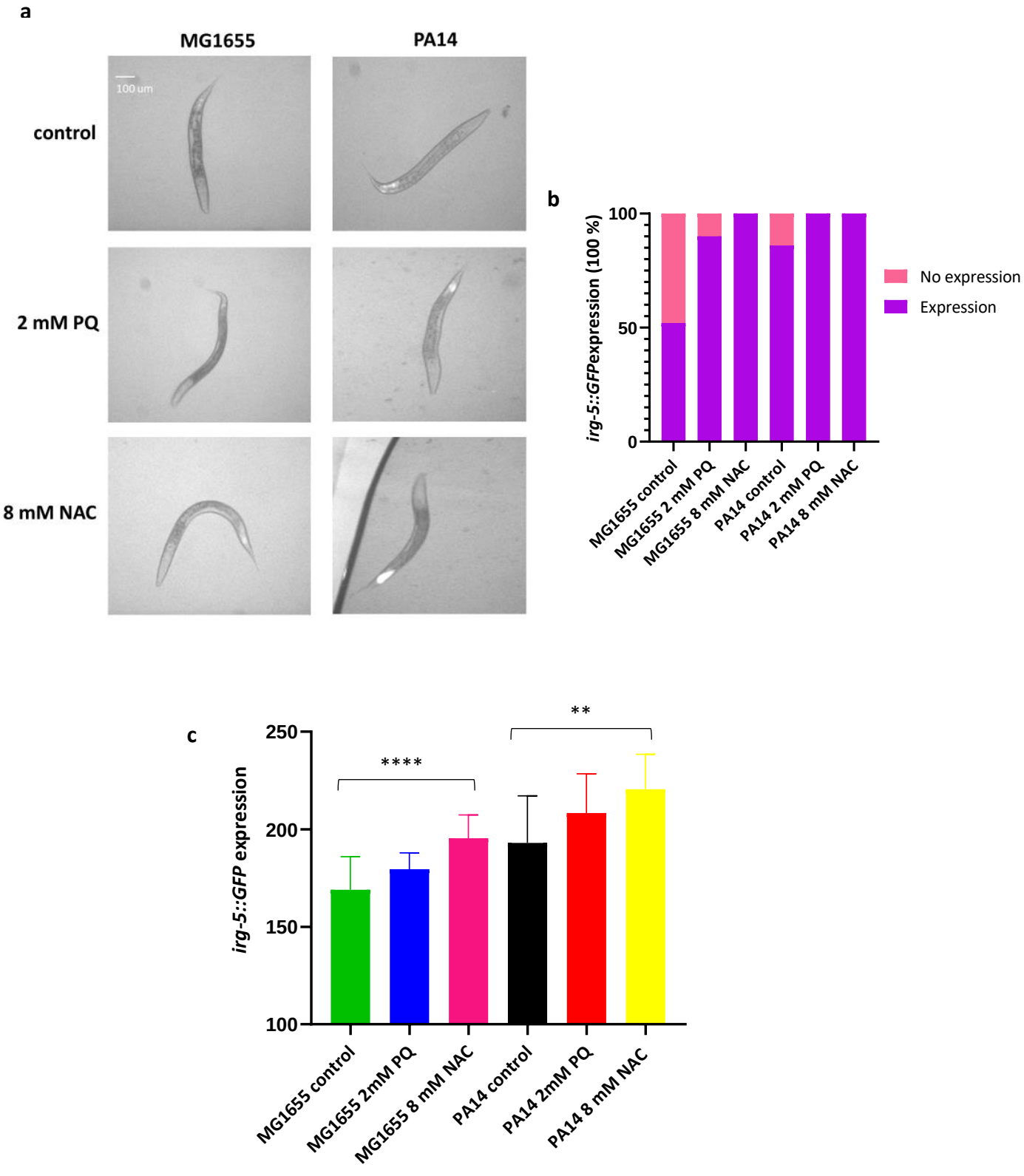


Figure 11: Expression of the immune gene *irg-5* after exposure PA14. a) Representative images of worms expressing the *irg-5::GFP* transgene, pretreated with paraquat or NAC and then maintained on SK plates seeded with MG1655 or PA14 for 5 hours. **b)** Percentage of worms that show expression of *irg-5*::GFP transgene is depicted. In these transgenic animals, either no or minimal expression of GFP is

observed in the presence of *E. coli* bacteria, but high levels of expression are seen in animals exposed to bacterial pathogens. AY101 worms that did not express GFP were not included in **c)**. **c)** analysis of *irg-5* expression from populations of at least 20 worms. The experiments were performed with the strain AY101 *acIs101*[F35E12.5p::GFP + *rol-6* (su1006)]. Statistical analysis was performed with the unpaired *t* test. **: p <0.01, ****: p <0.0001.

3.4 The NADPH oxidase Ce-Duox1/BLI-3 is important for host's survival during *P. aeruginosa* infection, whereas the COMPASS complex does not have an effect

Previous studies have shown that ROS are generated during infection with *E. faecalis* in *C. elegans* by the dual oxidase Ce-Duox1/BLI-3. This model describes the activation of p38 MAPK signaling, resulting in the nuclear localization of SKN-1 (van der Hoeven et al., 2011). To study the role of Ce-Duox1/BLI-3 specifically during *P. aeruginosa* infection, I performed RNA knockdown of *bli-3* (*bli-3* RNAi by feeding). Worms were grown from hatching to the L4 stage on bacteria expressing *bli-3* RNAi and then transferred to SK plates seeded with MG1655 or PA14. Due to reports of *bli-3* knockout being indispensable for worm development (van der Hoeven et al., 2011), I exposed worms to undiluted bacteria expressing *bli-3* RNAi or to bacteria expressing *bli-3* RNAi diluted (1:30) with control ones (bacteria expressing the vector only). Indeed, I observed that *bli-3* RNAi (undiluted) severely impacted worm survival on the MG1655 control bacteria, whereas the 1:30 *bli-3* RNAi did not. However, only *bli-3* RNAi (undiluted) was sufficient to decrease worm survival on PA14, confirming the protective role of *bli-3* against *P. aeruginosa* (**Fig.12**).

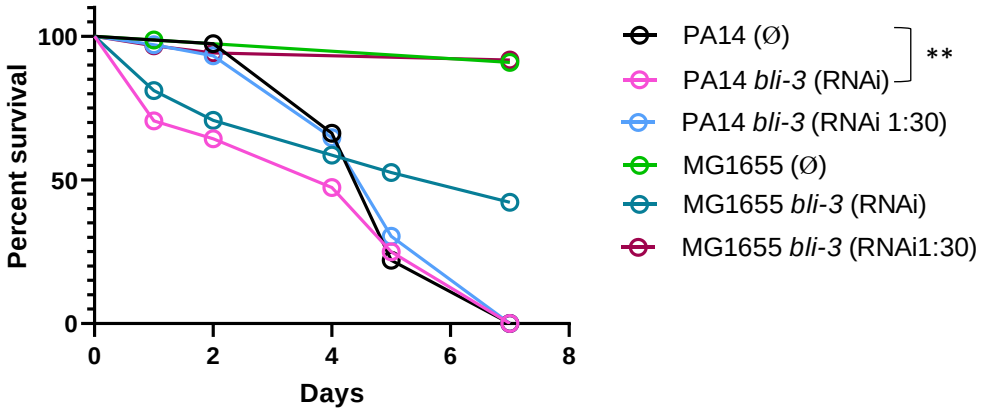


Figure 12: Survival of *bli-3* knockdown on worms after exposure to PA14. Survival curves of worms treated with *bli-3* RNAi and then maintained on SK plates seeded with MG1655 or PA14. Experiments were performed with the wild- type *C. elegans* strain, *N2*. Curves represent data from populations of *n*:20-100 worms. Statistical analysis was performed with the Gehan-Breslow-Wilcoxon test. **: p < 0.01.

Based on the observation that *bli-3* function (ROS generation) is important under *P. aeruginosa* infection and our previous transcriptomics analysis that shows that worms infected with *P. aeruginosa* resemble worms which are mildly oxidized and have an inactivated COMPASS complex (Bazopoulou et al., 2019), I proceeded with questioning whether inactivation of the COMPASS complex has a protective role on the infected host. To test this, I performed RNAi on a member on the COMPASS complex, *ash-2*, a condition known to inactivate the complex (**Fig. 13**). Contrary to my initial hypothesis, I found that *ash-2* RNAi (from hatching to the L4 stage) did not increase survival of worms transferred to SK plates seeded with PA14 compared to the vector control.

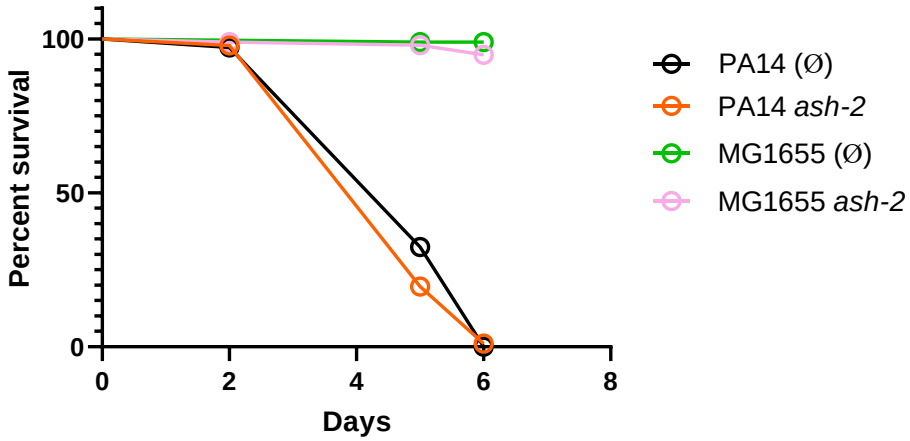


Figure 13: Survival of *ash-2* knockdown after exposure to PA14. Survival curves of worms treated with *ash-2* RNAi and then maintained on SK plates seeded with MG1655 or PA14. Experiments were performed with the wild-type *C. elegans* strain, *N2*. Curves represents data from populations of *n*:20-100 worms.

4. Conclusion/Discussion

In this master thesis I studied the role of ROS in host's innate immunity after infection with the human opportunistic bacterium *P. aeruginosa*. In previous studies of our lab, it was shown that ROS have a major role as pro-longevity and pro-health signals and variations in their endogenous levels, specifically early-in-life, represent one of the long-sought after, stochastic factors that individualize the aging process and susceptibility to age-related diseases (Bazopoulou et al., 2019; Oleson et al., 2021). Specifically, it was found that a mild oxidation (oxidative eustress) during early development increases stress resistance and extends lifespan in both *C. elegans* and mammals. Under these oxidative conditions, the redox-sensitive COMPASS complex is inactivated, resulting in low H3K4me3 levels, which correlate with increased longevity and stress resistance.

As I discussed in the introduction, ROS also play a critical role in host defenses against pathogens. In this thesis, by taking advantage of a powerful host-microbe model system, I sought to understand whether and how ROS and redox-regulated processes affect host fitness, at a whole organism level. Specifically, I studied how the redox state of *C. elegans* host is affected during infection with the human opportunistic bacterium *P. aeruginosa*. Preliminary transcriptomics data showed that *C. elegans* genes that are being upregulated during *P. aeruginosa* infection significantly overlap with genes being upregulated during oxidative eustress, suggesting that i) the redox environment may influence the host's susceptibility to infection and ii) redox signaling – potentially through the redox-regulated COMPASS - may play a significant role in innate immunity.

Based on the above observations, I hypothesized that an oxidizing intracellular environment may be beneficial for the survival of worms under infection conditions. For this purpose, I focused on an infection-like process that requires live bacteria ("slow killing") and exposed worms in the human opportunistic bacterium *P. aeruginosa* (PA14). As other studies have shown in the past, I found that the survival of *C. elegans* is severely impacted in the presence of these bacterium. Similarly to mammals, a single NADPH oxidase (NOX), the Ce-Duox1/BLI-3 is activated in *C. elegans* in response to pathogens which produces antimicrobial ROS. I hypothesized that this ROS burst will oxidize the host. Unexpectedly, I found that shortly after exposure, the *C. elegans* redox environment becomes significantly reduced. This may be a result of the well-characterized SKN-1-dependent antioxidant activities (van der Hoeven et al., 2011) in response to the function of BLI-3 and/or redox-modulating effectors or toxins produced form the pathogen. While this observation does not exclude an oxidation of the host during infection, a future time-course of redox monitoring would reveal any oxidation-reduction fluctuations.

Next, by exploiting the redox-critical L2 developmental stage in *C. elegans*, I tested oxidative and reducing manipulations and monitored their effects on host susceptibility to a subsequent PA14 infection. I found that early-in-life exposure to mild oxidation prolongs host survival, whereas exposure to antioxidants decreases it. Moreover, I found that an early-in-life oxidizing environment conferred indirectly, by

dietary bacteria, is also extending survival, further supporting my hypothesis that an oxidative eustress preconditions the host and protects against a later-in-life infection.

The activity of BLI-3 is vital for host survival during infection. According to existing bibliography worms with *bli-3* knockdown, when infected with *E. faecalis* showed increased susceptibility to this pathogen (Chávez et al., 2009). In agreement with this, I found that the *C. elegans* Ce-Duox1/BLI-3 oxidase is also important in the host defense against *Pseudomonas aeruginosa*, since its absence leads to reduced survival.

The transient increase in ROS levels during development which is sufficient to increase stress resistance and lifespan in *C. elegans* was studied mechanistically (Bazopoulou et al., 2019). Our lab found that these health benefits are due to ROS targeting SET-2, a homolog of the human SET-1/MLL-1 methyltransferase and a member of the histone 3 K4-trimethylation complex, also known as COMPASS complex. This results in the redox-dependent inactivation of SET-2, a biologically conserved event, and a global decrease in the levels of the H3K4me3 modification. Moreover, unpublished data from our lab (Karagianni *Bachelor Thesis*, 2023) showed that *ash-2* (which encodes a member of the COMPASS complex) when knock downed solely during development sufficiently increases lifespan in *C. elegans* under non-infection conditions. Based on these data, my hypothesis also includes that the redox-regulated COMPASS is part of host defense and inactivation of *ash-2* would be beneficial for the host during infection conditions. However, data did not confirm that and worms under *ash-2* knockdown conditions have no statistically significant difference in lifespan compared to the "control" worms, suggesting the opposite of my hypothesis. As a follow up, I intend perform RT-PCR to verify the efficiency of the knockdown and then repeat the *ash-2* knockdown experiment not only on "wild type" worms infected with *Pseudomonas* but also on worms with inactivated BLI-3 which sensitizes the host in the presence this bacterium. There is a possibility that by combining *ash-2* and *bli-3* knockdowns, the reduced survival of *bli-3* inactivation is reversed, suggesting that ASH-2 is indeed protective under this unfavorable for survival condition. Moreover, I could test the inactivation of other components of COMPASS including SET-2 and WDR5.1, in order study the role of this complex in host's defense mechanisms.

It is common knowledge that ROS are produced during infection, as part of host's immune system (Chávez et al., 2009). Previous studies have described several secreted factors such as proteins, toxins, enzymes and their secretion systems as the main regulators of *P. aeruginosa's* virulence (Azam & Khan, 2019). However, there are no reports on the impact of these factors on the function of BLI-3, ROS production and/or changes in the host's redox state (oxidation and/or reduction). For future studies, it would be interesting to identify such bacterial factors (i.e., through targeted genetic deletions or overexpression in *Pseudomonas*).

Another future direction of this line of research would be the search for redox-sensitive targets in the host which respond to infection and may consist part of the immune defense system. The range and complexity of host–bacteria dynamics requires systems-level approaches. The key here lies to "omics" studies that have already provided insights into the multiplicity of associations between members of a microbiota and host systems (Khan et al., 2019) Taking advantage of recent advances

in redox proteomics techniques (Knoke & Leichert, 2023), the goal would be to map oxidative post-translational modifications (OxiPTMs) and identify proteins that respond to ROS production induced by opportunistic and/or pathogenic bacteria. Combined with transcriptomics to identify subsequent gene expression changes, this unbiased approach will identify at the host-pathogen interface and at whole organism level redox-targeted biological processes that trigger gene expression changes to regulate host survival. Ultimately, this line of research will decipher, as I believe, the critical role of ROS signaling in host immune responses and identify conserved ROS-regulated targets with prophylactic and/or therapeutic potential during bacterial infections.

Supplementary data

Table 1: Statistical analysis for survival curves

Strain	Biological replicate #	Condition	Median survival	*p* value (Logrank, Mantel-Cox)	Significance	Figure in Text
N2	1	WT	6	*p* <0.0001	**** (vs MG1655)	Fig. 6a
N2	2	WT	5	*p* <0.0001	**** (vs MG1655)	Fig. 6a
N2	1	PFA	16	0.2903	ns (vs MG1655 PFA)	Fig. 6b
N2	1	2 mM PQ	6	*p* <0.0001	****(vs PA14 untreated)	Fig. 7a
N2	1	3 mM PQ	5	0.0392	*(vs PA14 untreated	Fig. 7a
N2	1	8 mM NAC	5	0.0168	*(vs PA14 untreated)	Fig. 7a
N2	1	1 mM PQ	5	0.3524	Ns (vs PA14 untreated)	Fig. 7b
N2	1	5 mM NAC	6	0.0047	** (vs PA14 untreated)	Fig. 7b
N2	1	*bli-3*	4	*Gehan-Breslow-Wilcoxon test 0.0038	** (vs PA14 untreated)	Fig. 12
N2	2	*bli-3*	5	0.4853	ns (vs PA14 untreated)	Fig. 12
N2	1	*ash-2*	5	0.1375	ns (vs PA14 untreated)	Fig. 13
N2	2	*ash-2*	4	0.0073	** (vs PA14 untreated)	Fig. 13

Table 2: Statistical analysis for survival curves

Strain	**Biological replicate #**	**Condition**	***p* value Unpaired *t* test**	**Significance**	**Figure in Text**
PB020	1	WT	0.0014	** (vs MG1655)	Fig. 9
PB020	2	WT	0.0373	* (vs MG1655)	Fig. 9
PB020	1	2 mM PQ	p <0.0001	**** (vs PA14 untreated)	Fig.10ab
PB020	1	8 mM NAC	0.2103	ns (vs PA14 untreated)	Fig. 10cd
AY101	1	2 mM PQ	0.0957	ns (vs PA14 untreated)	Fig. 11
AY101	2	2 mM PQ	0.9647	ns (vs PA14 untreated)	Fig. 11
AY101	1	8 mM NAC	0.0074	** (vs PA14 untreated)	Fig. 11

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