MODULATION OF PHARYNGEAL HEALTH IN BACTERIAL DIET-DEPENDENT SURVIVAL

by

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CHAPTER 1: INTRODUCTION

We interact and live together with various bacteria that affect our physiology and survival. Bacteria can colonize a variety of tissues in our bodies and exert diverse effects that can be harmful or beneficial (Hill and Round, 2021). Some bacteria infect certain tissues or open wounds and cause harm rapidly, whereas others colonize tissues and modulate an animal’s behavior by signaling from within the host (Hill-Yardin et al., 2021; Masuzzo et al., 2020). For an animal, the modulation of its response to the diverse bacteria that it encounters is essential for a healthy lifespan and the continuation of its species.

The animal’s nervous system senses, processes, and reacts to external and internal cues. It is only natural that it is a crucial element in the animal's interactions with different bacteria. Considering the complex nature of both the human nervous system and the potential bacterial cues, it is important to use a simpler model to elucidate host-microbe relationships. The roundworm *C. elegans*, with its naturally diverse bacterial food sources (Dirksen et al., 2016) and simple nervous system (White et al., 1986) that is capable of complex behaviors (Bargmann, 2012), has become an important model for studying host-microbe interactions (Kim and Flavell, 2020; Marsh and May, 2012; Zhang and Hou, 2013). Additionally, its bacterial food sources serve as a means to test the effects of food levels and/or food composition on the host-microbe relationship. Indeed, different bacterial food sources have distinct effects on physiology and survival, presumably by changing certain gene activities (Xiao et al., 2015; Yen and Curran, 2016), which can also be affected by host-microbe interactions.

Animals adapt to their environments and develop different strategies for different conditions and threats. Modulation of its nervous system gives the animal great flexibility in coping with changing conditions (Alcedo and Prahlad, 2020). Considering the very diverse nature of bacterial cues and their effects, neuromodulation is a great strategy for an animal to adapt to these cues. *C. elegans*, with its bacterial interactions and easy to
study nervous system, can be a great instrument in elucidating such strategies and the underlying molecular mechanisms.

**Brief history of *C. elegans* as a model organism**

Today, *C. elegans* is used widely as a model organism for research in many areas of biology, like neurology, aging, metabolism, immunity, stress, and evolution (Corsi et al., 2015). Our knowledge of the existence of *C. elegans* goes back to 1897, when it was described by French zoologist Emile Maupas (Nigon and Felix, 2017). In the following decades, several of its biological traits, like chromosomal content and sex determination, growth, genetics of heat resistance, were investigated by researchers, like Hikokura Honda, Victor Nigon, Ellsworth Dougherty, Helene Fatt (Nigon and Felix, 2017). Its rise as a widely accepted genetic model came in 1960s and 1970s, pioneered by Sydney Brenner (Brenner, 1974), who gained a Nobel Prize, alongside Robert Horvitz and John Sulston, for the discovery of the genetic regulation of organ development and programmed cell death (Sulston and Horvitz, 1977). In the following years, research using *C. elegans* pioneered a lot of fields in biology, leading to important biological concepts, such as RNA interference, the green fluorescent protein as a live imaging tool, microRNAs in gene regulation, and the insulin pathway’s role in lifespan (Chalfie et al., 1994; Corsi et al., 2015; Fire et al., 1998; Kenyon et al., 1993; Lee et al., 1993).

*C. elegans* is small, transparent, and easily cultured, which all were parts of the reason why Sydney Brenner picked it as a potential genetic model (Brenner, 1974). *C. elegans* has a fast life cycle with four larval stages and an alternative diapause stage called dauer, to which the animal enters under unfavorable environmental conditions (Figure 1.1). When food availability and population density become favorable again, the animal exits dauer stage and becomes a reproductive adult. *C. elegans* also has a short lifespan, which is a favorable trait when studying longevity.
Figure 1.1. Life cycle of C. elegans. [Image taken with permission from: (Wolkow and Hall, 2015)]

Bacterial diet of C. elegans and its effects on physiology

Diet affects a variety of physiological processes. Some of these effects can be acute, like short-term changes in blood sugar levels or various allergic reactions. Other effects may need longer times to manifest themselves. For example, consuming omega-3 fatty acids help with development of the brain and its neuroprotection (Zhang et al., 2011), or consuming high levels of saturated fatty acids promote dementia in people with certain genetic backgrounds (Kivipelto et al., 2008).

C. elegans eat bacteria, where their diet of diverse bacteria results in a rich microbiome (Dirksen et al., 2016). Much like different macro- and micronutrients in the human diet, there are beneficial and harmful bacteria in the C. elegans diet (Samuel et al.,
However, under laboratory conditions, the standard *C. elegans* diet is primarily *E. coli* OP50. The choice of *E. coli* OP50 as the primary food source was done mostly for practical reasons. OP50 is a uracil auxotroph, which grows slowly on the experimental agar plates to provide clear images and better handling of the worm (Brenner, 1974).

Bacteria other than OP50 have varying levels of benefit to *C. elegans* and provide answers to different scientific questions. For example, the use of non-pathogenic *Comamonas* DA1877 strain uncovered diet-dependent effects on *C. elegans* developmental rate, reproduction, and lifespan (MacNeil et al., 2013). On the other hand, using pathogenic bacteria, like *P. aeruginosa* and *S. marcescens*, which are also pathogenic to humans, helped us elucidate an inducible innate immune system and identify novel genes that regulate it (Mallo et al., 2002; Tan et al., 1999). *C. elegans* lacks a classical adaptive immune system that utilizes B cells and T cells, but its inducible innate immune system involves many conserved pathways, like p38 MAPK, insulin signaling, TGF-β, the unfolded protein response (UPR), and autophagy pathways (Martineau et al., 2021). *C. elegans* has also shown that these pathways have tissue-specific effects when they regulate innate immune responses.

OP50 as a beneficial food source to *C. elegans* has been a topic of debate. It was discovered that on rich Brain-Heart Infusion (BHI) medium, OP50 has increased pathogenicity to *C. elegans* compared to OP50 on regular nematode growth medium (NGM; (Garsin et al., 2001)). Recently, OP50 has also been found to be capable of colonizing the *C. elegans* pharynx, which leads to the death of the animal (Podshivalova et al., 2017; Zhao et al., 2017). This colonizing effect of OP50 is manifested only in a subpopulation of *C. elegans*. However, when colonization happens, the pharynges of these animals become swollen and a quick death follows.

Insulin/IGF signaling has emerged as an important regulator of this effect. A reduced *daf-2*/insulin receptor function increases the animal’s chance of avoiding
pharyngeal colonization and the resulting death (Podshivalova et al., 2017; Zhao et al., 2019). In addition, the effects of daf-2 depend on the pharyngeal-expressed isoforms of its downstream effector, daf-16/FOXO (Zhao et al., 2021).

DAF-16/FOXO also partly mediates the sensory influence on lifespan (Alcedo and Kenyon, 2004; Apfeld and Kenyon, 1999), which in turn depends on bacterial food type (Maier et al., 2010). Because the sensory system also regulates C. elegans avoidance from and resistance to pathogenic bacteria (Liu and Sun, 2021; Styer et al., 2008; Sun et al., 2011; Zhang et al., 2005), these data show the importance of the nervous system in the animal's responses to a variety of bacteria.

**C. elegans nervous system**

A hermaphrodite C. elegans nervous system has 302 neurons, accounting for almost one third of its 959 somatic cells (Hobert, 2005). Additionally, there are 56 glial cells that support these neurons (Hobert, 2005). The total number of nervous and glial cells goes up to 483 in C. elegans males, which have 1164 somatic cells (Emmons, 2005). These neurons belong to sensory, motor, interneuron, and polymodal categories and regulate all important physiological processes (Hobert, 2005; White et al., 1986). Sensory neurons specialize in receiving different cues, like chemicals or temperature, to allow the worm to move towards favorable environments or avoid hazards (Bargmann, 2006). Motor neurons relay signals to muscle cells and enable voluntary and involuntary motor functions (Desai et al., 1988; Liu et al., 2020). Interneurons connect other neurons to each other and integrate multiple signals from different cues (Guillermin et al., 2017; Hobert et al., 1997). Polymodal neurons are capable of doing multiples of the above functions (White et al., 1986).

Neurons employ a wide array of molecules to transmit signals that are essential for their functions. Some of these neurotransmitters are considered classical, like acetylcholine (ACh), γ-aminobutyric acid (GABA), monoamines such as serotonin (5-HT),
dopamine (DA), epinephrine, noradrenaline (NE), and histamine (Brownlee and Fairweather, 1999; Squire et al., 2003). *C. elegans* has acetylcholine as its most abundant neurotransmitter (Pereira et al., 2015) and interestingly lacks three monoamine neurotransmitters: epinephrine, NE, and histamine (Chase and Koelle, 2007). Instead of epinephrine and NE, *C. elegans* has the octopamine and tyramine (Chase and Koelle, 2007), which only exist in trace amounts in humans (Squire et al., 2003). This variety of neurotransmitters provide a wide range of sensory and behavioral capabilities, and monoamines can further extend this range through neuromodulatory mechanisms and improve the animal’s capacity to survive (Alcedo and Prahlad, 2020).

However, classical neurotransmitters have certain limitations that make neurons employ other types of molecules to communicate. These neurotransmitters usually transmit signals to neighboring cells and are secreted specifically from axonal synapses (Squire et al., 2003). Based on these two traits, signaling would follow the synaptic wiring network of an animal, which is sufficient for a lot of physiological processes. However, it is also important to be able to transmit neuronal signals over long distances and to have the flexibility of secreting transmitters from multiple parts of a cell. Neuropeptides provide these two capabilities; *C. elegans*, with its wide array of more than 250 neuropeptides (Li and Kim, 2008), is an excellent model to study them.

**Neuropeptides**

Neuropeptides are small peptides that are secreted from neurons and can transmit information over long distances (Squire et al., 2003). Neuropeptides bind to G protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), and ion channels (Li and Kim, 2008; Osmakov et al., 2019). They have important roles in communication between neurons (Squire et al., 2003). In *C. elegans*, these roles include immunity, feeding, reproduction, development, and sleep (Li and Kim, 2008; Lotti et al., 2014; Mieda and Yanagisawa, 2002).
The three classes of *C. elegans* neuropeptides are insulin-like peptides (ILPs), FMRFamide (Phe-Met-Arg-Phe-amide)-related peptides (FLPs), and neuropeptide-like peptides (NLPs) that are neither ILPs nor FLPs (Li and Kim, 2008). The three classes of neuropeptides share certain characteristics in how they are processed to become active peptides before they are secreted. After translation, the pre-propeptide is processed by a series of enzymatic cleavages and converted into the bioactive peptide (Figure 1.2). First, Figure 1.2. An example of how neuropeptides are processed. Different steps of cleavages and modifications using FLP-1 as an example are shown. [Image taken with permission from: (Li and Kim, 2008)].

a signal peptidase cleaves off the signal peptide from the pre-propeptide in the endoplasmic reticulum (ER), which results in the formation of the propeptide (Strand, 2003). Then, prohormone convertases and carboxypeptidases further cleave the
propeptide in the ER, the Golgi, and dense core vesicles [DCVs; (Jacob and Kaplan, 2003; Lindberg et al., 1998; Strand, 2003; Thacker and Rose, 2000)]. DCVs are derived from the trans-Golgi network and are analogous to synaptic vesicles that store and release classical neurotransmitters (Sieburth et al., 2007). DCVs are, however, bigger in size (Sieburth et al., 2007). Another difference between synaptic vesicles and DCVs is the spatial requirement for exocytosis. Synaptic vesicle exocytosis is restricted to the axonal synaptic zone, but DCVs are more widely distributed at the nerve terminal (Salio et al., 2006; Sieburth et al., 2007). Their exocytosis and the subsequent secretion of the neuropeptides are also not restricted to the synaptic zone (Salio et al., 2006; Sieburth et al., 2007). Finally, post-translational modifications are common in the processing of the neuropeptides and can provide protein stability and bioactivity (Kolhekar et al., 1997; Li and Kim, 2008; Schinkmann and Li, 1992). A common post-translational modification for NLPs and FLPs, but not for ILPs, is the amidation of their C-terminal glycine residue (Li and Kim, 2008).

As expected from the more than 250 peptides produced from 113 genes that belong to three distinct classes, *C. elegans* neuropeptides contribute to a wide range of biological processes. For example, the 40 ILP genes regulate developmental plasticity, reproduction, immunity, longevity, thermotolerance, and learning (Artan et al., 2016; Chen et al., 2013; Cornils et al., 2011; Duret et al., 1998; Fernandes de Abreu et al., 2014; Kawano et al., 2000; Wu et al., 2019). The 31 FLP genes also have roles in thermosensation, longevity, locomotion, egg laying, metabolism, avoidance, and stress responses (Chang et al., 2015; Chen et al., 2016; Nelson et al., 2014; Styer et al., 2008). On the other hand, NLPs are very diverse and are encoded by 42 genes. As expected, they also have many roles in regulating lifespan, survival, learning, immunity, feeding, sleep, and chemosensation (Chalasani et al., 2010; Cheong et al., 2015; Maier et al., 2010; Pujol et al., 2008b; Van der Auwera et al., 2020; Watteyne et al., 2020).
Despite all of their common features, the three classes of neuropeptides also have differences. Unlike NLPs and FLPs, ILPs do not get amidated at their C-terminus (Kirsz and Zieba, 2012). Structurally, ILPs and FLPs have distinctive features (Duret et al., 1998; Li et al., 1999), and NLPs are very diverse even within themselves (Nathoo et al., 2001). Another important difference is in the types of receptors to which they bind.

**Neuropeptide receptors**

Like their neuropeptide ligands, *C. elegans* neuropeptide receptors belong to different classes. ILPs are thought to bind to the insulin/insulin-like growth factor receptor ortholog DAF-2, an RTK (Hua et al., 2003)]. NLPs and FLPs typically bind to GPCRs; however, it is possible for some FLPs to bind ion channels (Bowman et al., 2002). GPCRs are seven-pass membrane receptors and their downstream signals are transduced through cAMP, the phosphatidylinositolues, or Rho GTPases (Figure 1.3). When a ligand

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**Figure 1.3. Canonical GPCR signaling.** Different Gα subunits can activate different downstream signaling cascades. [Image taken with permission from (Frooninckx et al., 2012)].
binds to a GPCR, this causes a conformational change in the receptor (Bastiani and Mendel, 2006). This change turns on the receptor’s guanine nucleotide exchange factor (GEF) activity, which in turn activates the receptor’s associated heterotrimeric Gαβγ-protein (Bastiani and Mendel, 2006). The GEF activity replaces the guanosine diphosphate (GDP) bound to the α subunit of the G protein heterotrimer with a guanosine triphosphate [GTP; (Bastiani and Mendel, 2006)]. The α subunit of the G protein then dissociates from the inhibitory β and γ subunits and is free to bind to downstream targets (Bastiani and Mendel, 2006). The β and γ subunits can also act through ion channels and the phospholipase C β [PLCβ; (Frooninckx et al., 2012; Smrcka et al., 2008)].

The different bacteria that C. elegans encounters in its habitat can be harmful. The animal uses neuropeptide receptors, in association with sensory neurons, as important tools to sense and respond to these bacteria (Bargmann, 2006; Liu and Sun, 2021; Styer et al., 2008; Zhang et al., 2005). For example, the octopamine receptor-1 (octr-1) gene, which encodes a protein orthologous to the human adrenergic receptors, acts in the sensory neuron ASH to negatively regulate innate immune reactions (Cao et al., 2017). In this case, the downstream effectors of OCTR-1 are the unfolded protein response (UPR) proteins (Cao et al., 2017).

Another important neuropeptide receptor that responds to bacterial pathogenicity is the C. elegans neuropeptide Y ortholog, NPR-1 (Neuropeptide Receptor-1). NPR-1 modulates both avoidance and innate immunity responses against pathogenic bacteria by suppressing the function of sensory neurons AQR, PQR, and URX (Aballay, 2009; Reddy et al., 2009; Styer et al., 2008).

The C. elegans neuromedin U receptor-1 (nmur-1) gene also encodes a conserved neuropeptide receptor that has been shown to recognize specific environmental cues (Maier et al., 2010; Watteyne et al., 2020). The ligand of its vertebrate ortholog, neuromedin U peptide, was first isolated from the pig spinal cord and named after its ability
to stimulate the uterus (Minamino et al., 1985). Together with their receptors, this peptide ligand and its homologs in other mammals are known to regulate stress responses, feeding behavior, inflammation, energy homeostasis, tumorigenesis, cell motility and adhesion signaling (Hanada et al., 2001; Iwai et al., 2008; Jethwa et al., 2005; Kirsz and Zieba, 2012; Lin et al., 2015; Lin et al., 2016; Moriyama et al., 2005). Humans possess two NMUR genes, NMUR1 and NMUR2 (Howard et al., 2000). Human NMUR1 is expressed primarily in the peripheral nervous system, whereas human NMUR2, which is slightly more closely related to *C. elegans* *nmur-1*, is expressed mostly in the central nervous system (Howard et al., 2000). The specific functions of these two human NMURs also differ, where NMUR2 affects feeding and energy homeostasis and NMUR1 regulates inflammatory responses (Howard et al., 2000; Ye et al., 2021).

In *C. elegans*, *nmur-1* is expressed in sensory neurons, motor neurons, and interneurons (Maier et al., 2010; Watteyne et al., 2020). *nmur-1* has also been shown to regulate worm lifespan in a food type-dependent manner (Maier et al., 2010). Recently, *nmur-1* in the sensory neuron AFD has been found to promote the retrieval of learned salt avoidance behavior (Watteyne et al., 2020). The *capa-1* gene that encodes the *C. elegans* NMU peptide orthologs acts with *nmur-1* from the sensory neuron ASG to promote this behavior (Watteyne et al., 2020).

There is some evidence to indicate that NMUR signaling might affect the insulin/IGF signaling pathway. Its food type-dependent regulation of lifespan is at least partly dependent on the *daf-16/FOXO* function (Maier et al., 2010). In addition, fly and mammalian NMU has been shown to suppress insulin secretion in certain systems (Alfa et al., 2015; Kuhre et al., 2019; Zhang et al., 2017; Zhang et al., 2020). This makes neuromedin U signaling the first candidate pathway for a “decretin” system, as opposed to an “incretin” system that stimulates insulin secretion (Alfa et al., 2015; Kuhre et al., 2019; Zhang et al., 2017; Zhang et al., 2020).
Insulin signaling

The first discovered and most widely known function of insulin signaling is to regulate the glucose levels in the blood (Banting et al., 1922). The hormone insulin increases the uptake of blood glucose into cells, which then can be used or stored as an energy source, a process that is essential for energy homeostasis in the body (Banting et al., 1922; Vogt and Bruning, 2013). Insulin signaling is highly conserved across species and has other crucial roles: it can regulate longevity, reproduction, development, and body weight and act as a messenger of energy levels (Belgardt and Bruning, 2010; Bruning et al., 2000; Das and Arur, 2017; Kenyon et al., 1993; Kloting and Bluher, 2005; Luo et al., 2010; Vogt and Bruning, 2013).

The *C. elegans* daf-16/FOXO gene is the primary downstream effector of the animal’s insulin receptor ortholog, DAF-2. When DAF-2 activity is reduced, a signaling cascade (Figure 1.4) is activated that ultimately translocates DAF-16 into the nucleus, where it regulates the activities of numerous genes that affect lifespan, development,

![Figure 1.4. Canonical insulin signaling.](image)

**Figure 1.4. Canonical insulin signaling.** Mammals and *C. elegans* have multiple insulin/insulin-like peptide ligands (Allen et al., 2015) that can bind and regulate the activity of a receptor tyrosine kinase and its downstream signaling cascade. For additional details, see text.
reproduction, stress resistance, fat metabolism, and pathogen response (Lee et al., 2003; Murphy et al., 2003; Tulet, 2015). Upon activation by its ligand, DAF-2 then activates phosphoinositide-3 (PI3) kinase AGE-1 (Dorman et al., 1995). In turn, AGE-1 activates the 3-phosphoinositide-dependent protein kinase PDK-1 (Paradis and Ruvkun, 1998). PDK-1 recruits protein kinase B orthologs AKT-1 and AKT-2, which phosphorylate DAF-16/FOXO (Paradis and Ruvkun, 1998). Phosphorylated DAF-16 is sequestered to the cytoplasm where it is inactive; thus, PDK-1 action on AKT-1 and AKT-2 leads to DAF-16 translocation into the nucleus (Lee et al., 2001).

A loss-of-function mutation in daf-2 can double the lifespan of C. elegans (Kenyon et al., 1993). Indeed, insulin signaling is a regulator of longevity in diverse species (Bluher et al., 2003; Hwangbo et al., 2004). However, it remains unclear how much of the insulin signaling-dependent lifespan extension is due to a slowing of aging and how much is due to an increase in stress resistance. For example, daf-2 affects both the longevity and immune response of C. elegans (Evans et al., 2008; Garsin et al., 2003; Podshivalova et al., 2017). Like its positive effect on longevity, a reduction-of-function mutation in daf-2 also improves the animal’s survival on various pathogenic bacteria, including the human pathogens P. aeruginosa, E. faecalis, and S. aureus (Garsin et al., 2003). The survival-promoting effect of daf-2 reduction-of-function mutations also applies to the animals’ responses to the pharyngeal colonization of C. elegans by OP50, an effect that may be considered as age-dependent pathogenicity (Podshivalova et al., 2017; Zhao et al., 2021). However, the effects of daf-2 on longevity and immunity can be uncoupled. Compared to wild type, the daf-2 mutation has been shown to increase longevity much more on bacteria that are more pathogenic (Garsin et al., 2003). This extension is completely daf-16-dependent in at least some of the bacteria tested (Garsin et al., 2003). This surely suggests that immune function plays an important role in insulin/IGF-dependent survival,
although the rate of lifespan increase in *daf-2* mutants on different pathogenic bacteria is variable (Garsin et al., 2003). On the other hand, *daf-2* mutants are still long-lived on non-pathogenic or dead bacteria (Garsin et al., 2003; Podshivalova et al., 2017). We also know that non-bacterial mammalian diet affects insulin/IGF signaling (Collier and O'Dea, 1983; Elrick et al., 1964). Taken together, this indicates that insulin/IGF signaling is not only a major determinant of survival in response to complex bacteria-derived cues, but also modulates diet-dependent survival.

Considering how crucial insulin signaling is in responses to complex and variable cues, it is to be expected that its activity will also be modulated. The DAF-2/DAF-16 signaling cascade can be inhibited by the PTEN DAF-18 through dephosphorylation of the phosphatidylinositol 3,4,5-trisphosphate (PIP₃), which consequently prevents the activation of the AKT proteins (Ogg and Ruvkun, 1998). Many aspects of insulin signaling, including the cells from which they function, are also likely subject to some forms of modulation.

**Neuromodulation**

Neuromodulation is the change in a neuron’s response to certain stimuli and is established by the neuron’s extracellular and intracellular environments in the presence of said stimuli (Bargmann, 2012). Neuromodulation can change the neuron’s excitability or synaptic nature (Bargmann, 2012), which will make the neuron more adaptable to a greater range of environmental cues and conditions (Alcedo and Prahlad, 2020). This adaptability would result in the better maintenance of homeostasis, and thus, improved survival and fitness (Alcedo and Prahlad, 2020).

Neuromodulators consist of different classes of molecules. In *C. elegans*, the monoamine neurotransmitters and neuropeptides can both function as neuromodulators with short- and long-range modulating capabilities that can be quite complex [reviewed by (Alcedo and Prahlad, 2020)]. A single neuromodulator can produce opposing responses
to a specific cue [reviewed by (Alcedo and Prahlad, 2020)]. However, the mechanisms that underlie this process remain unclear.

**Filamins: actin-binding protein scaffolds as potential modulators**

What other molecules can modulate the effects of DAF-2 and DAF-16 on longevity? One candidate is FILAMIN-2 (FLN-2), whose expression might be regulated by DAF-16 (Amrit et al., 2016). Interestingly, human FILAMIN has been shown to interact with the insulin receptor (He et al., 2003). *C. elegans* FLN-2 is proposed to act as a scaffold and alter the actin cytoskeleton (DeMaso et al., 2011). In mammals, filamins (i) interact with many different proteins through their filamin-type immunoglobulin-like repeat domains, (ii) crosslink actin filaments to form the cytoskeleton, (iii) anchor transmembrane proteins to the actin cytoskeleton, and (iv) translocate to the nucleus upon proteolytic cleavage (Popowicz et al., 2006). Filamins have roles in many cellular and molecular processes that include cell migration and adhesion [reviewed in (Nakamura et al., 2011)], immune responses (Hayashi and Altman, 2006; Muscolini et al., 2011; Tavano et al., 2006), and endothelial and viral barrier function (Griffiths et al., 2011; Malathi et al., 2014). In *C. elegans*, transcriptional or translational reporters show that *fln-2* is expressed in the pharynx, intestine, hypodermis, and gonad (DeMaso et al., 2011), where it can act as a scaffold that interact with multiple proteins and affect worm physiology in many ways.

**Scope of the thesis**

To ensure its survival, an animal must modulate its physiology in response to environmental cues, which can sometimes be detrimental to its health. One such environmental cue is the animal’s diet. *E. coli* OP50, a standard bacterial food source of *C. elegans* in the laboratory, has been shown to infect the animal’s pharynx with age and shorten its lifespan (Zhao et al., 2017). Since different bacterial diets have different effects on *C. elegans* lifespan (Maier et al., 2010; Soukas et al., 2009), I wanted to know if there are diets that prevent the infection of the animal’s pharynx. In Chapter 3, I show this to be
the case and find that the dietary influence on pharyngeal health depends on bacterial LPS structures. To understand why some diets suppress pharyngeal health, whereas others do not, I explored mechanisms through which *C. elegans* responds only to certain bacteria.

In Chapters 3 and 4, I show that the neuropeptide neuromedin U receptor NMUR-1 (Maier et al., 2010) and the potential actin-binding scaffold protein FILAMIN-2 [FLN-2; (DeMaso et al., 2011)] modulate *C. elegans* responses to *E. coli* OP50. While these two genes have little or no effect when *C. elegans* is on other bacteria. NMUR-1 and FLN-2 have opposing effects on worm pharyngeal health and longevity on OP50. In Chapter 3, I also discuss my surprising finding that NMUR-1 itself has two opposing effects on bacterial type-dependent pharyngeal infections, which depend on insulin signaling levels. This finding identifies NMUR-1, which is neuronally expressed, as a modulator of insulin signaling. In Chapter 4, I further show that the effect of FLN-2 on *C. elegans* longevity only partly involves pharyngeal infections and partly depends on insulin signaling. The dependence of FLN-2 on multiple factors to regulate worm longevity is consistent with its proposed scaffold function. Thus, pharyngeal health and its consequent effects on longevity are determined by insulin signaling (Podshivalova et al., 2017; Zhao et al., 2021), which in turn is modulated by a neuropeptide receptor and a scaffold protein (this work).

Both the *C. elegans* neuropeptide receptor NMUR-1 and the scaffold protein FLN-2 have orthologs in mammals (Howard et al., 2000; Popowicz et al., 2006). My findings suggest potential modulators of mammalian insulin signaling, which is also an important regulator of mammalian longevity (Kenyon, 2010). These modulators provide mechanisms through which insulin signaling can be adjusted as the animal adapts to its environment for its survival.
CHAPTER 2: MATERIALS AND METHODS

C. elegans strains and growth conditions

All C. elegans mutant strains used in this study were backcrossed at least three times to wild type. Mutant strains that were used in the survival assays and the pumping rate assay are reported in Tables S4.1 and S4.2 with their genotypes. The actin reporter strain NK1069 [qyIs198[inft-1p::moeABD::mCherry, unc-119(+); unc-119(ed4)] was a kind gift from Erin Cram (Wirshing and Cram, 2017). It was crossed to fln-2(ok1305ot611) mutants to create fln-2(ok1305ot611); qyIs198[inft-1p::moeABD::mCherry, unc-119(+)] and reported in Chapter 4 as Moesin::mCherry. All experiments were carried out at 25°C. All worms were grown at least two generations in the experimental temperature and bacterial food-source, unless the experiment involved an animal carrying the daf-2(e1368) mutation. For these latter experiments, control worms and daf-2 mutants were grown at the permissive temperature, 20°C, before they were shifted to the 25°C non-permissive temperature at the last larval (L4) stage to prevent dauer entry (Kenyon et al., 1993).

Bacterial strains and growth conditions

The bacterial strains that were used in the study are E. coli OP50, E. coli CS180, E. coli CS2198, and E. coli CS2429 [see (Maier et al., 2010); and references therein]. Bacterial strains were grown from single colonies in Luria-Bertani media at 37°C until the log-phase. For the experimental assays, 6-cm Nematode-Growth (NG) agar plates (Brenner, 1974) were seeded with approximately 250 μl of bacteria and streaked to cover the entire plate (full-lawn bacterial plates). I used full-lawn plates to prevent the confounding factor of worms avoiding the bacterial lawns during the lifespan assays (Reddy et al., 2009). Plates were incubated at 25°C overnight before they were used for any experiment.
Breaking the linkage between \textit{nmur-1(ok1387)} and \textit{fln-2(ot611)}

To break the linkage between \textit{nmur-1(ok1387)} and \textit{fln-2(ot611)} on chromosome X of the QZ58 \textit{C. elegans} strain, QZ58 was crossed to wild type. Among the subsequent progeny of the \textit{nmur-1 fln-2/+} + cross-progeny, we identified 2 recombination events out of 206 chromosomes: one progeny was homozygous for the \textit{fln-2(ot611)} mutation and heterozygous for \textit{nmur-1(ok1387)}; another animal was homozygous for \textit{nmur-1(ok1387)}, but not for \textit{fln-2(ot611)}. These animals were allowed to reproduce to isolate the \textit{nmur-1} single mutant and the \textit{fln-2} single mutant. The mutations were detected by PCR.

The \textit{ok1387} deletion was detected by using the primers: \textit{ok1387} fw (5’-ATA AGT GTC ATA GAT ACA GG-3’); \textit{ok1387} rv (5’-AAT ACA TAT ACT GAT TGA CC-3’); and \textit{ok1387} int rv (5’-AAT GCT ATG GCA GAG AAG TG-3’). The mutant was detected as a 441-bp band, whereas wild type was detected as a 602-bp band.

The \textit{ot611} point mutation was detected by using a forward primer whose 3’ end is complementary to the A point mutation and generates a 253-bp band with the \textit{ot611} reverse primer, 5’-CCT GTC ACA TGA GCA CTA ATG TC-3’. The wild-type allele of \textit{fln-2} was detected by using a forward primer whose 3’ end is complementary to C and generates a 253-bp band with the \textit{ot611} reverse primer. The presence or absence of the wild-type and \textit{ot611} alleles were further confirmed by sequencing. We used the \textit{ot611} F primer, 5’-GTC ACT ATA ATA GAC GCC GTA ATG TC-3’, and the \textit{ot611} reverse primer to generate a 536-bp fragment that was sequenced to determine whether position 301 of the fragment is a C or an A.

Lifespan assays

Worms were picked for all experiments at the late L4 stage and were transferred daily for the first 6 days of adulthood to prevent the mixing of subjects with their progeny. The details of the censoring during experiments are explained in the legends of Tables S4.1 and S4.2. Kaplan-Meier estimates were done using the JMP 8.0.1 software (SAS).
P values of both Wilcoxon and Log-rank tests are reported in Tables S4.1 and S4.2. Only the Wilcoxon test is reported in all figures, since it is the better measure of statistical significance when hazard ratios are not constant throughout an assay (Maier et al., 2010). For example, in most of our survival comparisons, one group showed more earlier deaths than the other (Maier et al., 2010).

For the survival assays that include the TGFβ *dbl-1* mutants, worms were initially treated with feeding-induced RNA-mediated interference (RNAi) against *egg-5* and its paralog *egg-4* (Parry et al., 2009) to prevent the high rate of matricide associated with the *dbl-1* mutation (de Lucas et al., 2021). RNAi treatment of wild type, *fln-2* or *dbl-1* single mutants, and *dbl-1; fln-2* double mutants against *egg-5* and *egg-4* were carried out during the first day of adulthood, as described previously (Entchev et al., 2015), except that the culture temperature was at 25°C. Animals were then shifted back to *E. coli* OP50 on the second day of adulthood.

**Necropsy analysis to determine P-deaths versus non-P deaths**

The pharynges of all the dead animals during the course of survival assays were imaged using a Nikon Eclipse Ni-U microscope and a Photometrics Coolsnap ES2 camera at 400x magnification. The surface area of the terminal pharyngeal bulb (see Figures 3.1C and 3.1D) was measured using the Image J software (Schneider et al., 2012). The surface area of the terminal bulb was then divided by the diameter of the body of the same animal at the region of the terminal bulb, which is also known as the grinder (areaₚ/diameterₒ). This normalization addressed the possibility that the general size of the animals affected the pharyngeal surface area.

Through a principal component analysis of dead wild-type animals on OP50 (n = 387) from 8 independent survival assays, I initially separated these animals into two clusters—one with swollen pharynges (P-deaths) and one without swollen pharynges (non-P deaths). Since P-deaths happen early in the lifespan of the population (Zhao et al.,...
2017), I used areaP/diameterG and the age of death as variables. The principal component analysis was carried out in the R 4.0.0 software (R Core Team, 2020), where I plotted the data using ggplot2 (Wickham, 2009) and ggfortify (Tang et al., 2016). From this plot (Figure 2.1), I determined the threshold areaP/diameterG that would separate the two clusters. This threshold value of 27 (dashed line in Figure 2.1) was then used to categorize animals that died with significant pharyngeal swelling (P-deaths) or with no pharyngeal swelling (non-P deaths) in all experiments.

*Figure 2.1. Principal component analysis using areaP/diameterG and age at death as variables. The red color represents cluster 1, the non-P deaths, and the turquoise color represents cluster 2, the P-deaths. The dashed line indicates the threshold areaP/diameterG value of 27, which was used to categorize P-deaths versus non-P deaths.*
Measurements of pharyngeal pumping rates

The pharyngeal pumping rate of each animal was measured on a Leica M165FC stereomicroscope on the first day of adulthood at 25°C, before the animal was gravid. Pharyngeal terminal bulb pumps were counted for 30 seconds and multiplied by two to reflect pumps per minute. The pharyngeal pumping in each animal was counted 3 times and averaged. The results were analyzed with the two-factor ANOVA test using the GraphPad Prism 6.04 software.

Visualization of actin

The actin cytoskeleton was imaged using a Nikon Eclipse Ni-U microscope and Photometrics Coolsnap ES2 camera. In three sets of experiments, I visualized the actin cytoskeletons of wild-type and fln-2(ok1305ot611) mutant animals that carry qyIs198[inft-1p::moeABD::mCherry, unc-119(+)]]. The qyIs198 transgene expresses the actin-binding domains of Moesin that have been fused to mCherry (Wirshing and Cram, 2017). For these experiments, 20 wild-type and 19 fln-2(ok1305ot611) mutant animals grown on OP50 were observed on day 2 of adulthood and 15 wild-type and 15 fln-2(ok1305ot611) mutant animals on OP50 were observed on day 4 of adulthood. I also observed wild type on OP50 and wild type on CS180 on day 4 (n = 14, OP50; n = 12, CS180), day 5 (n = 16, OP50; n = 16, CS180), and 6 (n = 12, OP50; n = 12, CS180) of adulthood. For phalloidin staining, 14 one-day old wild type and 7 one-day old fln-2 mutants on OP50 were fixed and stained with Texas Red-X phalloidin, as previously reported (DeMaso et al., 2011).
CHAPTER 3: NEUROPEPTIDE RECEPTOR MODULATION OF INSULIN SIGNALING IN BACTERIA-DEPENDENT SURVIVAL

Abstract

Bacterial food sources will differentially affect the physiology and survival of the worm *C. elegans*. *C. elegans* fed two *E. coli* strains—the B type OP50 versus the K12 type CS180—display different survival phenotypes. Wild-type *C. elegans* fed OP50 have a higher rate of early deaths compared to *C. elegans* fed CS180. The early deaths on OP50 are characterized by swollen pharynges (P-deaths) that result from bacterial accumulation within the tissue. In contrast, worms fed CS180 are more resistant to P-deaths. These diet-dependent differences in P-deaths depend on the bacterial lipopolysaccharide (LPS) structures and the activities of the *C. elegans* neuropeptide neuromedin U receptor *nmur-1*. I find that *nmur-1* acts in sensory neurons to inhibit P-deaths on OP50, but not on CS180. Interestingly, however, *nmur-1* promotes the opposite response when the insulin receptor *daf-2* has reduced activity—where *nmur-1* now promotes P-deaths on OP50. Since both effects of *nmur-1* appear dependent on the FOXO *daf-16* transcription factor, I propose that *nmur-1* acts as a modulator of insulin signaling. Thus, NMUR-1 ensures that the insulin receptor DAF-2 signals at the appropriate level to promote pharyngeal health and optimal survival in response to specific bacteria.
Introduction

Since organismal longevity is subject to diet (Skorupa et al., 2008; Weindruch et al., 1986), it is not surprising that *C. elegans* grown on one type of bacterial food source will live shorter than *C. elegans* grown on another type of food source (Maier et al., 2010; Soukas et al., 2009). The mechanisms through which food types modulate longevity are going to be many and complex. In *C. elegans*, one of these mechanisms involves bacterial colonization of some tissues, like its pharynx, which shortens animal longevity (Podshivalova et al., 2017; Zhao et al., 2017). However, while at least one bacterial food type promotes this pharyngeal infection and decreases worm survival (Podshivalova et al., 2017; Zhao et al., 2017), other food types might not.

Interestingly, the food-type effects on *C. elegans* lifespan have been shown to be mediated by sensory neurons (Maier et al., 2010). Specific mutations that affect certain sensory neurons cause the worms to live long on one bacteria, but live like wild type on other bacteria (Maier et al., 2010). This indicates that a given set of longevity-influencing sensory cues will only be present in some bacterial food sources and absent in others. To understand how food-derived cues modulate survival in a sensory neuron-dependent manner, I examined how the neuropeptide neuromedin U receptor *nmur-1* affects the bacterial colonization of *C. elegans* pharynges. *nmur-1* is expressed in several sensory neurons and in subsets of interneurons and motor neurons and has been implicated in food-dependent effects on physiology (Maier et al., 2010; Watteyne et al., 2020).

Previously, *nmur-1* deletion mutants have been found to live longer than wild type when fed the B type *E. coli* OP50, but live the same as wild type when fed K-12 type *E. coli* bacteria (Maier et al., 2010). This food type-dependent phenotype can be rescued by the extrachromosomal expression of the wild-type *nmur-1* genomic locus (Maier et al., 2010). However, the strain that carries the *nmur-1* deletion mutation also carries a tightly
linked mutation in a second gene, filamin-2 (fln-2), whose wild-type function has recently been shown to promote bacterial colonization of worm pharynges (Zhao et al., 2019).

Here I show that upon breaking the linkage between the nmur-1 mutation and the fln-2 mutation, I also find that both mutations affect worm survival in a food type-dependent manner. While wild-type fln-2 promotes pharyngeal infections and shortens lifespan (Zhao et al., 2019) on OP50 (see Chapter 4), I show that wild-type nmur-1 from sensory neurons inhibits pharyngeal infections and the associated mortality on OP50. However, both genes have little to no effect on another bacterial food source, the E. coli K-12 CS180 (also see Chapter 4). Surprisingly, I further find that nmur-1 has an opposite function upon downregulation of insulin receptor daf-2, a known regulator of pharyngeal infections (Podshivalova et al., 2017). In this context, nmur-1 now increases pharyngeal infections and the corresponding deaths on OP50. Because both activities of nmur-1 depend on the insulin signaling effector daf-16/FOXO, our data suggest that nmur-1 modulates the effects of insulin signaling on pharyngeal health and survival.

**Results**

**Bacterial food type modulates swollen pharynx-dependent deaths in C. elegans**

Wild-type C. elegans fed different E. coli diets have different lifespans (Maier et al., 2010; Soukas et al., 2009). Worms grown on E. coli OP50 lived shorter than worms grown on E. coli CS180 (Figure 3.1A; Table S3.1). Worms on OP50 also had a higher rate of early deaths compared to worms on CS180 (Figure 3.1A), which might mean that there was an early hazard present for worms on OP50 and was absent on CS180. One known cause for early deaths on OP50 was swollen pharynges (P-deaths) that result from bacterial colonization of the tissue [compare Figure 3.1C to Figure 3.1D; (Podshivalova et al., 2017; Zhao et al., 2017)]. Since worms on CS180 showed slower deaths early in adulthood (Figure 3.1A; Table S3.1), I tested if the P-death phenotype contributed to
Figure 3.1. Food type modulates swollen pharynx-dependent deaths in *C. elegans*. (A) Wild-type *C. elegans* fed *E. coli* OP50 had a higher rate of early deaths compared to *C. elegans* fed *E. coli* CS180. (B) The early deaths depended on swollen pharynges (P-deaths) in worms fed OP50 compared to worms fed CS180. (C) Image of an unswollen pharynx of a dead adult worm. (D) Image of a swollen pharynx of a dead adult worm. The statistical analyses of the survival assays in this and subsequent figures are shown in Table S3.1, unless otherwise stated.
the lifespan difference between worms on the two bacterial diets. Indeed, OP50-fed worms showed a faster rate of P-deaths compared to CS180-fed worms (Figure 3.1B; Table S3.1). In contrast, worms on CS180 were more resistant to P-deaths (Figure 3.1B; Table S3.1).

**LPS structure of the dietary bacteria modulates pharynx-dependent survival**

Next, I asked what bacterial cues contributed to the difference in the P-death rates between OP50-fed and CS180-fed *C. elegans*. OP50 is derived from the *E. coli* B strain (Dusenbery et al., 1975) and CS180 is derived from *E. coli* K-12 strain (Klena et al., 1992). Thus, they at least have different LPS structures, although both lack an O-antigen [Figure 3.2; (Maier et al., 2010)]. OP50 additionally lacks certain sugar residues and an N-acetylglucosamine residue on its outer-core LPS [Figure 3.2; (Maier et al., 2010)]. Because CS180 LPS truncations (CS2198 and CS2429; Figure 3.2) have been shown to shorten wild-type worm lifespan (Maier et al., 2010), I compared the number of P-deaths on both CS2198 and CS2429 to those on CS180. While I observed no significant difference between the P-death rates on CS2198 versus CS2429, these two bacterial strains with their shorter LPS caused more P-deaths than CS180 (Figure 3.2; Table S3.2). Thus, these data show that the type of bacterial diet influences pharyngeal health and that a change in the outer core LPS structure is sufficient to promote bacterial colonization of the pharynx and death of the animal.

**nmur-1 slows down pharynx-dependent deaths in a bacteria-dependent manner**

I then asked what host genetic factors influence the food type-dependent *C. elegans* P-deaths. The *C. elegans* QZ58 strain promotes increased early survival on OP50, but not on CS180 [Figure 3.3; Table S3.1; (Maier et al., 2010)]. However, QZ58 carries mutations in two tightly linked genes that are 1 map unit apart: a deletion, *ok1387*, in the neuropeptide receptor *nmur-1* and a nonsense mutation, *ot611*, in the potential
Figure 3.2. LPS is a modulator for swollen pharynx-dependent deaths in *C. elegans*. *E. coli* B strain OP50 has a shorter LPS compared to the *E. coli* K-12 strain CS180. *E. coli* CS2198 and CS2429 are derived from CS180 and have a truncated LPS. CS2198 and CS2429 also promoted P-deaths in *C. elegans*. The red scissors indicate the truncations of CS2198 and CS2429. The statistical analyses of the P-death ratios in this figure is shown in Table S3.2.
Figure 3.3. The QZ58 strain slows down pharynx-dependent deaths in a bacteria-dependent manner. Compared to wild type (wt), the strain QZ58 promoted increased early survival on OP50, but not on CS180 (Maier et al., 2010). QZ58 contains mutations in the neuropeptide receptor nmur-1 and the scaffold protein filamin-2 (fln-2), which suggests nmur-1 and/or fln-2 as candidate modulators of P-deaths.
actin-binding scaffold protein \textit{fln-2} (Figure 3.3). To study the effects that are specific to \textit{nmur-1} or to \textit{fln-2} on food type-dependent P-death phenotype, I broke the linkage between the \textit{nmur-1} and \textit{fln-2} mutations (see Chapter 2 for methods).

Compared to wild type, the isolated \textit{nmur-1(ok1387)} single mutant had more P-deaths and a shorter lifespan on OP50, but not on CS180, which shows that \textit{nmur-1} does function in a food type-dependent manner (Figures 3.4A and 3.4B; Table S3.1). The effects of \textit{nmur-1} on worm survival also appears to depend entirely on the pharynx. \textit{nmur-1} mutants behaved like wild type when I only counted deaths that are characterized by unswollen pharynges (non-P deaths; Figure 3.1C; Table S3.1). These findings also show that the long-life phenotype of QZ58 on OP50 is not due to the \textit{nmur-1} mutation, but is caused by the \textit{fln-2} mutation (see Chapter 4). This \textit{nmur-1} deletion phenotype can be rescued by the extrachromosomal expression of wild-type \textit{nmur-1} from its own promoter (Table S3.1) and from a sensory neuron-specific promoter (\textit{osm-6p}; Figure 3.4C; Table S3.1). Together these results suggest that wild-type \textit{nmur-1} in sensory neurons inhibits P-deaths in a bacteria-dependent manner.

\textit{nmur-1} has a second function, which accelerates P-deaths under low insulin receptor activity

The wild-type insulin receptor DAF-2 promotes deaths caused by bacterial colonization and swelling of the pharynx (Podshivalova et al., 2017; Zhao et al., 2021). In fly and mammalian systems, neuromedin U signaling has been shown to influence insulin signaling by suppressing insulin secretion (Alfa et al., 2015; Kuhre et al., 2019; Zhang et al., 2017; Zhang et al., 2020). Thus, I tested whether the P-death phenotype of \textit{nmur-1} depends on \textit{daf-2} activity. Unexpectedly, the \textit{daf-2(e1368)} reduction-of-function mutation not only suppressed the P death-promoting and lifespan-shortening effects of the \textit{nmur-1} mutation, but also revealed that \textit{nmur-1} has two opposing functions. In contrast to above (Figure 3.4; Table S3.1), the \textit{nmur-1} mutation now led to fewer P-deaths and increased
Figure 3.4. Wild-type *nmur-1* slows down pharynx-dependent deaths in a bacteria-dependent manner. (A-B) The *nmur-1* mutation, *ok1387*, which was isolated after recombining away the *fln-2* mutation, led to faster P-deaths on OP50 (A), but not on CS180 (B). (C) The *nmur-1* P-death phenotype was rescued by the extrachromosomal expression of *nmur-1* in sensory neurons from the *osm-6* promoter (*osm-6p::nmur-1*). In this figure and all other figures in this chapter, *P* values indicate a comparison to wild type on the same bacteria, unless otherwise stated. The statistical comparison to a different genotype is indicated as a subscript of *P*.
Figure 3.5. *nmur-1* has a second, opposing effect on P-deaths in the *daf-2* reduction-of-function mutant background. (A) Unlike wild type (black curves), reduced *daf-2* activity (grey curves; *e1368* mutation) decreased the deaths that are due to bacterial colonization of the pharynx [see also (Podshivalova et al., 2017)]. The *nmur-1* deletion (blue curves) further reduced the P-deaths of *daf-2* mutants. (B) The *nmur-1* mutation had no effect on CS180 in the absence (red curves) or presence of the *daf-2(e1368)* mutation (blue curves).
the lifespan of *daf-2* mutants on OP50 (Figure 3.5A; Table S3.1), but not necessarily on CS180 (Figure 3.5B; Table S3.1). These data suggest that wild-type *nmur-1* adjusts insulin signaling activity as it exerts its effects on the pharynx on certain bacterial food sources, like OP50.

**The effects of *nmur-1* on pharyngeal health completely depend on the *daf-16*/FOXO transcription factor**

Because the FOXO transcription factor *daf-16* is the downstream effector for many *daf-2* functions [reviewed by (Kenyon, 2010)], I tested if *nmur-1* also depends on *daf-16* function. As previously reported (Zhao et al., 2019), I found that loss of *daf-16* increased the number of P-deaths and suppressed the effect of *daf-2* on P-deaths (Figures 3.6A and 3.6B; Table S3.1). Both *nmur-1*-dependent P-death phenotypes were also suppressed by the *daf-16* loss-of-function mutation (Figures 3.6A and 3.6B; Table S3.1). This suggests that wild-type *nmur-1* modulates P-deaths either by acting through or in parallel to insulin receptor signaling and *daf-16* (Figure 3.7).

**Discussion**

As we age, we become more vulnerable to multiple diseases and stressors (Butcher and Lord, 2004; Hirokawa et al., 1992; Schmucker et al., 2003). Aging *C. elegans* also undergoes many detrimental changes to its physiology: for example, proteomic aggregation, loss of intestinal integrity, locomotory defects, neuronal dysfunction and tumorigenesis (Herndon et al., 2002; McGee et al., 2011; Tank et al., 2011; Walther et al., 2015; Wang et al., 2018). A source for at least one of these changes is the animal’s diet, such as *E. coli* OP50, its most common food source in the laboratory. As the worm ages, OP50 starts to accumulate in its pharynx, eventually causing this tissue to swell, which kills the animal (Zhao et al., 2017). Many wild-type *C. elegans* adults are inefficient in preventing OP50-induced damage to their pharyngeal tissues at an early age (Zhao et al., 2017). Pharyngeal infections and swelling can also be induced by known pathogens, such
Figure 3.6. The effects of *nmur-1* completely depend on the *daf-16* FOXO transcription factor. (A) The *daf-16*(*mu86*) loss of function increased P-deaths more than wild type and the *nmur-1* single mutant. The *daf-16; nmur-1* double mutant behaved like *daf-16* single mutants. (B) The *daf-16*(*mu86*) mutation suppressed both the *daf-2* mutant phenotype and the *daf-2; nmur-1* phenotype.
Figure 3.7. Models for how *nmur-1* modulates pharyngeal-dependent deaths. See text for an explanation of the two models.
as *Salmonella enterica*, which additionally infects humans (Tenor and Aballay, 2008). Thus, the P-death phenotypes of *C. elegans* serve as a good model in which to elucidate age-dependent tissue degeneration and pathogenesis.

Bacterial LPS can act as an important stimulator for the host immune system (Lamping et al., 1998; Medzhitov et al., 1997; Shahin et al., 1987; Zhou et al., 1999). Here, I show that the LPS structure of the dietary bacteria also determines the pharyngeal infection rate of *C. elegans* (Figure 3.2). The LPS structure might affect bacterial adherence to the pharyngeal tissues. However, the *E. coli* strains used in this study lack an O-antigen [Figure 3.2; see (Maier et al., 2010) and references therein]. Unlike the O-antigen, the bacterial core LPS has been shown to be less adhesive, but it may regulate the expression of adherence proteins (Genevaux et al., 1999; Strauss et al., 2009; Zhang et al., 2006).

Another possibility is that the shorter LPS of CS2198, CS2429 and OP50 might be recognized by certain *C. elegans* proteins, like the Toll-like receptor TOL-1 or various C-type lectins (Pees et al., 2017; Tenor and Aballay, 2008; Utarabhand et al., 2017). For example, the N-acetylglucosamine that is absent in both CS2429 and CS2198 bacteria (Figure 3.2) serves as a ligand for a human C-type lectin (Zhang et al., 2006). Interactions between different bacterial LPS and *C. elegans* proteins might stimulate or hinder specific immune responses in the animal. Indeed, the expression of genes involved immune responses change between *C. elegans* fed OP50 and CS2429 versus CS180 (W. Maier, J. Alcedo, personal communication).

A pathway that has been implicated in regulating the animal’s immune responses is insulin signaling. When insulin signaling has been reduced or hyperactivated, this can be suboptimal for the animal. Insulin receptor *daf-2* null mutants exhibit lethality or embryonic and larval arrest (Gems et al., 1998), whereas a gain of function in *daf-2* inhibits the DAF-16 transcription factor and results in short-lived animals that are vulnerable to
various stressors (Garsin et al., 2003; Yanase et al., 2002). These studies suggest the importance of maintaining insulin signaling levels, which should not be too high or too low. Neuromodulation provides a mechanism for fine-tuning insulin signaling activity, which is affected by the animal's fluctuating environments [reviewed by (Alcedo and Prahlad, 2020)].

The NMUR-1 neuropeptide receptor can serve as a potential modulator of the insulin pathway in regulating pharyngeal health (Figure 3.7). First, *nmur-1* is expressed in neurons (Maier et al., 2010; Watteyne et al., 2020), some of which also express *daf-2* and/or its insulin-like peptide ligands (Chalasani et al., 2010; Li et al., 2014). Second, mammalian neuromedin U signaling has been reported to affect insulin secretion from pancreatic β-cells (Zhang et al., 2020). Here I show that wild-type *C. elegans* NMUR-1 promotes insulin receptor DAF-2 activity, which inhibits healthy pharynges (Figure 3.7), leading to pharyngeal infections and death (Figure 3.5). Interestingly, this NMUR-1 activity opposes another function of this protein in the presence of a wild-type *daf-2* gene: wild-type NMUR-1 also promotes healthy pharynges (Figure 3.7) and prevents infections and deaths (Figures 3.4 and 3.5). The two opposing activities of NMUR-1 is a feature of neuromodulators, where they can change their activities depending on the animal’s external and internal environments (Alcedo and Prahlad, 2020). The opposing roles of NMUR-1 in pharyngeal health and longevity might be mediated by insulin signaling-dependent and -independent mechanisms (Model 1 in Figure 3.7). However, an intriguing possibility is that NMUR-1 directly modulates insulin signaling itself (Model 2 in Figure 3.7). In this second model, NMUR-1 ensures that the insulin pathway signals appropriately, where the insulin receptor is neither hyperactive nor hypoactive in response to specific bacterial diets (Figure 3.7). Thus, this model highlights a mechanism that prevents large deviations in insulin pathway activity, which is necessary in optimizing pharyngeal health and animal survival.
<table>
<thead>
<tr>
<th>Condition: all deaths</th>
<th>Animals observed/Total animals (trials)</th>
<th>Mean ± SEM (% difference)*</th>
<th>P value of Wilcoxon*</th>
<th>P value of Log-rank*</th>
<th>Fig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type on OP50</td>
<td>188/320 (4)</td>
<td>11.94 ± 0.37</td>
<td></td>
<td></td>
<td>3.1</td>
</tr>
<tr>
<td>Wild type on CS180</td>
<td>219/320 (4)</td>
<td>13.39 ± 0.21 (+9)^OP50</td>
<td>&lt; 0.0001^OP50</td>
<td>0.568^OP50</td>
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</tr>
<tr>
<td>Condition: P-deaths (Days 1-15)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type on OP50</td>
<td>106/320 (4)</td>
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<td></td>
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<td>3.1</td>
</tr>
<tr>
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<td>&lt; 0.0001^OP50</td>
<td>&lt; 0.0001^OP50</td>
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<tr>
<td>Condition: all deaths</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type on OP50</td>
<td>40/70 (1)</td>
<td>13.12 ± 0.67</td>
<td></td>
<td></td>
<td>3.3</td>
</tr>
<tr>
<td>QZ58 on OP50</td>
<td>40/70 (1)</td>
<td>16.79 ± 0.44 (+28)^wt_OP50</td>
<td>0.0001^wt_OP50</td>
<td>0.0001^wt_OP50</td>
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</tr>
<tr>
<td>Wild type on CS180</td>
<td>47/70 (1)</td>
<td>14.75 ± 0.44</td>
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<td></td>
<td></td>
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<tr>
<td>QZ58 on CS180</td>
<td>50/70 (1)</td>
<td>15.15 ± 0.58 (+3)^wt_CS180</td>
<td>0.508^wt_CS180</td>
<td>0.508^wt_CS180</td>
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<tr>
<td>Condition: all deaths</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Wild type on OP50</td>
<td>195/380 (3)</td>
<td>10.66 ± 0.33</td>
<td></td>
<td></td>
<td>3.4</td>
</tr>
<tr>
<td>nmur-1(ok1387) on OP50</td>
<td>228/380 (3)</td>
<td>9.38 ± 0.31 (-12)^wt_OP50</td>
<td>&lt; 0.0001^wt_OP50</td>
<td>0.0015^wt_OP50</td>
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<tr>
<td>Wild type on CS180</td>
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<td></td>
<td>3.4</td>
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<tr>
<td>nmur-1(ok1387) on CS180</td>
<td>108/140 (2)</td>
<td>14.41 ± 0.32 (+6)^wt_CS180</td>
<td>0.038^wt_CS180</td>
<td>0.079^wt_CS180</td>
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<td>Condition: P-deaths (Days 1-15)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type on OP50</td>
<td>134/380 (3)</td>
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<td></td>
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<td>nmur-1(ok1387) on OP50</td>
<td>176/380 (3)</td>
<td>-</td>
<td>&lt; 0.0001^wt_OP50</td>
<td>0.0004^wt_OP50</td>
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</tr>
<tr>
<td>Wild type on CS180</td>
<td>15/160 (2)</td>
<td>-</td>
<td></td>
<td></td>
<td>3.4</td>
</tr>
<tr>
<td>nmur-1(ok1387) on CS180</td>
<td>11/140 (2)</td>
<td>-</td>
<td>0.277^wt_CS180</td>
<td>0.283^wt_CS180</td>
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</table>
Table S3.1 cont.

<table>
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<tr>
<th>Condition: non-P deaths</th>
<th>Animals observed/Total animals (trials)</th>
<th>Mean ± SEM (% difference)*</th>
<th>P value of Wilcoxon*</th>
<th>P value of Log-rank*</th>
<th>Fig.</th>
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<td>Wild type on OP50</td>
<td>59/380 (3)</td>
<td>15.54 ± 0.40</td>
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<tr>
<td>nmur-1(ok1387) on OP50</td>
<td>54/380 (3)</td>
<td>15.11 ± 0.35 (-3)wt_OP50</td>
<td>0.198 wt_OP50</td>
<td>0.528 wt_OP50</td>
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</tr>
<tr>
<td>Wild type on CS180</td>
<td>91/160 (2)</td>
<td>14.24 ± 0.32</td>
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<td></td>
</tr>
<tr>
<td>nmur-1(ok1387) on CS180</td>
<td>97/140 (2)</td>
<td>14.95 ± 0.31 (+5)wt_CS180</td>
<td>0.076 wt_CS180</td>
<td>0.131 wt_CS180</td>
<td></td>
</tr>
</tbody>
</table>

Condition: P-deaths (Days 1-15)

| Wild type on OP50       | 53/176 (1)                             | -                         |                     | < 0.0001 wt_OP50    | 0.0003 wt_OP50          |
| nmur-1(ok1387) on OP50  | 75/176 (1)                             | -                         | < 0.0001 osm-6p**   | < 0.0001 osm6p**    |     |
| nmur-1(ok1387); osm-6p::nmur-1 on OP50 | 37/176 (1) | -                        | 0.292 wt_OP50       | 0.147 wt_OP50       |     |

Condition: all deaths

<p>| Wild type on OP50       | 268/540 (4)                            | 10.47 ± 0.28              |                     |                     |     |
| nmur-1(ok1387) on OP50  | 323/540 (4)                            | 9.46 ± 0.26 (-10)wt_OP50  | &lt; 0.0001 wt_OP50    | 0.0026 wt_OP50      |     |
| daf-2(e1368) on OP50    | 226/760 (4)                            | 16.2 ± 0.52 (+54)wt_OP50  | &lt; 0.0001 wt_OP50    | &lt; 0.0001 wt_OP50    |     |
| daf-2(e1368); nmr-1(ok1387) on OP50 | 206/540 (4) | 18.41 ± 0.49 (+76)wt_OP50 | &lt; 0.0001 wt_OP50    | &lt; 0.0001 wt_OP50    |     |
| (daf-2 OP)              |                                          |                           | &lt; 0.0001 daf-2 OP   | &lt; 0.0001 daf-2 OP   |     |
| Wild type on CS180      | 106/160 (2)                            | 13.54 ± 0.32              |                     |                     |     |
| nmur-1(ok1387) on CS180 | 108/140 (2)                            | 14.41 ± 0.32 (+6)wt_CS180 | 0.038 wt_CS180      | 0.079 wt_CS180      |     |
| daf-2(e1368) on CS180   | 104/160 (2)                            | 23.90 ± 0.49 (+76)wt_CS180| &lt; 0.0001 wt_CS180   | &lt; 0.0001 wt_CS180   |     |
| daf-2(e1368); nmur-1(ok1387) on CS180 | 105/160 (2) | 23.73 ± 0.41 (+75)wt_CS180 | &lt; 0.0001 wt_CS180   | &lt; 0.0001 wt_CS180   |     |
| (-0.7) daf-2 CS        |                                          |                           | 0.417 daf-2 CS      | 0.540 daf-2 CS      |     |</p>
<table>
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<tr>
<th>Condition: P-deaths (Days 1-15)</th>
<th>Animals observed/Total animals (trials)</th>
<th>Mean ± SEM (% difference)*</th>
<th>P value of Wilcoxon*</th>
<th>P value of Log-rank*</th>
<th>Fig</th>
</tr>
</thead>
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<td>Wild type on OP50</td>
<td>179/540 (4)</td>
<td>-</td>
<td>&lt; 0.0001&lt;sub&gt;wt_OP50&lt;/sub&gt;</td>
<td>0.0002&lt;sub&gt;wt_OP50&lt;/sub&gt;</td>
<td>3.5</td>
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<tr>
<td>nmur-1(ok1387) on OP50</td>
<td>244/540 (4)</td>
<td>-</td>
<td>&lt; 0.0001&lt;sub&gt;wt_OP50&lt;/sub&gt;</td>
<td>&lt; 0.0001&lt;sub&gt;wt_OP50&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>daf-2(e1368) on OP50</td>
<td>100/760 (4)</td>
<td>-</td>
<td>&lt; 0.0001&lt;sub&gt;wt_OP50&lt;/sub&gt;</td>
<td>&lt; 0.0001&lt;sub&gt;wt_OP50&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>daf-2(e1368); nmur-1(ok1387) on OP50</td>
<td>56/540 (4)</td>
<td>-</td>
<td>&lt; 0.0001&lt;sub&gt;wt_OP50&lt;/sub&gt;</td>
<td>&lt; 0.0001&lt;sub&gt;wt_OP50&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>Wild type on CS180</td>
<td>15/160 (2)</td>
<td>-</td>
<td>0.277&lt;sub&gt;wt_CS180&lt;/sub&gt;</td>
<td>0.283&lt;sub&gt;wt_CS180&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>nmur-1(ok1387) on CS180</td>
<td>10/140 (2)</td>
<td>-</td>
<td>0.0002&lt;sub&gt;wt_CS180&lt;/sub&gt;</td>
<td>0.0002&lt;sub&gt;wt_CS180&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>daf-2(e1368) on CS180</td>
<td>1/160 (2)</td>
<td>-</td>
<td>0.0002&lt;sub&gt;wt_CS180&lt;/sub&gt;</td>
<td>0.0001&lt;sub&gt;wt_CS180&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>daf-2(e1368); nmur-1(ok1387) on CS180</td>
<td>1/160 (2)</td>
<td>-</td>
<td>0.876&lt;sub&gt;daf-2_CS&lt;/sub&gt;</td>
<td>0.89&lt;sub&gt;daf-2_CS&lt;/sub&gt;</td>
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<td>Condition: P-deaths on OP50 (Days 1-15)</td>
<td>54/176 (1)</td>
<td>-</td>
<td>0.026&lt;sup&gt;wt&lt;/sup&gt;</td>
<td>0.069&lt;sup&gt;wt&lt;/sup&gt;</td>
<td>3.6</td>
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<tr>
<td>nmur-1(ok1387)</td>
<td>64/176 (1)</td>
<td>-</td>
<td>0.017&lt;sup&gt;wt&lt;/sup&gt;</td>
<td>0.0086&lt;sup&gt;wt&lt;/sup&gt;</td>
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<tr>
<td>daf-2(e1368)</td>
<td>33/176 (1)</td>
<td>-</td>
<td>&lt; 0.0001&lt;sup&gt;wt&lt;/sup&gt;</td>
<td>&lt; 0.0001&lt;sup&gt;wt&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>daf-2(e1368); nmur-1(ok1387)</td>
<td>20/176 (1)</td>
<td>-</td>
<td>0.0147&lt;sup&gt;daf-2&lt;/sup&gt;</td>
<td>0.0228&lt;sup&gt;daf-2&lt;/sup&gt;</td>
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<tr>
<td>daf-16(mu86)</td>
<td>68/176 (1)</td>
<td>-</td>
<td>0.991&lt;sup&gt;daf-16&lt;/sup&gt;</td>
<td>0.978&lt;sup&gt;daf-16&lt;/sup&gt;</td>
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<tr>
<td>daf-16(mu86); daf-2(e1368)</td>
<td>81/176 (1)</td>
<td>-</td>
<td>0.360&lt;sup&gt;daf-16&lt;/sup&gt;</td>
<td>0.282&lt;sup&gt;daf-16&lt;/sup&gt;</td>
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</tr>
<tr>
<td>Condition: P-deaths on OP50 (Days 1-15)</td>
<td>Animals observed/Total animals (trials)</td>
<td>Mean ± SEM (% difference)*</td>
<td>P value of Wilcoxon*</td>
<td>P value of Log-rank*</td>
<td>Fig.</td>
</tr>
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<td>----------------------------------------</td>
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<td>---------------------</td>
<td>---------------------</td>
<td>------</td>
</tr>
<tr>
<td>daf-16(mu86); nmur-1(ok1387)</td>
<td>73/176 (1)</td>
<td>-</td>
<td>0.0009^wt</td>
<td>0.0215^wt</td>
<td>3.6</td>
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<tr>
<td></td>
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<td></td>
<td>0.540^{daf-16}</td>
<td>0.479^{daf-16}</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.131</td>
<td>0.077</td>
<td></td>
</tr>
<tr>
<td>v. daf-16; daf-2; nmur-1</td>
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<td></td>
<td>v. daf-16; daf-2; nmur-1</td>
<td>0.077</td>
<td></td>
</tr>
<tr>
<td>daf-16(mu86); nmur-1(ok1387)</td>
<td>73/176 (1)</td>
<td>-</td>
<td>0.0009^wt</td>
<td>0.0215^wt</td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
<td>0.541^{daf-16}</td>
<td>0.479^{daf-16}</td>
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<tr>
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<td></td>
<td>0.131</td>
<td>0.077</td>
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<tr>
<td>v. daf-16; daf-2; nmur-1</td>
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<td>v. daf-16; daf-2; nmur-1</td>
<td>0.077</td>
<td></td>
</tr>
<tr>
<td>daf-16(mu86); daf-2(e1368); nmur-1(ok1387)</td>
<td>83/176 (1)</td>
<td>-</td>
<td>&lt; 0.0001^wt</td>
<td>&lt; 0.0001^wt</td>
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<tr>
<td></td>
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<td></td>
<td>0.382^{daf-16}</td>
<td>0.304^{daf-16}</td>
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</table>

Wild-type and mutant worms were assayed in parallel in independent trials. Statistics from the cumulative experiments on *E. coli* OP50 and *E. coli* CS180 are shown. For the conditions where all deaths were measured (observed), the total number of animals (second column) include worms that were censored during the assay. Worms that crawled off the plate, exploded, or bagged were censored at the time of the event, which allowed incorporation of these worms into the data set until the censor date, thereby avoiding loss of information. For the conditions where only P-deaths were measured, only the animals that died with swollen pharynges (Figure 3.1D) were counted during the first day to the fifteenth day of adulthood. For the conditions where only non-P deaths were measured, only the animals that died with unswollen pharynges (Figure 3.1C) and did not explode or bag were counted throughout the duration of the lifespan assay. *, indicates the strains on a specific bacteria to which a particular set of animals are compared. **, osm-6p indicates nmur-1(ok1387); osm-6p::nmur-1 worms, in which wild-type nmur-1 is expressed in the sensory neurons of nmur-1 deletion mutants. The following indicates: OP, OP50; CS, CS180; ^, versus.
Table S3.2. The ratios of pharyngeal-dependent deaths on CS180, CS2429 and CS2198

| P-death ratios | Animals observed/ Total animals (trials) | Animals with P-death | % P-deaths | \( P \) value based on \( \chi^2 \)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Wild type on CS180</td>
<td>64/80 (1)</td>
<td>17</td>
<td>26.56</td>
<td></td>
</tr>
</tbody>
</table>
| Wild type on CS2429 | 61/80 (1) | 35 | 57.37 | \( 0.0005^{CS180} \)
| Wild type on CS2198 | 65/80 (1) | 46 | 70.76 | \( < 0.0001^{CS180} \)  
| \text{ } | \text{ } | \text{ } | \text{ } | \( 0.12^{CS2429} \) |

The percentage of deaths (P-deaths) caused by swollen pharynges were counted throughout the lifespan of each population. Again, the total number of animals (second column) include worms that were censored during the assay (see the legend for Table S3.1). *, indicates the wild-type worms on a specific bacteria to which a particular set of animals are compared.
CHAPTER 4: THE POTENTIAL ACTIN-BINDING SCAFFOLD PROTEIN FILAMIN-2 REGULATES C. ELEGANS SURVIVAL ON SPECIFIC BACTERIAL FOOD SOURCES

Introduction

Human filamins are actin-binding scaffold proteins that can bind many different proteins and participate in many signaling processes (Griffiths et al., 2011; Hayashi and Altman, 2006; Malathi et al., 2014; Muscolini et al., 2011; Nakamura et al., 2011; Popowicz et al., 2006; Tavano et al., 2006). C. elegans has two filamin genes (DeMaso et al., 2011), one of which, filamin-2 (fln-2), modulates swollen pharynx-dependent deaths (P-deaths) when C. elegans are fed an E. coli OP50 diet (Zhao et al., 2019). Because the C. elegans strain QZ58 that carries mutations in fln-2 and the neuropeptide receptor nmur-1 has fewer P-deaths than wild type on OP50, but not on E. coli CS180 (Figure 3.3), I wished to know if fln-2 modulates P-deaths in a bacteria-dependent manner.

Here I show that deletion and nonsense mutations in fln-2 behave like the QZ58 strain, which shows that wild-type fln-2 promotes P-deaths in response to specific bacterial diets. The effects of fln-2 on P-deaths are not fully dependent on a specific regulator of bacterial-dependent responses in the worm. This suggests that fln-2 might regulate multiple immunity or stress response pathways, consistent with its proposed function as a protein scaffold (DeMaso et al., 2011). Since fln-2 also promotes deaths independent of swollen pharynges, which involves insulin signaling and Toll-like receptor signaling, this suggests that FLN-2 acts as a general scaffold for longevity-regulating pathways.

Results

fln-2 promotes pharynx-dependent deaths in a bacteria-dependent manner

The linked mutations in the fln-2 gene locus, ok1305 and ot611, extended lifespan and decreased P-deaths on OP50 (Figure 4.1A; Table S4.1). In this fln-2 mutant, ok1305 is a deletion of 106 amino acids at the N-terminal region of FLN-2, whereas ot611 is a nonsense mutation that truncated most of the FLN-2 protein (Zhao et al., 2019). The ot611
Figure 4.1. Wild-type *fln-2* promotes pharynx-dependent deaths in a bacteria-dependent manner. (A) The *fln-2* mutation, *ok1305ot611*, caused fewer P-deaths on OP50, and (B) to a lesser extent on CS180, when compared to wild type (wt). In this figure and all other figures, *P* values indicate a comparison to wild type on the same bacteria, unless otherwise stated. The statistical analyses of the survival assays in this and subsequent figures are also shown in Table S4.1, unless again stated otherwise.
nonsense mutation alone, which I isolated from QZ58, and an independently generated C-terminal deletion in fln-2, tm4687, also behaved similar to ok1305ot611 (Figure 4.2; Table S4.1). Together these data suggest that wild-type fln-2 promotes P-deaths on OP50.

On CS180, the long-life effect of the fln-2 mutations on the overall lifespan of the population was completely absent (Figure 4.1B, left panel; Table S4.1). However, when I only analyzed the P-deaths on CS180, I found a small, but still significant decrease of P-deaths in fln-2 mutants in comparison to wild type (Figure 4.1B, right panel; Table S4.1). This showed that wild-type fln-2 can promote P-deaths on CS180, but only to a much smaller extent when compared to fln-2 mutants on OP50. However, the fln-2 effect on CS180 is negligible when all deaths are considered. Since the QZ58 strain, which contains both nmur-1 and fln-2 mutations, also behaved like the fln-2 single mutant, this suggests that fln-2 is epistatic to nmur-1 (Figure 4.2; Table S4.1). Thus, fln-2 acts either in parallel and/or downstream of nmur-1 to regulate P-deaths.

**daf-2 may act in parallel to fln-2 to regulate P-deaths**

Human filamins are known to interact with the insulin receptor (He et al., 2003). As I have shown and discussed previously [see Chapter 3; (Podshivalova et al., 2017; Zhao et al., 2021)], the C. elegans insulin receptor daf-2 modulates P-deaths together with nmur-1. To test if fln-2 also interacts with daf-2 when modulating P-deaths, I analyzed the P-deaths of daf-2; fln-2 double mutants alongside the daf-2 single and fln-2 single mutants. I found that the daf-2; fln-2 double mutants had much fewer P-deaths on OP50 than either single mutants (Figure 4.3; Table S4.1), which suggests that daf-2 may act in parallel to fln-2.

Next, I asked how fln-2 interacts with both daf-2 and nmur-1 in regulating P-deaths on OP50. I found that the daf-2; nmur-1 fln-2 triple mutant behaved exactly like the daf-2; fln-2 double mutant on OP50 (Figure 4.4; Table S4.1). The presence of the nmur-1(ok1387) mutation had no effect on the combined mutations of daf-2(e1368) and
Figure 4.2. *fln-2* is epistatic to *nmur-1*. The QZ58 strain contains both the *nmur-1(ok1387)* mutation and the *fln-2(ot611)* mutation. The effect of QZ58 on deaths that are due to swollen pharynges (P-deaths) is indistinguishable from the effect of *fln-2(ot611)*. This is consistent with *fln-2* acting in parallel and/or downstream of *nmur-1*. The statistical comparison between the *fln-2* mutant vs the QZ58 survival curves is indicated in blue \[P_{(fln-2)}\].

![Graph showing survival curves for different conditions](image1)

Figure 4.3. *daf-2* may act in parallel to *fln-2*. The *daf-2(e1368); fln-2(ok1305ot611)* double mutant lived longer and had fewer P-deaths than the *daf-2(e1368)* reduction-of-function single mutant or the *fln-2(ok1305ot611)* single mutant.

![Graph showing survival curves for different conditions](image2)
Figure 4.4. *fln-2* is epistatic to both *nmur-1* and *daf-2*. The *daf-2(e1368); nmur-1(ok1387) fln-2(ot611)* triple mutants, depicted as “*daf-2 + QZ58*” (orange survival curve), had the same amount of P-deaths as the *daf-2(e1368); fln-2(ot611)* double mutants (blue survival curve) on OP50.
*fln-2*(ot611) (Figure 4.4; Table S4.1). Together, these data again suggest that *fln-2* acts either downstream and/or parallel to *nmur-1* and *daf-2* on this food source.

**fln-2 is not completely dependent on a single immunity or stress response pathway**

Since human filamins interact with signaling pathways that have also been implicated in *C. elegans* innate immune or stress responses (D'Addario et al., 2002; He et al., 2003; Sasaki et al., 2001; Shifrin et al., 2012), I asked whether wild-type *fln-2* promotes P-deaths by suppressing *C. elegans* immunity or stress resistance. Thus, I tested if the *fln-2* mutation could still suppress P-deaths in the absence of the wild-type p38 MAPK *pmk-1*, the TGF-β *dbl-1*, or the FOXO transcription factor *daf-16* [Figure 4.5; Table S4.2; reviewed by (Gravato-Nobre and Hodgkin, 2005; Kenyon, 2010)]. In addition, I tested two genes that have been shown to affect *C. elegans* pharyngeal immunity: (i) *tol-1*, an ortholog of the human Toll-like receptors that can act as a receptor for LPS; and (ii) *ced-1*, a receptor that mediates cell corpse engulfment and the unfolded protein response (Tenor and Aballay, 2008). Interestingly, I found that loss of *fln-2* function suppressed P-deaths in all of the conditions that I tested, including the P-deaths that were caused by a shorter LPS structure (Figure 4.5; Table S4.2). This suggests that *fln-2* is not dependent on one immunity or stress-response pathway in *C. elegans*, which would be expected for a scaffold protein.

**fln-2 also promotes deaths independent of swollen pharynges**

FLN-2 is predicted to act as a protein scaffold, like the human filamins (DeMaso et al., 2011). Thus, it is possible that *fln-2* will bind multiple proteins and affect many processes. Indeed, I further observed that when *daf-16* activity was reduced, *fln-2* mutants lived longer even when P-deaths were censored (Figures 4.6A and 4.6B; Table S4.1). This effect was also independent of the bacterial food source, since it was present on both OP50 and CS180 (Figures 4.6A and 4.6B; Table S4.1).
Figure 4.5. *fln-2* affects P-deaths at least partly independently of *daf-16* or other major immunity pathways in *C. elegans.* (A-C) The amount of P-deaths in wild type (wt) and different single or double mutants on *E. coli* OP50 are shown. In (C), because the *dbl-1* mutation causes a high percentage of matricide (de Lucas et al., 2021), the wild type and the *fln-2, dbl-1,* and *dbl-1; fln-2* mutants were initially treated with feeding-induced RNA-mediated interference (RNAi) against *egg-4* and *egg-5* during the first day of adulthood. These animals were then transferred back to OP50 after one day of treatment. Knockdown of the *egg-4/egg-5* protein kinases killed all or most of the embryos, preventing a high rate of matricide [(See Chapter 2 on Materials and Methods); (Entchev et al., 2015; Green et al., 2011; Piano et al., 2002)]. The statistical analyses of P-death ratios in this figure is shown in Table S4.2. The following indicates: *, ≤ 0.05; **, ≤ 0.01; and ***, ≤ 0.001.
*fln-2* also affected deaths independent of swollen pharynges, but only on certain bacteria. In animals lacking the Toll-like receptor ortholog *tol-1*, loss of *fln-2* also led to fewer deaths that are not characterized by swollen pharynges (non-P deaths; Figure 4.7; Table S4.1). However, the effect of *fln-2* on non-P deaths in the *tol-1* mutant background was only evident on CS180, but not on OP50 (Figure 4.7; Table S4.1). Together, my findings show that *fln-2* interacts with multiple signaling pathways and affect lifespan through a number of mechanisms.

**Discussion**

*fln-2* is expressed in the pharynx, intestine and gonadal tissues and has been proposed to bind the actin cytoskeleton, based on its homology to the human actin-binding filamin proteins (DeMaso et al., 2011). The idea that a potential actin-binding protein promotes pharyngeal degeneration and death in response to specific bacterial diets is intriguing, since this might suggest that the actin cytoskeleton is involved in modulating diet-dependent longevity. However, the pharyngeal actin cytoskeletons of wild-type and *fln-2(ok1305ot611)* mutant adults grown on the two bacteria at 25°C are indistinguishable from each other, when stained with phallolidin or visualized with the actin-binding Moesin::mCherry reporter [data not shown; see Chapter 2 on Materials and Methods; (Wirshing and Cram, 2017)]. This suggests that the actin cytoskeleton does not directly regulate the pharyngeal swelling that leads to the animal’s death. Another actin-binding protein, which is encoded by *fln-1* and is expressed in other tissues (DeMaso et al., 2011; Kovacevic and Cram, 2010), also had no effect on *C. elegans* longevity (Table S4.1). Thus, these data suggest that the actin cytoskeleton is less likely to be a direct regulator of longevity.

*fln-2* has also been predicted to function as a scaffold (DeMaso et al., 2011), which is consistent with its interactions with multiple genes. *fln-2* might act in parallel to multiple genes that regulate the animal’s immunity or stress responses (Figure 4.5; Tables S4.1
Figure 4.6. Loss of daf-16 un masks a pharynx-independent and bacteria-independent effect of fln-2 on survival. (A) In daf-16(mu86) mutants on OP50, fln-2(ok1305ot611) also decreased deaths that were independent of swollen pharynges (non-P deaths). (B) In daf-16(mu86) mutants on CS180, fln-2(ok1305ot611) also reduced swollen pharynx-independent deaths.
Figure 4.7. Loss of tol-1 un masks a pharynx-independent, but bacteria-dependent effect of fln-2 on survival. (A) In tol-1(nr2033) mutants on OP50, fln-2(ok1305ot611) decreased P-deaths, but had no effect on non-P deaths. (B) In tol-1(nr2033) mutants on CS180, fln-2(ok1305ot611) decreased both P-deaths and non-P deaths.
and S4.2). In addition, I show that fln-2 might act in parallel with another gene that modulates pharyngeal-dependent deaths (Figures 4.2 and 4.4; Table S4.1), the neuropeptide neuromedin U receptor nmur-1 (see Chapter 3). nmur-1 is capable of both promoting and suppressing pharyngeal swelling and death, based on the genetic background of the animal and/or its diet (Chapter 3). A parallel interaction between the two genes and the modulatory nature of nmur-1 might explain why nmur-1 overexpression in the fln-2 mutant background can rescue the fln-2 long-life phenotype (Maier et al., 2010).

Interestingly, fln-2 also affects longevity independent of pharyngeal swelling (non-P deaths; Figures 4.6 and 4.7; Table S4.1). This additional role of fln-2 depends on daf-16 and tol-1. Loss of either daf-16 or tol-1 unmask the stimulatory effect of wild-type fln-2 on non-P deaths (Figures 4.6 and 4.7; Table S4.1). Thus, while daf-16 and tol-1 appear to act parallel to wild-type fln-2 in inhibiting healthy pharynges and promoting P-deaths, daf-16 and tol-1 inhibit fln-2 function in non-P deaths (Figure 4.8). Human Toll-like receptors have been shown to modulate JNK signaling (Faure et al., 2000; Wang et al., 2012). C. elegans JNK signaling modulates DAF-16 translocation to affect lifespan (Oh et al., 2005). This suggests a possibility that C. elegans TOL-1 reacts to bacterial-derived cues and regulates DAF-16 to inhibit FLN-2 in one of its functions as a potential scaffold protein.
Figure 4.8. A model for how \textit{fln}-2 regulates pharynx-independent deaths. \textit{fln}-2 acts in parallel to \textit{daf}-16 and \textit{tol}-1 to regulate pharyngeal swelling in response to a specific bacteria (top panel). However, \textit{daf}-16 and \textit{tol}-1 inhibit \textit{fln}-2 from exerting its effects on pharynx-independent survival (bottom panel).
Table S4.1. Cumulative statistics of deaths on different bacteria

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<tr>
<th>Condition: all deaths</th>
<th>Animals observed/Total animals (trials)</th>
<th>Mean ± SEM (% difference)*</th>
<th>P value of Wilcoxon*</th>
<th>P value of Log-rank*</th>
<th>Fig.</th>
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Table S4.1. cont.

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<td>(+27)</td>
<td>&lt; 0.0001&lt;sup&gt;wt_daf-16&lt;/sup&gt;</td>
<td>&lt; 0.0001&lt;sup&gt;wt_daf-16&lt;/sup&gt;</td>
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<tr>
<td><strong>Wild type on OP50</strong></td>
<td>92/160 (2)</td>
<td>12.48 ± 0.58</td>
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<td>4.7</td>
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<tr>
<td><strong>fln-2(ok1305 ot611)</strong> on OP50</td>
<td>87/160 (2)</td>
<td>16.61 ± 0.48 (+33)&lt;sup&gt;wt.OP&lt;/sup&gt;</td>
<td>&lt; 0.0001&lt;sup&gt;wt.OP&lt;/sup&gt;</td>
<td>&lt; 0.0001&lt;sup&gt;wt.OP&lt;/sup&gt;</td>
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<tr>
<td><strong>tol-1(nr2033)</strong> on OP50</td>
<td>89/160 (2)</td>
<td>12.37 ± 0.61 (+34)&lt;sup&gt;wt.OP&lt;/sup&gt;</td>
<td>0.667&lt;sup&gt;wt.OP&lt;/sup&gt;</td>
<td>0.4798&lt;sup&gt;wt.OP&lt;/sup&gt;</td>
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Table S4.1. cont.

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<th>Animals observed/Total animals (trials)</th>
<th>Mean ± SEM (% difference)*</th>
<th>P value of Wilcoxon*</th>
<th>P value of Log-rank*</th>
<th>Fig.</th>
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<td>tol-1(nr2033); fln-2(ok1305ot611) on OP50</td>
<td>100/160 (2)</td>
<td>17.28 ± 0.5 (+40)*&lt;sup&gt;wt_OP&lt;/sup&gt;</td>
<td>&lt; 0.0001&lt;sup&gt;wt_OP&lt;/sup&gt;</td>
<td>&lt; 0.0001&lt;sup&gt;wt_OP&lt;/sup&gt;</td>
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<tr>
<td>Wild type on CS180</td>
<td>121/160 (2)</td>
<td>13.71 ± 0.28</td>
<td>&lt; 0.0001 v. &lt;sup&gt;tol-1&lt;/sup&gt;_OP&lt;sup&gt;CS&lt;/sup&gt;</td>
<td>&lt; 0.0001 v. &lt;sup&gt;tol-1&lt;/sup&gt;_OP&lt;sup&gt;CS&lt;/sup&gt;</td>
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<td>fln-2(ok1305ot611) on CS180</td>
<td>134/160 (2)</td>
<td>13.63 ± 0.27 (-0.6)&lt;sup&gt;wt_CS&lt;/sup&gt;</td>
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<td>0.948&lt;sup&gt;wt_CS&lt;/sup&gt;</td>
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<td>tol-1(nr2033) on CS180</td>
<td>132/160 (2)</td>
<td>12.87 ± 0.29 (-7.4)&lt;sup&gt;wt_CS&lt;/sup&gt;</td>
<td>0.0081&lt;sup&gt;wt_CS&lt;/sup&gt;</td>
<td>0.1007&lt;sup&gt;wt_CS&lt;/sup&gt;</td>
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<td>tol-1(nr2033); fln-2(ok1305ot611) on CS180</td>
<td>120/160 (2)</td>
<td>14.79 ± 0.29 (+8)&lt;sup&gt;wt_CS&lt;/sup&gt;</td>
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<td>0.0052&lt;sup&gt;wt_CS&lt;/sup&gt;</td>
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<tr>
<td>fln-2(ok1305ot611) on OP50</td>
<td>29/80 (1)</td>
<td>16.03 ± 0.57 (+3)&lt;sup&gt;wt_OP50&lt;/sup&gt;</td>
<td>0.4333&lt;sup&gt;wt_OP50&lt;/sup&gt;</td>
<td>0.769&lt;sup&gt;wt_OP50&lt;/sup&gt;</td>
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<td>daf-16(mu86) on OP50</td>
<td>26/80 (1)</td>
<td>11.84 ± 0.58 (-24)&lt;sup&gt;wt_OP50&lt;/sup&gt;</td>
<td>0.0137&lt;sup&gt;wt_OP50&lt;/sup&gt;</td>
<td>0.0007&lt;sup&gt;wt_OP50&lt;/sup&gt;</td>
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<td>daf-16(mu86); fln-2(ok1305ot611) on OP50</td>
<td>34/80 (1)</td>
<td>13.82 ± 0.45 (-11)&lt;sup&gt;wt_OP50&lt;/sup&gt;</td>
<td>0.1834&lt;sup&gt;wt_OP50&lt;/sup&gt;</td>
<td>0.0323&lt;sup&gt;wt_OP50&lt;/sup&gt;</td>
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<td>Wild type on CS180</td>
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<td>fln-2(ok1305ot611) on OP50</td>
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<td>0.9049&lt;sup&gt;wt_CS&lt;/sup&gt;</td>
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<td>8.52 ± 0.24 (-33)&lt;sup&gt;wt_CS&lt;/sup&gt;</td>
<td>&lt; 0.0001&lt;sup&gt;wt_CS&lt;/sup&gt;</td>
<td>&lt; 0.0001&lt;sup&gt;wt_CS&lt;/sup&gt;</td>
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<td>Animals observed/Total animals (trials)</td>
<td>Mean ± SEM (% difference)*</td>
<td>$P$ value of Wilcoxon*</td>
<td>$P$ value of Log-rank*</td>
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<td>daf-16(mu86); fln-2(ok1305ot611) on CS180</td>
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<td>90/150 (2)</td>
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<td>17.68 ± 0.52</td>
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<td>fln-2(ok1305ot611) on OP50</td>
<td>68/160 (2)</td>
<td>18.11 ± 0.45 (+2)$^{wt_OP}$</td>
<td>0.6841$^{wt_OP}$</td>
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<td>tol-1(nr2033); fln-2(ok1305ot611) on OP50</td>
<td>84/160 (2)</td>
<td>18.60 ± 0.46 (+5)$^{wt_OP}$</td>
<td>0.3035$^{wt_OP}$</td>
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<td>105/160 (2)</td>
<td>14.38 ± 0.26</td>
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<td>fln-2(ok1305ot611) on CS180</td>
<td>128/160 (2)</td>
<td>13.84 ± 0.27 (-4)$^{wt_CS}$</td>
<td>0.2258$^{wt_CS}$</td>
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<td>tol-1(nr2033) on CS180</td>
<td>103/160 (2)</td>
<td>13.92 ± 0.30 (-3)$^{wt_CS}$</td>
<td>0.0697$^{wt_CS}$</td>
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<tr>
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<td>tol-1(nr2033); fln-2(ok1305ot611) on CS180</td>
<td>112/160 (2)</td>
<td>15.21 ± 0.28 (+6)$^{wt_CS}$</td>
<td>0.0104$^{wt_CS}$</td>
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<td>(v. tol-1$^{CS}$)</td>
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<td>Wild type on OP50</td>
<td>150/80 (1)</td>
<td>12.69 ± 0.82</td>
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<td>fln-2(ot611) on OP50</td>
<td>150/80 (1)</td>
<td>17.99 ± 0.63 (+42)$^{wt_OP}$</td>
<td>$&lt; 0.0001^{wt_OP}$</td>
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<td>Condition:</td>
<td>Animals observed/ Total animals (trials)</td>
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<td>P value of Log-rank*</td>
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<td><strong>Wild type on CS180</strong></td>
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<td>0.6925\text{wt_CS}</td>
<td>0.6057\text{wt_CS}</td>
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<td><strong>Wild type on OP50</strong></td>
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<tr>
<td><strong>fln-2(ot611) on OP50</strong></td>
<td>7/80 (1)</td>
<td>-</td>
<td>&lt; 0.0001\text{wt_OP}</td>
<td>&lt; 0.0001\text{wt_OP}</td>
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<td><strong>Wild type on CS180</strong></td>
<td>7/80 (1)</td>
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<td><strong>fln-2(ot611) on CS180</strong></td>
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<td>0.01109\text{wt_CS}</td>
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<td><strong>Condition: all deaths</strong></td>
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<tr>
<td><strong>Wild type on OP50</strong></td>
<td>39/80 (1)</td>
<td>10.48 ± 0.66</td>
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<tr>
<td><strong>fln-2(tm4687) on OP50</strong></td>
<td>65/80 (1)</td>
<td>14.74 ± 0.53 (+41)\text{wt_OP}</td>
<td>&lt; 0.0001\text{wt_OP}</td>
<td>&lt; 0.0001\text{wt_OP}</td>
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<td><strong>Condition: P-deaths (Days 1-15)</strong></td>
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<tr>
<td><strong>Wild type on OP50</strong></td>
<td>11/80 (1)</td>
<td>-</td>
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<tr>
<td><strong>fln-2(tm4687) on OP50</strong></td>
<td>6/80 (1)</td>
<td>-</td>
<td>0.0153\text{wt_OP}</td>
<td>0.0087\text{wt_OP}</td>
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<td><strong>Condition: all deaths</strong></td>
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<td><strong>Wild type on OP50</strong></td>
<td>51/80 (1)</td>
<td>11.14 ± 0.6</td>
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<tr>
<td><strong>fln-1(ok2611) on OP50</strong></td>
<td>46/80 (1)</td>
<td>11.47 ± 0.55 (+3)\text{wt_OP}</td>
<td>0.3372\text{wt_OP}</td>
<td>0.8705\text{wt_OP}</td>
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<td><strong>fln-2(ok1305ot611) on OP50</strong></td>
<td>57/80 (1)</td>
<td>16 ± 0.48 (+44)\text{wt_OP}</td>
<td>&lt; 0.0001\text{wt_OP}</td>
<td>&lt; 0.0001\text{wt_OP}</td>
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<td>Animal conditions</td>
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<td>Mean ± SEM (% difference)*</td>
<td>P value of Wilcoxon*</td>
<td>P value of Log-rank*</td>
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</tr>
<tr>
<td>fin-1(ok2611); fin-2(ok1305ot611) on OP50</td>
<td>43/80 (1)</td>
<td>16.14 ± 0.55 (+45) &lt;sup&gt;wt_OP&lt;/sup&gt;</td>
<td>&lt; 0.0001&lt;sup&gt;wt_OP&lt;/sup&gt;</td>
<td>&lt; 0.0001&lt;sup&gt;wt_OP&lt;/sup&gt;</td>
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<tr>
<td>Condition: P-deaths (Days 1-15)</td>
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<td>(0.8) v&lt;sup&gt;fin-2_OP&lt;/sup&gt;</td>
<td>0.9326&lt;sup&gt;fin-2_OP&lt;/sup&gt;</td>
<td>0.6458&lt;sup&gt;fin-2_OP&lt;/sup&gt;</td>
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<tr>
<td>Wild type on OP50</td>
<td>25/80 (1)</td>
<td>-</td>
<td>0.5094&lt;sup&gt;wt_OP&lt;/sup&gt;</td>
<td>0.8601&lt;sup&gt;wt_OP&lt;/sup&gt;</td>
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<tr>
<td>fin-1(ok2611) on OP50</td>
<td>25/80 (1)</td>
<td>-</td>
<td>&lt; 0.0001&lt;sup&gt;wt_OP&lt;/sup&gt;</td>
<td>&lt; 0.0001&lt;sup&gt;wt_OP&lt;/sup&gt;</td>
</tr>
<tr>
<td>fin-2(ok1305ot611) on OP50</td>
<td>4/80 (1)</td>
<td>-</td>
<td>&lt; 0.0001&lt;sup&gt;wt_OP&lt;/sup&gt;</td>
<td>&lt; 0.0001&lt;sup&gt;wt_OP&lt;/sup&gt;</td>
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<td>fin-1(ok2611); fin-2(ok1305ot611) on OP50</td>
<td>2/80 (1)</td>
<td>-</td>
<td>&lt; 0.0001&lt;sup&gt;wt_OP&lt;/sup&gt;</td>
<td>&lt; 0.0001&lt;sup&gt;wt_OP&lt;/sup&gt;</td>
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Wild-type and mutant worms were again assayed in parallel in independent trials. Statistics from the pooled experiments on *E. coli* OP50 and *E. coli* CS180 are listed. In measuring all deaths (observed), the total number of animals (second column) include censored worms during the assay. Worms that crawled off the plate, exploded, or bagged were censored at the time of the event to avoid loss of information, since this allowed incorporation of these worms into the data set until the censor date. When only P-deaths were measured, only the animals that died with swollen pharynges (Figure 3.1D) were counted during the first day to the fifteenth day of adulthood. When only non-P-deaths were measured, only the animals that died without swollen pharynges (Figure 3.1C) were counted throughout the duration of the lifespan assay. These non-P deaths did not include worms that exploded or bagged. *, indicates the strains on a specific bacteria to which a particular set of animals are compared. The following indicates: OP, OP50; CS, CS180; v., versus.
Table S4.2. Ratios of pharyngeal-dependent deaths

<table>
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<tr>
<th>Experiment 1 on OP50</th>
<th>Animals observed/ Total animals (trials)</th>
<th>Animals with P-death</th>
<th>% P-deaths</th>
<th>P value based on χ²*</th>
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<td>Wild type</td>
<td>55/80 (1)</td>
<td>24</td>
<td>0.44</td>
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<td>fin-2(ok1305ot611)</td>
<td>46/80 (1)</td>
<td>5</td>
<td>0.11</td>
<td>0.0003&lt;sub&gt;WT_OP50&lt;/sub&gt;</td>
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<td>pmk-1(km25)</td>
<td>17/80 (1)</td>
<td>8</td>
<td>0.47</td>
<td>0.8053&lt;sub&gt;WT_OP50&lt;/sub&gt;</td>
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<tr>
<td>pmk-1(km25); fln-2(ok1305ot611)</td>
<td>21/80 (1)</td>
<td>2</td>
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<td>0.0099&lt;sub&gt;pmk-1.OP50&lt;/sub&gt;</td>
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<td>tol-1(nr2033)</td>
<td>52/80 (1)</td>
<td>35</td>
<td>0.67</td>
<td>0.0143&lt;sub&gt;WT_OP50&lt;/sub&gt;</td>
</tr>
<tr>
<td>tol-1(nr2033); fln-2(ok1305ot611)</td>
<td>49/80 (1)</td>
<td>8</td>
<td>0.16</td>
<td>&lt;0.0001&lt;sub&gt;tol-1.OP50&lt;/sub&gt;</td>
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<tr>
<td>ced-1(e1735)</td>
<td>61/80 (1)</td>
<td>29</td>
<td>0.48</td>
<td>0.67&lt;sub&gt;WT_OP50&lt;/sub&gt;</td>
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<tr>
<td>ced-1(e1735); fln-2(ok1305ot611)</td>
<td>54/80 (1)</td>
<td>8</td>
<td>0.15</td>
<td>0.0002&lt;sub&gt;ced-1.OP50&lt;/sub&gt;</td>
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<th>Experiment 2 on OP50</th>
<th>Animals observed/ Total animals (trials)</th>
<th>Animals with P-death</th>
<th>% P-deaths</th>
<th>P value based on χ²*</th>
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<td>fin-2(ok1305ot611)</td>
<td>35/80 (1)</td>
<td>1</td>
<td>0.03</td>
<td>0.002&lt;sub&gt;WT_OP50&lt;/sub&gt;</td>
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<tr>
<td>daf-16(mu86)</td>
<td>63/80 (1)</td>
<td>29</td>
<td>0.46</td>
<td>0.04&lt;sub&gt;WT_OP50&lt;/sub&gt;</td>
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<tr>
<td>daf-16(mu86); fln-2(ok1305ot611)</td>
<td>36/80 (1)</td>
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<td>0.06</td>
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<th>Experiment 3 on OP50**</th>
<th>Animals observed/ Total animals (trials)</th>
<th>Animals with P-death</th>
<th>% P-deaths</th>
<th>P value based on χ²*</th>
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<td>Wild type&lt;sup&gt;L4440&lt;/sup&gt;</td>
<td>54/80 (1)</td>
<td>48</td>
<td>0.89</td>
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<tr>
<td>fin-2(ok1305ot611)&lt;sup&gt;L4440&lt;/sup&gt;</td>
<td>54/80 (1)</td>
<td>21</td>
<td>0.39</td>
<td>&lt;0.0001&lt;sub&gt;L4440.OP&lt;/sub&gt;</td>
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<tr>
<td>Wild type&lt;sup&gt;egg&lt;/sup&gt;</td>
<td>62/80 (1)</td>
<td>51</td>
<td>0.82</td>
<td>0.3158&lt;sub&gt;L4440.OP&lt;/sub&gt;</td>
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<tr>
<td>fin-2(ok1305ot611)&lt;sup&gt;egg&lt;/sup&gt;</td>
<td>62/80 (1)</td>
<td>26</td>
<td>0.42</td>
<td>&lt;0.0001&lt;sub&gt;egg.OP&lt;/sub&gt;</td>
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<tr>
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<td>64/160 (1)</td>
<td>43</td>
<td>0.67</td>
<td>0.0529&lt;sub&gt;egg.OP&lt;/sub&gt;</td>
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<tr>
<td>dbl-1(nk3); fln-2(ok1305ot611)&lt;sup&gt;egg&lt;/sup&gt;</td>
<td>38/160 (1)</td>
<td>10</td>
<td>0.26</td>
<td>&lt;0.0001&lt;sub&gt;dbl-1.egg.OP&lt;/sub&gt;</td>
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<th>Experiment 4 on the CS strains</th>
<th>Animals observed/ Total animals (trials)</th>
<th>Animals with P-death</th>
<th>% P-deaths</th>
<th>P value based on χ²*</th>
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<td>Wild type on CS180</td>
<td>126/170 (2)</td>
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<td>0.17</td>
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<tr>
<td>fin-2(ok1305ot611) on CS180</td>
<td>143/170 (2)</td>
<td>6</td>
<td>0.04</td>
<td>0.0006&lt;sub&gt;WT_CS180&lt;/sub&gt;</td>
</tr>
<tr>
<td>Experiment 4 cont.</td>
<td>Animals observed/ Total animals (trials)</td>
<td>Animals with P-death</td>
<td>% P-deaths</td>
<td>P value based on χ²*</td>
</tr>
<tr>
<td>--------------------</td>
<td>----------------------------------------</td>
<td>---------------------</td>
<td>------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Wild type on CS2429</td>
<td>126/170 (2)</td>
<td>70</td>
<td>0.56</td>
<td>&lt;0.0001&lt;sup&gt;WT_CS180&lt;/sup&gt;</td>
</tr>
<tr>
<td>fln-2(ok1305ot611) on CS2429</td>
<td>138/170 (2)</td>
<td>19</td>
<td>0.14</td>
<td>0.5125&lt;sup&gt;WT_CS180&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The percentage of deaths (P-deaths) caused by swollen pharynges were counted throughout the lifespan of each population. The total number of animals (second column) include worms that were censored during the assay (see the legend for Table S4.1). *, indicates the *C. elegans* strain to which a particular set of animals are compared. **, indicates animals that were treated on day 1 of adulthood with an empty vector control (L4440) or RNAi against *egg-4* and *egg-5* (egg) to prevent excessive matricide (see legend of Figure 4.5; also see Chapter 2 on Materials and Methods). The following also indicate: WT, wild type; _OP50 or _OP, compared to the indicated strain on OP50; WT(L4440)_OP, compared to wild type that were exposed to L4440 during the first day of adulthood; *(egg)_OP, compared to animals that were exposed to egg-4 and egg-5 RNAi during the first day of adulthood; and _CS180, compared to the indicated strain on CS180.
CHAPTER 5: CONCLUSIONS AND PERSPECTIVES

The *C. elegans* pharynx is a tubular organ that moves substances through its lumen via the rhythmic contractions of bi-nucleated muscles, which resemble the mammalian heart (Mango, 2007). The pumping of both *C. elegans* pharynx and mammalian heart are controlled by similar electrical circuits (Mango, 2007). The *C. elegans* pharynx can also get infected and is a life-threatening condition like human heart infections (Feldman and McNamara, 2000; Mylonakis and Calderwood, 2001; Troughton et al., 2004; Zhao et al., 2017). These similarities make the *C. elegans* pharynx a potential model to identify factors that affect human heart infections and health.

In this work, I have identified the neuropeptide neuromedin U pathway as a regulator of *C. elegans* pharyngeal health, which involves the diet-dependent modulation of insulin signaling and its downstream effector, DAF-16/FOXO (Chapter 3). In mammals, FOXO transcription factors affect cardiac muscle remodeling and insulin resistance, which are associated with heart disease and damage (Ni et al., 2007; Riehle and Abel, 2016; Tremblay and Giguere, 2008). Interestingly, I find that the effects of the neuromedin U receptor *nmur-1* on *C. elegans* lifespan are exclusively through pharyngeal health (Table S3.1). In addition, I have shown that another regulator of pharyngeal health, *fln-2* (Zhao et al., 2019), an ortholog of mammalian filamins (DeMaso et al., 2011), affects lifespan in a bacterial diet-dependent manner (Chapter 4). Furthermore, *fln-2* regulates *C. elegans* survival independent of the pharynx and is subject to negative regulation by insulin signaling during this pharynx-independent process (Chapter 4).

The interactions between insulin signaling, *nmur-1* and *fln-2* emphasize that insulin signaling is a major determinant of pharyngeal health and survival. At the same time, the dependence of *nmur-1* and *fln-2* on bacterial diet highlights the importance of these two genes in an animal’s response to its environment. Different bacteria are major components of the *C. elegans* environment, which can affect animal health through a variety of cues
(Dirksen et al., 2016; Samuel et al., 2016). Here I have found that the LPS structure is a bacterial cue that affects worm pharyngeal health (Chapter 3).

**Potential mechanisms through which nmur-1 modulates insulin signaling**

Sensory neurons have been shown to affect lifespan at least partly through the insulin signaling effector DAF-16/FOXO (Alcedo and Kenyon, 2004; Apfeld and Kenyon, 1999). NMUR-1 regulates diet-dependent pharyngeal health by acting in sensory neurons and by modulating insulin signaling (Chapter 3). Thus, NMUR-1 in sensory neurons presumably integrates the information about the quality of the animal’s diet, while it modulates insulin pathway activities.

Mammalian neuromedin U signaling has been shown to regulate glucose-stimulated insulin secretion in pancreatic β-cells, possibly in an autocrine/paracrine manner (Zhang et al., 2020). Through the Gα<sub>q</sub> protein, neuromedin U signaling reduces cAMP levels and Ca<sup>2+</sup> influx and promotes mitochondrial dysfunction during this process (Zhang et al., 2020; Zhang et al., 2021). Neuromedin U regulates the expression of genes that affect mitochondrial dynamics, biogenesis and mitophagy (Zhang et al., 2020).

Mammalian neuromedin U signaling also induces endoplasmic reticulum (ER) stress and the expression of the ER stress-activated transcription factor XBP-1s, possibly through disrupted Ca<sup>2+</sup> dynamics in the cell (Zhang et al., 2020). Intriguingly, in *C. elegans daf-2* insulin receptor mutants, DAF-16/FOXO facilitates the XBP-1-mediated activation of an increased number of longevity genes (Henis-Korenblit et al., 2010). The proteins encoded by these longevity genes then negatively feed back onto XBP-1 to boost ER homeostasis (Henis-Korenblit et al., 2010).

It is possible that neuronal NMUR-1 and the ER stress response work together to ensure that DAF-2 activity is at the appropriate level to promote pharyngeal health and survival on specific bacterial diets (Figure 3.7). The NMUR-1 modulation of the DAF-2 receptor might involve ER stress-mediated secretion of the insulin-like peptide (ILP)
ligands. ER stress can alter intracellular Ca^{2+} levels, which are important for exocytosis of ILPs from dense core vesicles [DCVs; (Squire et al., 2003; Voets et al., 2001)]. Thus, any changes in intracellular calcium levels due to NMUR-1 activity and/or ER stress will also affect ILP release from the DCVs and the subsequent signaling of the DAF-2 receptor through DAF-16/FOXO. Some ILPs promote and other ILPs inhibit DAF-2 signaling (Cornils et al., 2011; Fernandes de Abreu et al., 2014). This raises the possibility that NMUR-1 modulates DAF-2 signaling by stimulating the secretion of ILP agonists in certain conditions and ILP antagonists in other conditions.

Another question to consider is how NMUR-1 senses the state of insulin signaling. It is possible that some DAF-16 targets also signal back to NMUR-1 and/or its ligands. Through such a feedback mechanism, DAF-16 and insulin signaling will modulate their own activity through NMUR-1.

**Potential mechanisms through which fln-2 modulates pharyngeal health**

fln-2 is expressed in several *C. elegans* tissues, including the pharynx, and has actin-binding domains that can potentially affect pharyngeal architecture and function (DeMaso et al., 2011). This raises the hypothesis that fln-2 affects pharynx-dependent deaths by changing the pumping rates of *C. elegans* pharynges. However, I have not

![Figure 5.1. C. elegans pharyngeal pumping rates of wild type and fln-2 mutants on the two bacterial diets.](image)

There are no significant differences in the pharyngeal pumping rates of wild type (OP50, n = 14; CS180, n = 14) and fln-2 mutants (OP50, n = 14; CS180, n = 14) at 25°C, based on two-way ANOVA.
observed a change in the pumping rates between wild type or fln-2 mutants, fed either OP50 or CS180 (Figure 5.1), which argues against this hypothesis.

Human filamins can promote barrier function against different pathogens, but sometimes the filamin interactions with various signaling proteins can also facilitate pathogen entry (Griffiths et al., 2011; Malathi et al., 2014; Young et al., 1992). *C. elegans* FLN-2 lacks the canonical dimerization domains (DeMaso et al., 2011). Although I have not observed a change in the gross actin cytoskeletal structures of fln-2 mutants (Chapter 4), the worm FLN-2 does have conserved actin-binding domains and filamin-type immunoglobulin-like domains. Thus, FLN-2 might still bind actin, in addition to other proteins, in regulating pharyngeal health versus pharyngeal infections.

Pharyngeal-dependent deaths might be influenced by the healing of the pharynx after physical injury (Zhao et al., 2017). *C. elegans* has previously been shown to have the capacity to heal its wounds, at least of its cuticle (Pujol et al., 2008a). In older *C. elegans*, scar tissue that indicates wound healing has been observed at sites where bacterial invasion of the pharynx seemed to have occurred (Zhao et al., 2017). Interestingly, human filamins affect wound closure (Mohammadi et al., 2015). Considering that fln-2 is expressed in the pharynx (DeMaso et al., 2011), the wound healing phase might involve FLN-2 activity during pharyngeal infections.

**Potential mechanisms of LPS-dependent pharyngeal health**

One mechanism through which LPS might affect *C. elegans* pharyngeal infections would be to change the pharyngeal pumping rates, which are influenced by bacterial diet (Maier et al., 2010). However, as mentioned earlier, wild-type animals have similar pumping rates on OP50 and CS180 (Figure 5.1), both of which lack the O antigen, but have different core LPS structures (Maier et al., 2010; and references therein). The core LPS of *E. coli* is less adherent than the O antigen (Genevaux et al., 1999; Strauss et al., 2009; Zhang et al., 2006), but the sugars present in the different core LPS might recognize
different host proteins. For example, the N-acetylglucosamine that is present in the core LPS of CS180, but absent in OP50, binds the human C-type lectin CD209 (Zhang et al., 2006). *C. elegans* has a large family of C-type lectins, some of which affect the animal’s immune responses (Miltsch et al., 2014; Pees et al., 2021; Schulenburg et al., 2008). Some C-type lectins that have structural similarities to CD209 are also differentially expressed in worms fed OP50 versus CS180 (W. Maier, J. Alcedo, personal communication). However, because of the high number of paralogs and potential redundancy in the C-type lectin family in *C. elegans*, it might be difficult to test certain C-type lectins for their specific phenotypes (Pees et al., 2021; Woollard, 2005).

![Figure 5.2. A part of the *C. elegans* fatty acid synthesis pathway. Red circles indicate fatty acids that are potentially affected in *C. elegans* in response to bacteria with different LPS structures. Monounsaturated fatty acid is abbreviated as MUFA.](image)

A binding interaction between the bacterial LPS and a *C. elegans* transmembrane protein can presumably change gene expression patterns in the animal. In addition to immunity genes, which are exemplified by lectins, another class of genes that are differentially expressed between OP50-fed and CS180-fed worms are genes involved in fatty acid synthesis, *fat-5* and *fat-7* (W. Maier, J. Alcedo, personal communication). FAT-5 and FAT-7 produce monounsaturated fatty acids (Figure 5.2), which have been shown to
affect *C. elegans* lifespan (Han et al., 2017). The product of FAT-7 activity, oleic acid, can also be processed into polyunsaturated fatty acids, which have been shown to affect *C. elegans* immunity and sensory neuron functions (Anderson et al., 2019; Kahn-Kirby et al., 2004; Nandakumar and Tan, 2008). Thus, changes in the LPS structure of dietary bacteria might alter *C. elegans* fat metabolism, immune responses and neuronal activities, all of which have been linked to *C. elegans* survival (Alcedo and Kenyon, 2004; Anderson et al., 2019; Apfeld and Kenyon, 1999; Nandakumar and Tan, 2008).

**Modulating the modulators: maintaining homeostasis, while regulating tissue-specific health**

It is important for an animal to modulate the health of its crucial tissues and organs in response to its environment. Infection of the *C. elegans* pharynx, like the evolutionarily related vertebrate heart, is detrimental for the animal (Feldman and McNamara, 2000; Mylonakis and Calderwood, 2001; Troughton et al., 2004; Zhao et al., 2017). My study shows that the health of *C. elegans* pharynx is modulated by multiple parallel signaling pathways that can act in opposite ways (Figure 5.3). One of these pathways is insulin signaling, which promotes pharyngeal health and is a regulator of many important biological functions (Lee et al., 2003; Murphy et al., 2003; Tullet, 2015). Given its pleiotropic functions, overactivity or underactivity of insulin signaling can be detrimental to animal physiology. I have shown that NMUR-1 suppresses or promotes insulin signaling depending on the signaling levels of the latter, thereby maintaining homeostasis and preventing potentially bad outcomes.

Parallel to NMUR-1 and insulin signaling, FLN-2, which might also mediate the effects of bacterial LPS, acts to suppress pharyngeal health (Figure 5.3). FLN-2 can suppress longevity independent of pharyngeal health when DAF-16 activity is reduced (Figure 4.8), which again reflects the tight control over proteins that modulate tissue-specific health. Thus, multiple pathways influence the health of critical tissues, where
Neuromodulation is an important safeguard to prevent these pathways from deviating too much from their normal activity at the wrong time and potentially harm the animal.

Figure 5.3. Pharyngeal health is modulated by several parallel pathways that are also subject to modulation. Insulin signaling promotes pharyngeal health and is modulated by nmur-1, whereas fln-2 can act parallel to both. LPS is a bacterial cue for pharyngeal health, whose effects might be mediated by fln-2.
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ABSTRACT

MODULATION OF PHARYNGEAL HEALTH IN BACTERIAL DIET-DEPENDENT SURVIVAL

by

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August 2021

Advisor: Dr. Joy Alcedo

Major: Biological Sciences

Degree: Doctor of Philosophy

Both diet and bacterial microbiome modulate insulin signaling, which regulates key physiological processes that are important for survival. However, the mechanisms through which diet and the microbiome modulate insulin signaling remain unclear. To understand these mechanisms, I turned to the nematode worm \textit{C. elegans}, whose diet consists of different types of bacteria. Like humans and other animals, \textit{C. elegans} has to modulate its responses to its diet and to bacteria to optimize its survival. Because \textit{C. elegans} is highly tractable to genetics and exhibits a large degree of conservation with other animals, including humans, \textit{C. elegans} is an excellent model organism in which to explore both dietary influences on physiology and bacteria-host relationships.

To study how bacterial diet affects \textit{C. elegans} physiology, I measured the survival of \textit{C. elegans} fed two \textit{E. coli} strains—the B type OP50 and the K-12 type CS180. Wild-type \textit{C. elegans} fed OP50 has a higher rate of early deaths compared to \textit{C. elegans} fed CS180. These early deaths on OP50 depend on swollen pharynges (P-deaths), and worms fed CS180 are largely resistant to P-deaths. Since P-deaths are characterized by bacterial accumulation in the pharynx, this suggests that bacterial cue(s) present in OP50, but not in CS180, promote P-deaths. I have identified that at least one of these cues involves the lipopolysaccharide (LPS) structure of the bacteria.
Since I found that a reduction in the animal’s insulin receptor daf-2 activity leads to fewer P-deaths on both bacterial diets, this indicates that wild-type insulin receptor signaling promotes P-deaths in a bacterial type-independent manner. Thus, to define the molecular mechanisms that mediate the bacterial type-dependent effects on C. elegans P-deaths, I focused on the C. elegans strain QZ58, which has been shown to increase early survival on OP50, but not on CS180. QZ58 carries mutations in the neuropeptide neuromedin U receptor nmur-1 and the scaffold protein filamin-2 (fln-2), which suggests nmur-1 and/or fln-2 as candidate modulators of P-deaths in response to bacterial food types. I showed that loss of nmur-1 alone leads to increased P-deaths on OP50, but not on CS180, which implies that wild-type nmur-1 inhibits P-deaths in a bacterial diet-dependent manner. In contrast, loss of fln-2 alone leads to fewer P-deaths on OP50 and, to a lesser extent, on CS180, which indicates that wild-type fln-2 promotes P-deaths in response to certain bacterial food sources.

Interestingly, however, nmur-1 facilitates an opposite response when daf-2 has reduced activity, where loss of nmur-1 in a daf-2 reduction-of-function mutant background now leads to a further reduction in P-deaths. The opposing effects of nmur-1, which are both bacterial type-dependent, are also dependent on the insulin pathway effector daf-16, which encodes a FOXO transcription factor. Together these suggest that NMUR-1 ensures that the insulin pathway signals at the appropriate level to promote pharyngeal health and optimal survival in response to specific bacteria. On the other hand, the P-death-related effects of fln-2 do not depend on insulin signaling or any of the major C. elegans immunity pathways, which suggests that multiple pathways regulate the animal’s pharyngeal health.

The control of host responses to bacteria and/or its diet is crucial for a healthy lifespan. The fine-tuning of these responses become more crucial over the course of aging, where the host develops increased susceptibility to infection, like in the case of P-
deaths. Insulin signaling would be an important pathway to fine-tune, since it plays important roles in metabolism, immunity, and other diverse processes, like development, reproduction, and aging. Insulin pathway over- or underactivity will significantly impact an organism’s survival. Thus, elucidating the modulation of insulin signaling is going to be crucial in understanding how animals react and adapt to their changing environments.
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“Neuropeptide modulation of insulin signaling in bacteria-dependent survival”

Selected Meeting presentations (Poster)
Jun 2021 23rd International C. elegans Conference, Online
“Neuropeptide modulation of insulin signaling in bacteria-dependent survival”
Jun 2017 21st International C. elegans Conference, UCLA, CA
“The actin-binding and scaffold protein filamin-2 affects C. elegans survival in response to different bacterial food sources”
Jun 2015 20th International C. elegans Conference, UCLA, CA
“The role of the neuropeptide neuromedin U signaling in the sensory influence on C. elegans physiology via food-type recognition”
Apr 2015 3rd Midwest C. elegans Meeting, Van Andel Institute, Grand Rapids, MI
“The role of the neuropeptide neuromedin U signaling in the sensory influence on C. elegans physiology via food-type recognition”
Jul 2014 C. elegans Topic Meeting: Aging, Metabolism, Stress, Pathogenesis and Small RNAs, University of Wisconsin, Madison, WI
“The role of the neuropeptide neuromedin U pathway in the sensory influence on lifespan via food-type recognition”