Chemosensory neuron morphology and ivermectin resistance in *Caenorhabditis elegans*

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Abstract

Nematode parasitic infections present a great disease burden to livestock and human populations in developing countries. Ivermectin is one of the most successful nematocidal antiparasitic drugs however, its success is threatened by the development of drug resistance among parasites. This project investigates a genetic marker of resistance affecting the morphology of chemosensory neurons, so-called dye-filling defective (*dyf*) genes. Using the model nematode *Caenorhabditis elegans,* I examined two hypotheses explaining why Dyf mutations confer drug resistance. Firstly, to test whether Dyf-mediated ivermectin resistance is a result of defective drug entry, I designed a novel technique for measuring the internal concentration of ivermectin using tritium-labelled ivermectin. Secondly, I constructed various *stress response gene;dyf* double mutants to assess whether Dyf-mediated ivermectin resistance occurs via activation of an inappropriate stress response that counteracts drug effects. Despite previous assumptions that Dyf-mediated ivermectin resistance is a result of defective drug entry, my results suggest that drug entry is not affected in Dyf mutants. Instead, my results suggest that *nhr-8,* a nuclear hormone receptor involved in xenobiotic detoxification, and *tbc-2,* a RAB-5 GTPase-activating protein involved in aging and stress resistance, may be required for ivermectin resistance in the *dyf-7* mutant. Hopefully my findings can help unravel the mechanisms underlying ivermectin resistance to combat its spread and inform the development of new antiparasitic drugs.

Résumé

Les infections parasitaires à nématodes représentent une charge de morbidité grand pour le bétail et les populations humaines dans les pays en développement. L'ivermectine est l'un des médicaments antiparasitaires nématocides les plus efficaces, mais son succès est menacé par le développement d'une résistance aux médicaments chez les parasites. Ce projet étudie un marqueur génétique de résistance affectant la morphologie des neurones chimiosensoriels, appelés gènes remplissage-de-teinture dysfonctionnels (*dyf*). En utilisant le nématode modèle Caenorhabditis elegans, j'ai examiné deux hypothèses expliquant pourquoi les mutations Dyf confèrent une résistance aux médicaments. Premièrement, pour tester si la résistance à l'ivermectine médiée par les gènes Dyf est le résultat d'une entrée défectueuse du médicament, j'ai conçu une nouvelle technique pour mesurer la concentration interne de l'ivermectine dans le ver en utilisant de l'ivermectine marquée au tritium. Deuxièmement, j'ai construit divers *gènes de réponse au stress*;*dyf* doubles mutants pour évaluer si la résistance à l'ivermectine médiée par Dyf se produit via l'activation d'une réponse au stress inappropriée qui neutralise les effets des médicaments. Malgré les hypothèses précédentes selon lesquelles la résistance à l'ivermectine médiée par Dyf est le résultat d'une entrée défectueuse du médicament, mes résultats suggèrent que l'entrée du médicament n'est pas affectée chez les mutants Dyf. Au lieu de cela, mes résultats suggèrent que *nhr-8*, un récepteur hormonal nucléaire impliqué dans la détoxification xénobiotique, peut être nécessaire pour la résistance à l'ivermectine chez le mutant *dyf-7*. Espérons que les résultats de ce projet pourront aider à démêler les mécanismes sous-jacents à la résistance à l'ivermectine pour lutter contre sa propagation et éclairer le développement de nouveaux médicaments antiparasitaires.

Acknowledgements

First and foremost, I would like to thank my wonderful supervisor Joseph Dent, Ph.D. for his support, mentorship, trust, endless patience, and most importantly, for letting me have fun with science when I didn't have the energy to be serious. I would also like to thank my two fantastic supervisory committee members, Michael Hendricks, Ph.D., and Stephanie Weber, Ph.D., for their advice and guidance, and for keeping my ambitions grounded in reality. Additionally, I want to thank Jeremy Van Raamsdonk, Ph.D. for his advice and mentorship, and for providing one of the strains used in this project. Penultimately, I would like to thank Jeremy Seltzer, Fabiola Wu, Quisha San Andres, Flynn Baker, and Daniel Kwon; undergraduate students in the lab who provided wonderful conversation and whose presence kept my moral high. Finally, I would like to thank my partner Luc Bourbonnais, B.Eng. for his support and patience, and for genuinely trying to understand the difference between a gene and a protein.

Preface

The following text is a thesis prepared in accordance with the guidelines set out by the McGill Office of Graduate and Postdoctoral Studies.

My thesis consists of four chapters: Chapter 1 includes an outline of my project's rationale and motivation, and a comprehensive review of relevant background information and the related literature; Chapter 2 describes the methods and materials used in my project; Chapter 3 presents and summarizes my results; Chapter 4 includes a comprehensive discussion of all of my findings and a general conclusion.

I was solely responsible for data collection, data analysis, data representation, and for writing and revising my thesis. Myself along with my supervisor Joseph dent constructed strains used in my experiments and collaborated on experimental design.

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List of abbreviations

ABC: ATP-binding cassette

ANOVA: analysis of variance

APOC: African Programme for Onchocerciasis Control

ATP: adenosine triphosphate

Dyf: dye-filling defective

GABA: gamma-aminobutyric acid

GAP: GTPase-activating protien

GluCl: glutamate gated chloride channel

ER: endoplasmic reticulum

IFT: intraflagellar transport system

IVM: Ivermectin

mf: microfilariae

mrp: multi-drug resistance protein

nACHR: nicotinic acetylcholine receptor

Pgp: P-glycoprotein

WT: wild type

1 Introduction and literature review

1.1 Project outline and motivation

Nematode parasitic infections present a large disease burden to livestock worldwide and to human populations in developing countries. The issue was addressed in the early 1970s with the development of ivermectin – one of the most effective veterinary therapeutic agents ever and the foundation for one of the most successful public health programs in history [1]. Unfortunately, its unparalleled success is threatened by the development of ivermectin resistance among parasites [2-8]. Using the non-parasitic nematode *Caenorhabditis elegans* as a model, my project aims to uncover the genetic and mechanistic basis of ivermectin resistance, to slow the spread of resistance and restore the efficacy of ivermectin.

A subset of chemosensory neurons, known as dye-filling neurons for their ability to uptake lipophilic dye, have been implicated in ivermectin resistance in *C. elegans* [9, 10] and in nematode parasites such as *Haemunchus contortus* [11]. Previous work in the lab has identified mutations affecting the morphology of chemosensory neurons, so-called dye-filling defective (Dyf) genes, that eliminate the dye-filling phenotype and cause resistance. To elucidate the mechanisms underlying the relationship between neuron Dyf genes and ivermectin resistance, I examined two hypotheses: 1) defective sensory neurons activate an inappropriate stress response that counteracts drug effects, or 2) affected sensory endings are important for drug entry.

To assess whether Dyf-mediated ivermectin resistance is a result of an inappropriate stress response, I looked for epistasis between mutants defective in candidate stress response pathways and Dyf mutants. I generated *stress response gene;dyf* double mutants to assess whether candidate genes were necessary for Dyf-mediated resistance. If candidate stress response genes are important for resistance, then *dyf;candidte gene* double mutants should be

less resistant than Dyf single mutants. To select candidate genes, I searched the literature for genes implicated in ivermectin resistance, but not yet tested in the context of Dyf-mediated resistance. Additionally, after realizing that there is surprisingly little information on whether Dyf mutants are resistant to types of stress other than ivermectin, I performed an osmotic stress assay to potentially highlight new genes of interest. The results from these assays are discussed in 2.1-2.4.

Should Dyf-mediated ivermectin resistance result from defective drug entry, as suggested by the second hypothesis, we have two predictions: 1) Dyf mutants should take up less ivermectin than wild-type (WT) worms, and 2) the level of resistance should correspond to the number of sensory neurons that dye-fill. To address the first prediction, I designed a novel technique using radiolabelled ivermectin to assess the amount of drug entering the worm. The results from this experiment are discussed in 3.5. To address the second prediction, I helped construct various Dyf strains with dye-filling rescued in specific neurons. Currently Flynn Baker, an undergraduate student in the lab, is performing ivermectin sensitivity assays on the *dyf*-rescue strains. Unfortunately, the assays are not complete, and the results are not discussed here.

1.2 Ivermectin

History and success of ivermectin

Since the first papers on avermectins were published in 1979, ivermectin has continued to prove why it's deserving of the title 'wonder' drug. Ivermectin was first commercialized in 1981, entering veterinary and agricultural markets as an antiparasitic drug [1, 12]. At the time, economic losses due to parasitic infections in livestock were greater than $9 billion (adjusted for inflation) annually in the United States alone [13]. Among many other targets, Ivermectin exhibits potent anthelminthic activity against nematodes and arthropods, the two phyla of animal

parasites responsible for the majority of economic losses [13]. Within two years of being on the market, ivermectin became the veterinary antiparasitic market leader – a spot it has maintained ever since, with annual sales upwards of $1 billion USD [1, 14].

When ivermectin emerged onto the veterinary medicine market, onchocerciasis (river blindness) had long been a major public health concern [1]. Onchocerciasis is caused by the nematode parasite *Onchocerca volvulus* and is pervasive in tropical sub-Saharan Africa, and to a lesser extent in Central and South America [15]. The disease is characterized by skin lesions with severe itching, eye infections, and without proper treatment, permanent blindness [15]. Prior to the discovery and isolation of ivermectin, the only drugs available to treat onchocerciasis were suramin, a toxic molecule administered via a series of injections, and diethylcarbamazine, which had to be administered over multiple weeks and was associated with severe side effects [1, 16]. By the 1970s it became clear that both medications worsened eye lesions caused by onchocerciasis, and they stopped being used as treatments [1].

In the 1980s, large-scale clinical trials of ivermectin began in human populations affected by onchocerciasis [17]. Over multiple trials, treatment with a single oral dose of ivermectin significantly reduced the number of skin and ocular *O. volvulus* microfilariae [17, 18]. At the administered dose, patients treated with ivermectin experienced minimal side effects and severe adverse reactions were rare enough for the drug to be considered for mass use [17]. Throughout the clinical trials, public and private sectors realized that the end-users of the drug were largely individuals in developing countries, many of whom lived in poverty without consistent access to healthcare [1]. Because of the benefit ivermectin promised to provide to marginalized communities, the company that owned the rights to ivermectin promised to provide the drug free of charge to treat onchocerciasis, thus creating one of the biggest drug donation programs in

history [1, 19]. To tackle the issue of accessibility, pioneering research began on community-based treatment using ivermectin [1, 20].

In 1995, the African Programme for Onchocerciasis Control (APOC) was established with the goal of creating sustainable community-directed distribution programs with ivermectin [1]. When the APOC was created, the population at risk was 79.8 million people, with 21.9% being afflicted with onchocerciasis [21]. The disease burden was extreme, with an estimated 2.5 million disability-adjusted life years (DALYs) lost by the time the APOC was established. By 2020, after a successful rollout of mass drug administration programs, the total population at risk had increased to 180 million, however, only 5.1% were afflicted with onchocerciasis [21]. Should this trend continue, it is predicted that by 2030 only 1.8% of the population at risk will be afflicted, and elimination of onchocerciasis in most, if not all, affected countries is considered feasible [21, 22]. However, the future success of mass ivermectin administration programs is threatened by the development of resistance among *O. volvulus* and other ivermectin targets [23].

Ivermectin is a drug for the poor – alleviating the burden presented by neglected tropical diseases in underdeveloped countries around the world [12, 14]. Not only has it drastically improved the health of human populations, but the use of ivermectin in veterinary medicine has greatly increased the efficiency of the agricultural industry [1]. However, despite being on the market for over 40 years, there is still much to be understood about how ivermectin exerts its antiparasitic effect [12]. With the development of resistance among parasites threatening the future of programs such as the APOC [23], it is imperative that we develop a holistic understanding of the drug. Hopefully, through interdisciplinary collaboration between public health agencies, researchers, and industry, ivermectin can remain a ‘wonder’ drug.

Emergence of ivermectin resistance

Over the last 40 years, increasing reports of ivermectin resistance have emerged in animal and human populations [23]. Because ivermectin presents little to no toxicity to the host, overuse is common and has accelerated the evolution of resistance [24]. Between 2002 and 2006 a study analyzing the presence of anthelminthic resistance in *Haemonchus contortus,* a nematode parasite, was conducted on 46 sheep and goat farms in the United States [25]. Of the 46 farms studied, ivermectin resistance in *H. contortus* was detected on 76%. In a later study conducted by the same lab between 2007 and 2009, the incidence of ivermectin resistance in *H. contortus* increased to 97% of the farms studied [26]. Another study done in 2006 on cattle farms in New Zealand reported ivermectin resistance in 92% of 61 farms studied [27]. Similar studies have been conducted on goat, sheep, and cattle farms in Africa, Argentina, Australia, and Brazil, all of which mirror the results above [26].

Ivermectin resistance in human parasites has been slower to develop, potentially due to tighter regulations on drug distribution and administration. Nevertheless, ivermectin resistance in *O. volvulus* has been reported in Cameroon and Ghana [4, 28, 29]. In Cameroon, adult *O. volvulus* reproductive capacity was compared between two cohorts, one with no history of large-scale treatments and one who had received frequent treatment over 13 years [29]. In both groups, the reproductive capacity of adult worms was significantly decreased following treatment with ivermectin. However, when the number of viable and degenerating *O. volvulus* microfilariae (mf) were compared, the previously untreated group showed a significantly greater increase in degenerative mf following treatment with ivermectin [29]. Although ivermectin's embryostatic effect remained in both groups, its efficacy in preventing mf repopulation appears to have decreased in the frequently treated group. Similarly, a longitudinal study in 10 Ghanaian

communities observed significantly greater mf repopulation in 4 out of 9 previously treated communities compared to an ivermectin-naïve community [28].

Despite evidence suggesting the ivermectin resistance is emerging in *O. volvulus,* it is worth noting that poor treatment coverage is an issue among the communities studied and could explain observations of mf repopulation following ivermectin treatment [30]. Without consistent treatment, reinfection with new worms can occur and transmission can increase [30]. Despite, the challenges of studying ivermectin resistance in human parasites, the evidence from studies in livestock is concerning. Should the trends of increasing ivermectin resistance observed in the agricultural industry appear in human populations, millions of lives would be at risk.

1.3 Mechanisms of ivermectin resistance

Mechanism of action of ivermectin

Ivermectin is a macrocylic lactone belonging to the Avermectin family, a class of compounds derived from *Streptomyces avermitilis* that display strong antiparasitic activity [31, 32]. Avermectins exert their antiparasitic effects by binding to glutamate-gated chloride channels (GluCls) expressed in invertebrate neurons and muscle cells [33-35]. To a lower degree, avermectins also bind to other ligand-gated channels including GABA, glycine, and nicotinic acetylcholine [12, 34, 36, 37]. In invertebrates, ivermectin irreversibly binds and activates GluCls, inducing an inhibitory influx of chloride ions into the postsynaptic neuron that results in locomotor and pharyngeal paralysis, and subsequent death of the parasite [14, 38]. In mammalian hosts, ivermectin is minimally toxic as vertebrates lack GluCls [12], and binding affinity for other ligand-gated channels is low [34].

Mechanisms of drug resistance

Mechanisms of drug resistance typically fall into two broad categories: pharmacokinetic mechanisms and pharmacodynamic mechanisms. Pharmacokinetic mechanisms decrease the concentration of drug reaching its target, whereas pharmacodynamic mechanisms involve changes to the drug-drug site interaction. Three common pharmacokinetic mechanisms of drug resistance are 1) upregulation of cellular efflux mechanisms, 2) increased rate of drug metabolism, and 3) decreased drug absorption [39]. Cancer drugs are particularly susceptible to these mechanisms, with elevated efflux of anticancer agents considered the main cause of chemotherapy resistance [40, 41]. Drug efflux is regulated by transmembrane transporters, particularly the ATP-binding cassette (ABC) transporter superfamily [42]. ABC transporters are involved in intrinsic pathways that protect against environmental toxins by pumping them out of the cell, thus decreasing intracellular concentrations [41]. However, overexpression of ABC transporters in tumor cells has been observed in a variety of cancers, decreasing the efficacy of chemotherapeutic agents, leading to resistance [40, 41, 43].

Pharmacokinetic mechanisms have also been implicated in antibiotic resistance, particularly through upregulation of enzymes that metabolize the drugs [44]. In particular, resistance to penicillins and cephalosporins has been attributed to increased production of ß-lactamases, enzymes that inactivate ß-lactams [45].

Pharmacodynamic mechanisms of drug resistance typically include 1) mutations affecting the drug receptor site, and/or 2) decreased expression/availability of drug receptor sites [39]. For example, mutations in the active site of HIV-1 proteases are implicated in resistance to antiviral agents used to treat HIV [46]. These mutations inhibit the drug from binding to HIV-1 proteases, the target, while still allowing the proteases to retain their enzymatic function [47]. Similarly, resistance to antiviral therapies targeted at the Hepatitis C virus occurs through

mutations in the drug receptor site that interrupt the electrostatic network required for drug binding [48].

Resistance to the antiparasitic drug levamisole is also thought to occur via pharmacodynamic mechanisms. Normally, levamisole binds to nicotinic acetylcholine receptor (nACHR) targets in parasites [49]. In resistant worms, changes in the binding site structure and availability compromise the ability of the drug to interact with its target [50-52]. These changes occur through a variety of mechanisms including truncated nACHR subunit genes, reduced transcription of nACHR subunit genes, and reduced transcription of genes required for assembling nACHRs [49].

Of the known mechanisms of drug resistance, many have been proposed to contribute to ivermectin resistance. The magnitude of explanations comes from incongruence between experiments done *in situ* and findings *in natura.* While manipulation of any of the mechanisms described above is likely to induce resistance in an organism, what is observed in the field is another story altogether.

Mechanisms of ivermectin resistance in parasite populations

Early experimental studies on ivermectin resistance focused heavily on alterations to the drug target site, particularly α and ß GluCl subunits. These studies mostly looked at resistance in *Haemonchus contortus,* a common nematode parasite that presents a substantial disease burden to ruminants [53]. Comparison of the α-subunit gene between four strains of *H. contortus,* two selected on ivermectin and two not, revealed an increase in the frequency of one allele in resistant strains [54]. The same study found that the frequency of another allele was reduced in resistant strains, suggesting that it may be associated with susceptibility. Subsequent studies

showed that mutations in GluCls subunits affect ivermectin binding [55, 56], and increase resistance in *H. contortus* [57]. Taken together, these results highlight a possible resistance mechanism involving genetic variability in GluCl subunit genes. However, a link between GluCl subunit genes and ivermectin resistance has not been observed in field-derived nematode parasites, including field populations of *H. contortus* [49, 52].

Studies looking at ivermectin resistance in field-derived nematode parasites suggest an alternative mechanism involving P-glycoprotein (Pgp) drug efflux pumps. Early molecular analyses of Pgp genes in sensitive and resistant strains of *H. contortus* detected polymorphisms thought to be associated with resistance [53, 58, 59]. Interestingly, similar allelic variations were observed in *O. volvulus* isolated from populations treated with ivermectin [60]. Furthermore, Pgp genes were found to be upregulated in resistant field isolates of *H. contortus* [52, 58], and exposure of worm larvae to ivermectin resulted in overexpression of several Pgp genes both *in vitro* [61], and *in vivo* [49, 61, 62].

In addition to correlative evidence, experimental manipulation of Pgp activity supports their role in ivermectin resistance. Co-administration of ivermectin and Pgp inhibitors has been shown to reverse resistance in *H. contortus* [63], and increase the efficacy of ivermectin in sheep [64] and jirds [58, 65]. Nevertheless, neither experimental nor correlative research into the role of Pgps in ivermectin resistance has been able to provide a definitive mechanism capable of explaining the observed field resistance. While changes in Pgp activity can affect ivermectin sensitivity, these changes are not ubiquitous or consistent in resistant field isolates [49].

Recently, a study by Urdaneta-Marquez *et al.* [11] provided compelling evidence for a mechanism of resistance involving changes to the anatomy and function of amphid sensory organs [49]. Their study looked at *Hco-dyf-7,* the *H. contortus* homologue of *dyf-7,* a *C. elegans*

gene involved in the amphid Dyf phenotype observed in ivermectin resistant worms [49]. *H. contortus* strains selected for ivermectin resistance were enriched in an *Hco-dyf-7* haplotype that correlated with decreased *Hco-dyf-7* expression, sensory neuron defects, and the Dyf phenotype. Furthermore, screening for the Dyf phenotype reliably predicted resistance and the presence of the *Hco-dyf-7* haplotype [11]. Resistant field isolates from 5 continents were also enriched in the haplotype, supporting its relevance and pervasiveness [11]. Not only do the findings from Urdaneta-Marquez *et al.* present the most consistent resistance mechanism to date, they also provide a resistance-marker with significant implications for resistance-monitoring diagnostics and future research.

1.4 C. elegans and ivermectin resistance

C. elegans as a biological model organism

The roundworm nematode *Caenorhabditis elegans* is well established as a powerful biological model organism. Although originally introduced to study development and neurobiology, *C. elegans* is now used to study a wide range of biological processes including apoptosis, ageing, sex differentiation, cell signalling, metabolism, and gene regulation [66-68]. Its pervasiveness as a model organism comes from several key advantages not present in other common biological systems [66]. First, *C. elegans* feeds on various bacteria and is easily raised in the laboratory on agar with a lawn of *Escherichia coli.* Second, it reproduces prolifically and develops from an egg to a 1.3mm reproductive adult within 3 days. Third, *C. elegans* are transparent, allowing for easy use of fluorescent markers to track processes such as axonal migration and embryogenesis, and label various cellular components. Fourth, despite having only 959 somatic cells, adult hermaphroditic *C. elegans* are complex multicellular organisms with

different organs and tissues including muscle, hypodermis, a reproductive system, and a nervous system containing 302 neurons [66, 67]. Finally, the *C. elegans* genetic toolbox is extensive, made even more powerful by the completion of its whole genome sequence in 1998 [69].

C. elegans as a model for studying ivermectin resistance

Aside from its general use as a biological model organism, *C. elegans* is particularly well suited as a model for nematode parasites in the context of the present research. It is a powerful system for pharmacological investigation that has been used to elucidate the mechanisms of action and targets of a wide spectrum of drugs [66]. Although *C. elegans* is not parasitic and is genetically divergent from many nematode parasites [70, 71], the mechanism of action of ivermectin is the same in parasitic and nonparasitic nematodes [72]. In fact, GluCls were discovered and characterized in *C. elegans* before being identified as the target of ivermectin in nematode parasites [33, 38, 55].

The application of *C. elegans* as a system to study ivermectin resistance has been validated in many studies. After GluCls were identified as the target of ivermectin, Dent *et al.* showed that simultaneous mutation of three genes encoding GluCl α-type subunits in *C. elegans* conferred high level resistance to ivermectin [9]. Subsequent studies in *H. contortus* found a similar relationship between GluCl function and ivermectin resistance. [7, 52, 57]. Furthermore, Glendinning *et al.* [73] showed that expression of ivermectin sensitive GluCl subunits from *H. contortus* was able to rescue ivermectin sensitivity in a resistant strain of *C. elegans.* Although an ivermectin resistance mechanism involving GluCls has not been found *in natura*, findings from this line of investigation highlighted the value of *C. elegans* as a model for studying ivermectin resistance.

Aside from GluCl-mediated ivermectin resistance, research in *C. elegans* has provided a second, ecologically relevant, mechanism referred to as Dyf-mediated resistance. Dyf-mediated resistance results from mutations in members of a large family of genes called the Dyf genes [74]. Dyf genes are involved in regulating sensory neuron structure and are associated with the dye-filling defective phenotype. Normally, when worms are soaked in a lipophilic dye, the worms will uptake the dye through specialized sensory neuron structures. In dye-filling defective (Dyf) mutants, the dye-filling phenotype is no longer observed. Not only do mutations in Dyf genes result in ivermectin resistance [10], but these mutations evolve in strains selected for ivermectin resistance [75]. Previous work in the lab analyzing IVR6 and IVR10, two *C. elegans* strains selected for ivermectin resistance over multiple generations, identified a frame shift mutation in *dyf*-7 as the cause of resistance [76]. Furthermore, in a study by Page *et al.* [75], *C. elegans* mutagenesis resulted in 17 different ivermectin resistant strains; of these 17 strains, 15 exhibited the Dyf phenotype, while the other two strains displayed positive, but inconsistent dye-filling. Most importantly, as discussed in 1.3, Dyf mutations are observed in ivermectin resistant nematode parasites isolated from the field [11].

Although Dyf mutations are promising as a mechanism of ivermectin resistance, and as a potential resistance marker, little is known about why mutations in Dyf genes cause resistance. Fortunately, the correlates between Dyf-mediated resistance in *C. elegans* and in nematode parasites allow us to take advantage of the superior experimental toolbox available in *C. elegans* to study its mechanism.

Ivermectin resistance and sensory neuron structure

Of the proposed mechanisms of ivermectin resistance in *C. elegans,* the most ecologically relevant and promising of them involves mutations affecting sensory neuron morphology. The

body of *C. elegans* is nearly impenetrable to drugs except at the amphid and phasmid channels, where sensory neurons are exposed to the environment (Fig. 1) [10]. The amphid channels are found at the head of the worm and contain 8 pairs of chemosensory neurons that incorporate environmental cues to regulate behaviours such as chemotaxis, rapid avoidance, and changes in motility (Fig. 2) [77]. In addition to the amphid neurons, *C. elegans* possess bilateral sensory organs in the tail known as phasmids (Fig.1), each containing two classes of chemosensory neurons, PHA and PHB [78]. PHA and PHB detect toxic chemicals in the environment, hyperosmotic solutions, and mechanical stimulation, and work antagonistically with amphid neurons to initiate proper escape behaviours [79].

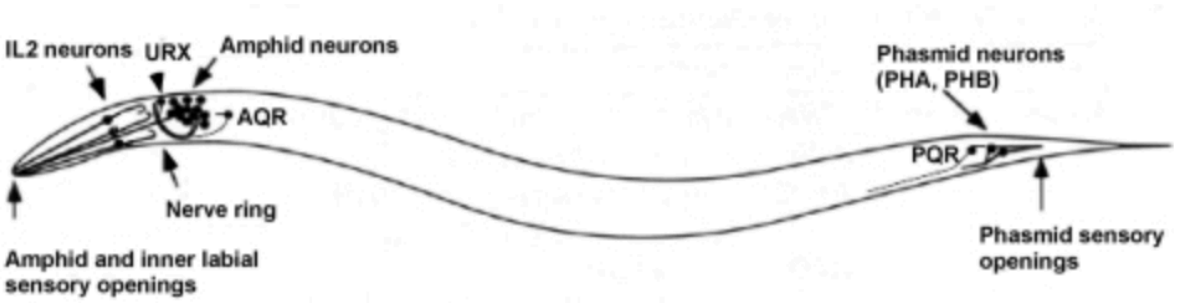

Figure 1 *Sensory system of C. elegans. Amphid neurons are at the head of the worm, phasmid neurons are at the tail. IL2 neurons are a subset of the ciliated amphid neurons that access the environment. (Adapted from [77])*

Sensory neurons in the amphid and phasmid channels have cilia extending from their dendritic endings. A subset of ciliated sensory neurons have cilia that are exposed to the environment; these neurons play a crucial role in integrating external information to guide metabolic and stress responsive changes [80]. The interaction between these neurons and the environment is evident from their ability to take up externally applied lipophilic dyes, such as FITC and DiI (Fig. 2). In ivermectin resistant Dyf mutants, the interaction between ciliated sensory neurons and the environment is compromised due to neuronal morphology defects. Because exposed sensory neuron cilia offer one of few points of access for drugs entering the

worm, one hypothesis explaining Dyf-mediated ivermectin resistance is that Dyf mutations inhibit both the dye and the drug from entering the worm. However, the drug entry hypothesis is mostly speculative, largely based on the dye and ivermectin being both large, bulky, lipophilic compounds with similar chemical properties.

Ciliated neuron	Exposed to the environment?	Embedded in	Dye-fills?	General Role
ASE	Yes	-	No	Chemoattraction
ADF	Yes	-	Yes	Dauer entry
ASG	Yes	-	No	Dauer entry, Chemoattraction
ASH	Yes	-	Yes	Mechanosensory, chemorepulsion, osmo-avoidance
ASI	Yes	-	Yes	Dauer entry, Chemoattraction
ASJ	Yes	-	Yes	Dauer exit/recovery
ASK	Yes	-	Yes	Chemoattraction
ADL	Yes	-	Yes	Chemorepulsion
AWA	No	Sheath cell	No	Chemoattraction
AWB	No	Sheath cell	No	Chemorepulsion
AWC	No	Sheath cell	No	Chemoattraction
AFD	No	Sheath cell	No	Thermosensation
ADE	No	Subcuticle	No	Mechanosensation
IL1	No	Subcuticle	No	Mechanosensation
IL2	Yes	-	Occasionally	Chemosensory
OLQ	No	Subcuticle	No	Mechanosensation
OLL	No	Subcuticle	No	Mechanosensation
CEP	No	Subcuticle	No	Mechanosensation
CEM	Yes	-	No	Mechanosensation
BAG	No	Behind cuticle	No	
FLP	No	Behind cuticle	No	Mechanosensation

Figure 2 *Sensory neurons in and around the amphid channels with their dye-filling status, exposure, and general role. The sheath cell is one of two non-neuronal cells in the amphids, and the cuticle is the C. elegans exoskeleton. (Adapted from Inglis et al. [81] and Lipari [10])*

Ivermectin resistance, dye-filling, and relevant Dyf genes

Although the dye-filling phenotype is well documented in *C. elegans,* little is known about how it works, and even less is known about how it relates to ivermectin resistance. One theory concerning the mechanism of dye-filling is that it involves a combination of passive diffusion and active transport through the plasma membrane [81]. Recently, Razzouti *et al.* [81] showed that disrupting cellular trafficking by depleting intracellular ATP with sodium azide inhibited dye-filling of ciliated sensory neurons, suggesting that dye-filling is ATP dependent. Whether or not disrupted active transport in dye-filling neurons correlates with the mechanism underlying Dyf-mediated ivermectin resistance is unknown.

There are many different Dyf proteins that confer ivermectin resistance in *C. elegans* [23]; however, in the present research there are two proteins of interest: DYF-3 and DYF-7. DYF-7 is an anchoring protein that anchors sensory dendrites during cell migration [82]. In WT worms, sensory neurons originate at the amphid sensory opening (Fig 1) and extend toward the nerve ring [82]. Loss of function mutations in *dyf-7* disrupt the anchoring of sensory dendrites, causing the dendrites to migrate with the cell body towards the nerve ring [82]. In *dyf-7* worms, we observed shortened dendrites in conjunction with the Dyf phenotype [82]. In addition to being Dyf, *dyf*-7 worms exhibit defective chemosensation and a 3-4 fold increase in ivermectin resistance compared to WT [10]. DYF-7 is of particular interest in the context of the present research because its homolog has been implicated in ivermectin resistance in nematode parasites [11].

In addition to DYF-7, the proteins encoded by *dyf-3* are also of interest. *dyf-3* encodes three proteins, DYF-3A, DYF-3B, and DYF-3C [83], which form a subcomplex in the intraflagellar transport (IFT) system. The subcomplex formed by DYF-3 proteins transports cargo necessary for ciliogenesis in subsets of sensory neurons [84]. Disruption of DYF-3 activity

results in truncated sensory cilia and abnormal posterior projections [83]. In addition to defective sensory neuron morphology, *dyf-3* worms exhibit the Dyf phenotype, defective chemosensation, and a 3-4 fold increase in ivermectin resistance compared to WT [10, 83]. Although *dyf-3* has not been implicated in ivermectin resistance in nematode parasites, it is still of interest for two reasons. Firstly, the sensory neuron morphology defects observed in *dyf-3* worms are representative of those observed in numerous other Dyf mutants. In contrast to *dyf-7,* which exhibits shortened sensory dendrites, most Dyf mutants exhibit truncated sensory cilia similar to *dyf-3* worms [10]. Secondly, working with *dyf-3* worms allows us to rescue dye-filling in specific sensory neurons. Cell-specific rescue of dye-filling cannot be done in *dyf-7* worms because DYF-7 is secreted [82], so rescue in one neuron rescues dye-filling in all neurons.

Dyf-mediated ivermectin resistance and relevant stress response genes

Sensory neurons incorporate information from the environment to direct changes in metabolism and stress responses. In *C. elegans,* chemosensory neurons modulate entry into and exit from dauer, an alternative stress-resistant developmental stage triggered by environmental cues in adverse environments [80]. In Dyf mutants, the stress response responsible for dauer entry no longer occurs, resulting in normal development under dauer-inducing conditions [77]. As xenobiotic detoxification and dauer formation involve overlapping stress response pathways [10], researches have hypothesized that *dyf*-mediated ivermectin resistance may be a result of an altered stress response.

C. elegans respond to xenobiotic compounds such as ivermectin in three phases: I) solubilization of lipophilic xenobiotics with drug metabolizing enzymes such as cytochrome P450 superfamily of microsomal enzymes, II) detoxification of harmful substances such as reactive oxygen species or compounds targeted in phase I, and III) removal of xenobiotics by

transporters such as the ATP-binding cassette (ABC) transporters and multi-drug resistance protein (mrp) transporters [10, 85]. Disrupting any of the three phases of xenobiotic detoxification in *C. elegans* has been shown to affect sensitivity to anthelmintics, including ivermectin [86-88]; however, the role of xenobiotic detoxification genes has not been assessed in the context of Dyf-mediated ivermectin resistance.

In the current project, there were three xenobiotic detoxification proteins of interest PMK-1, SKN-1, and NHR-8. PMK-1 is a p38 MAP kinase that positively regulates the transcription factor SKN-1 in response a variety of stressors, including xenobiotic compounds and oxidative stress [89]. SKN-1 upregulates expression of phase II detoxification enzymes in response to xenobiotic compounds and oxidative stress [87, 90]. Its activity is necessary for the increased longevity of certain dauer mutants and may be upregulated when dauer entry is inhibited [91], such as in Dyf mutants. Furthermore, manipulation of SKN-1 activity has been shown to affect the sensitivity of *C. elegans* to anthelmintics. Peddibhotla *et al.* [92] found that a selective inhibitor of the SKN-1 pathway sensitized *C. elegans* to ivermectin and levamisole, but not to albendazole, another type of anthelmintic. Interestingly, Fontaine *et al.* [87] found that direct manipulation of SKN-1 activity through either loss-of-function or gain-of-function mutations, significantly altered sensitivity to albendazole in *C. elegans.* The contradiction between findings from Peddibhotla *et al.* [92] and Fontaine *et al.* [87] could suggest that that the activity of albendazole is less sensitive to changes in SKN-1 activity than ivermectin and levamisole. Alternatively, ivermectin detoxification and albendazole detoxification may involve different pathways.

NHR-8 was of particular interest because of its role in upregulating Pgps and multidrug resistance proteins (mrps) involved in phase III of xenobiotic detoxification. [88, 93]. Increased expression of Pgps has been observed in the ivermectin resistant and Dyf *C. elegans* strain, *osm-*

3 [93]. Similarly, Pgps are overexpressed in the ivermectin resistant *C. elegans* strain IVR10 [94]. Interestingly, Guerrero *et al.* [93] found that disruption of *nhr-8* in *osm-3* worms rescued wild-type expression of most Pgps and significantly increased sensitivity to ivermectin. In WT worms, disruption of *nhr-8* similarly increased sensitivity to ivermectin and decreased expression of several Pgps and mrps [88]. Additionally, Menez *et al.* [88] found that when WT and *nhr-8* worms were selected for ivermectin resistance over multiple generations, nearly all WT worms evolved the Dyf phenotype, whereas almost none of the *nhr-8* worms did. The finding that disrupting *nhr-8* inhibits the evolution of the Dyf phenotype when worms are selected for ivermectin resistance could indicate that Dyf is no longer advantageous in an *nhr-8* background.

TBC-2, an endosomal trafficking protein, was another protein of interest in assessing the role of stress response genes in Dyf-mediated ivermectin resistance. TBC-2 has not been previously linked to ivermectin resistance; however, it is linked to the regulation of sensory cilia length [95] and rescues the stress response of long lived mitochondrial mutants [96]. Selection of *tbc-2* as a gene of interest was partially motivated by the desire to assess whether mitochondrial function is involved in the stress resistance observed in Dyf mutants. Because disruption of *tbc-2* rescues wild-type stress responses in mitochondrial mutants, I thought it may be a good place to start looking at whether mitochondrial dysfunction was involved in Dyf-mediated ivermectin resistance.

2 Materials and methods

2.1 Worm strains

C. elegans strains were maintained at 20 ºC on nematode growth media agar plates seeded with *E. coli* strain HB101 [68]. Unless otherwise mentioned here, strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). *tbc-2(tm2241)* was a kind gift from Jeremy Van Raamsdonk (Research Institute of the McGill University Centre, Montreal, QC). *nhr-8;dyf-7, pmk-1;dyf-7,* and *tbc-2;dyf-7* were constructed by me as described in 2.2.

2.2 Construction of *stress response gene*;*dyf-7* double mutants

Construction of nhr-8;dyf-7

To construct the *nhr-8;dyf-7* mutant I needed a balancer chromosome to be able to identify the presence of *nhr-8(ok186).* As such, I crossed PD4792 males to *dyf-7(m537)*; PD4792 expresses GFP on chromosome IV, the same chromosome as *nhr-8*. F1 progeny were dye-filled as described in 2.3 and non-Dyf, GFP-expressing F1 progeny were singled onto NGM agar plates seeded with *E. coli*. Once the F2 generation had reached L4, they were dye-filled and Dyf, GFP-expressing progeny were singled onto seeded NGM agar plates. I then assessed the F3 generation for ubiquitous GFP expression, signifying homozygosity of the dominant transgene. Since *dyf-7* is recessive, it was safe to assume that F2 and F3 worms selected for the Dyf phenotype were homozygous for *dyf-7.*

Once the *dyf-7*;PD4792 strain was constructed and homozygous for the balancer chromosome, I crossed *nhr-8* males to *dyf-7;*PD4792 worms. F1 progeny were dye-filled and non-Dyf, GFP-expressing worms were singled as before. Once the F2 generation had reached

L4, they were dye-filled and Dyf, non-gfp-expressing worms were singled. The absence of GFP expression indicates that the worms are homozygous for *nhr-8*; however, it is possible that *nhr-8* was lost due to recombination during meiosis. To verify that *nhr-8;dyf-7* worms were homozygous for *nhr-8(ok186),* I singled one worm and allowed it to lay eggs. Once the progeny were adults, I performed single worm PCR on 14 worms with primers for *nhr-8* and ran the PCR products on a gel to ensure that all 14 worms had the band corresponding to the length of the *nhr-8(ok186)*.

Construction of pmk-1;dyf-7

pmk-1 and *nhr-8* are on the same chromosome, so I used the same process as in the construction of *nhr-8;dyf-7* described above. The mutant *pmk-1* allele used was km25.

Construction of tbc-2;dyf-7

WT males were crossed to *dpy-10(e128)* hermaphrodites; males from the F1 generation were crossed with *dyf-7* hermaphrodites. Use of WT males to generate males heterozygous for *dpy-10* was necessary as neither *dyf-7* nor *dpy-10* males mate. Non-Dyf, non-dpy hermaphrodites were singled from F1 generation of the cross between heterozygous *dpy-10* males and *dyf-7.* F2 progeny were scored for Dyf and dpy.

dpy-10;dyf-7 hermaphrodites and crossed to *tbc-2(tm2241)* males. Non-Dyf, non-dpy progeny were singled from the F1 generation, and their progeny were scored for Dyf, non-dpy and singled. F3 progeny were scored for the absence of dpy worms, signifying they were homozygous for *tbc-2(tm2241).* Genotype was confirmed with PCR.

2.3 Generation of males from heat shock

5-6 L4 hermaphrodite worms were transferred NGM agar plates seeded with *E. coli* HB101. 6 plates were used per strain. Plates were placed in an incubator set to 32 ºC for 5 hours. After 96 hrs at 20 ºC, plates were scored for males and males were transferred to a mating plate with 2-3 hermaphrodites to propagate.

2.4 Worm freezing

An egg preparation was performed as described in 2.4, however, eggs were placed on an enriched peptone plate [97] seeded with *E. coli* HB101. Eggs were left to hatch and grow until the worms were starved and there were mostly L1s and L2s on the plate. Worms were washed off the plate with 4ml M9 into a 15ml Falcon tube and placed on ice. Once worms had sunk to the bottom of the tube the volume was adjusted to 3ml, 3ml FSA was added, and the mixture was homogenized by pipetting up and down several times. Three cryovials were labelled with the date and strain name and 2ml of the worm mixture was added to each tube. Cryovials were stored in a -80 ºC Freezer.

2.5 Dye-filling of sensory neurons

Worms were washed of their plate into a 1.5ml Eppendorf tube using 1ml M9 buffer. To wash bacteria off worms, tubes were placed in a microcentrifuge for 1 minute at 1000 rpm. The supernatant was removed and discarded, and the worms were resuspended in 1ml M9. 1ul of 3.75mg/ml stock solution of DiI was added to the tube and the worms were placed on a rocker for 1 hour at 120 rpm. Worms were washed again as described above and resuspended in 200ul

of M9. The worm suspension was then pipetted onto an unseeded agar plate and left to dry for approximately 10 minutes before visualizing under the fluorescent microscope.

2.6 Egg preparation for L1 synchronization

Gravid adult worms were washed as described in 2.3. 200ul of bleach and 200ul of 2M NaOH were added to the Eppendorf tube with the worms. The tube was hand-shaken for 5-10 minutes until 90% of the worm bodies were dissolved. The eggs were then spun down at 10 000 rpm for 15 seconds supernatant was removed, and eggs were resuspended in 1ml M9. The eggs were spun down at 10 000 rpm again for 15 seconds and the supernatant was removed. Eggs were resuspended in 200ul of M9 and pipetted onto an unseeded plate. Eggs were left on the unseeded plate for 24 hrs at 20 ºC for eggs to hatch and arrest at L1.

2.7 Ivermectin sensitivity assays

Assays were performed with at least two biological replicates per strain. An egg preparation was performed as described in 2.6*. Once eggs had hatched and arrested as L1s the worms were washed off their plate with 1ml M9 into 1.5ml Eppendorf tubes and volume was adjusted to 1ml. The solution was then titrated to ascertain the concentration of worms per ml by shaking the solution to homogenize, adding 30ul of the worm suspension to an unseeded plate, and counting the number of worms. Since worms are prone to adhere to plastic, to ensure we were getting an accurate measurement of the concentration of worms in solution, the Eppendorf tubes and all pipette tips coming in contact with the worms were coated in LB, a protein mixture that stops the worms from adhering [98].

If the total number of worms in solution was greater than 1050, an equal volume of worm suspension containing at least 50 worms was transferred to 21 seeded ivermectin plates, 3 plates per concentration (0.01ng/ml, 0.5ng/ml, 1ng/ml, 2ng/ml, 5ng/ml, 10ng/ml, and 20ng/ml). For assays with different strains, the volume of solution added to plates was adjusted so that there would be the same number of worms per plate for each strain. The plates were then placed at 20 ºC for 72 hrs before counting the number of gravid adult worms on each plate. A visual representation of the assay procedure can be found in Figure 3.

*For dose response assays involving stress response mutants, eggs were titrated as above and added directly to seeded ivermectin plates to minimize the influence of starvation on ivermectin sensitivity.

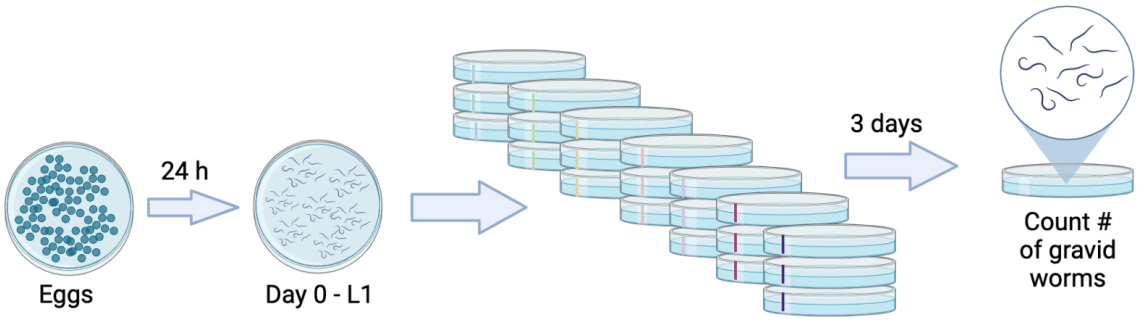

Figure 3 *Ivermectin sensitivity assay procedure. Worms are growth synchronized by performing an egg preparation and allowing eggs to hatch on an unseeded plate and arrest at L1. Equal numbers of worms are then placed on 3 plates at 7 different concentrations (0.01ng/ml, 0.5ng/ml, 1ng/ml, 2ng/ml, 5ng/ml, 10ng/ml, and 20ng/ml). After three days the number of surviving worms that have reached adulthood (gravid worms) are counted for each plate.*

2.8 Osmotic stress assay

25 L4 worms were transferred to seeded 450mM or 550 mM NaCl plates and stored at 20 ºC for 24 hrs. After 24 hrs survival was scored on each plate. Three plates per concentration were used for replicate. 2 biological replicates were completed per strain.

2.9 ^{3}H-Ivermectin assay

I would like to thank Reza Salavati (Institute of Parasitology, McGill University, Montreal, QC) for supplying the tritium labelled ivermectin used in this experiment.

An egg preparation was performed as described in 2.6, however eggs were placed directly onto an enriched peptone plate seeded with *E. coli* HB101. Plates were placed at 20 ºC for 48 hrs until worms reached the L3/L4 stage. 24hrs after the initial egg preparation, 1ul 16Ci/mmol ^{3}H-IVM in EtOH was transferred to a borosilicate tube (1 tube per strain) and the EtOH was allowed to evaporate overnight. 48hrs after the initial egg preparation 1ul DMSO was added to the borosilicate tube to solubilize the ^{3}H-IVM.

Worms were washed off the plate into a 1.5 ml Eppendorf tube with 2ml milliQ H_2O and were spun down in a microcentrifuge at 1000 rpm for 1 min. The supernatant was removed, 1 ml milliQ H_2O was added to the tube, the tube was shaken and spun down as before and this step was repeated twice more. The supernatant was removed to 135 ul and the worm suspension was transferred to a borosilicate tube containing ^{3}H-IVM, along with 0.1 ul DiI. The opening of the borosilicate tube was covered with parafilm, placed in a Styrofoam holder, and rocked for 1 hr at approximately 120 rpm. After 1 hr the worm-^{3}H-IVM solution was transferred to a 1.5 ml Eppendorf tube and 1 ml milliQ H_2O was added. The worms were washed with 3 times with

milliQ H_2O as described above. The supernatant from each wash was retained for analysis. After three washes 100 ul milliQ H_2O was added to the tube and the worm suspension was transferred to an enriched peptone plate. The peptone plate was allowed to dry at room temperature for ~20 minutes before being placed at 20 ºC for 24 hrs.

In between the L4 and adult developmental stages, the worms undergo a final molt where they shed and replace their cuticle. As IVM and the cuticle are strongly hydrophobic, allowing worms to undergo a molt during their 24 hrs on food is crucial for avoiding measuring radioactivity from drug stuck to the cuticle. After 24hrs on food, worms are washed with milliQ H_2O twice and titrated three times to determine the concentration of worms before adding them to 15 ml Ultima Gold scintillation fluid. The first wash after 24 hrs on food is retained to assess whether the molt occurred and whether it was effective in removing ^{3}H-IVM. All samples are placed in the scintillation counter and radioactivity is counted for 10 minutes per sample using a program designed to measure radioactivity due to the presence of tritium.

To ensure that the worms are not consuming the drug, the amount of dye in the intestine is assessed before and after being on food. Worms with dye in their intestine are removed from the sample. It is crucial that the presence of dye in the intestine is assessed both before and after being on food as the presence of dye before being on food does not correlate with the presence after 24hrs, yet is indicative of intestinal ivermectin, greatly affecting the measurement (H. J. Shields, unpublished observation). Although worms with intestinal DiI are removed from the sample, there is no evidence to support a direct correlation between intestinal DiI and intestinal ivermectin. As such, it is possible that residual intestinal ^{3}H-IVM accounts for a percentage of the radioactivity in the sample.

At least four biological replicates were performed per strain.

2.10 Statistical analysis

Ivermectin sensitivity assays

Data from ivermectin sensitivity assays was analyzed in MATLAB using the bootstrap technique. Bootstrapping provides the SEM and the lower and upper 95% confidence intervals. Significance was assessed by looking for overlap between the confidence intervals. For comparison of changes in EC_{50} values error was propagated and confidence intervals were obtained using the following formula:

$$\mathrm{CI} = \Delta \mathrm{EC}_{50} \pm (1.96)(\mathrm{SEM})$$

Overlap of confidence intervals indicated that values were insignificantly different.

Osmotic stress assay

Generation of figures and statistical analysis of data from osmotic stress assays was performed in GraphPad Prism. Comparison of means was done by a one-way analysis of variance (ANOVA) with $P \leq 0.05$ indicating significantly different means.

^{3}H-IVM assays

To account for the efficiency of the scintillation counter, measurements of radioactivity in counts per minute beta (CPMB) were converted to disintegrations per minute (DPM) using the following equation:

$$DPM = \frac{CPMB}{\%\ efficiency\ of\ counter}$$

Efficiency values were found by comparing the quenching value (tSIE) of each sample to the quench curve of the scintillation counter (tSIE vs. efficiency).

The effect of variations in wash efficiency and dose on sample measurements was analyzed by linear regression. Each dataset was fit to the linear model:

$$y = \beta_1 x_1 + \beta_2 x_2 + \cdots + \epsilon_i$$

For confounding variables x_i with non-zero β_i ($P \leq 0.05$), the dependent variable, y, was corrected to remove the effect of x_i:

$$y_i^* = y_i - \beta_i x_i$$

Comparison of the mean internal ivermectin concentration per worm in the ^{3}H-IVM assay was done by a one-way ANOVA with $P \leq 0.05$ indicating significantly different means. Measurements were corrected for confounding effects of variations in dose and wash efficiency as described above. Corrected DPM measurements were converted into nanograms of ivermectin:

$$nanograms\ IVM = (DPM)(2.32458 \times 10^{-5})$$

To estimate the internal concentration of ivermectin per unit volume, the internal concentration was divided by $3 \times 10^{-6} ml$, an estimate of the total body volume of L4 worms [99]. SEM was calculated for the number of worms in each replicate and the error was propagated.

3 Results

3.1 NHR-8 is required for dyf-mediated ivermectin resistance

I was interested in assessing the role of NHR-8 in Dyf-mediated ivermectin resistance because of its function as a regulator of Pgp expression in response to xenobiotic compounds. Increased Pgp expression has been linked to xenobiotic resistance in both WT and Dyf worms [93]. Before testing the role of individual Pgps in Dyf-mediated resistance, I wanted to first investigate whether NHR-8 was involved. To this end, I constructed an *nhr-8;dyf-7* double mutant and assessed its level of resistance compared to *dyf-7* worms.

Results from the dose response assays with *dyf-7* and *nhr-8* mutants show that in *dyf-7* mutants, disruption of *nhr-8* significantly increased ivermectin sensitivity (Fig 4A). The level of resistance is indicated by the EC_{50}; the concentration of ivermectin at which half the worms do not develop into gravid adults. Interestingly, disruption of *nhr-8* in *dyf-7* worms does not rescue ivermectin sensitivity to WT levels (Fig 4A), suggesting that upregulation of *nhr-8* is not entirely responsible for Dyf-mediated ivermectin resistance. It is possible that certain *pgp* genes are overexpressed independent of NHR-8; however, further experiments are required to determine the contribution of other genes.

In line with past research [88], disruption of *nhr-8* alone increased ivermectin sensitivity compared to WT (Fig 4A). To assess whether the effect of *nhr-8* disruption on Dyf-mediated resistance was actually evidence of epistasis between the mutants, and not an additive effect, I compared the magnitude of the change in sensitivity between *nhr-8;dyf-7* and *dyf-7* with the change between WT and *nhr-8* (Fig 4B). I found that the increase in sensitivity resulting from disruption of *nhr-8* in *dyf-7* worms is significantly greater than that resulting from disruption of

nhr-8 in WT worms (Fig 4B). The significant difference between the changes in sensitivity implicates NHR-8 in the mechanism underlying ivermectin resistance in *dyf-7* worms.

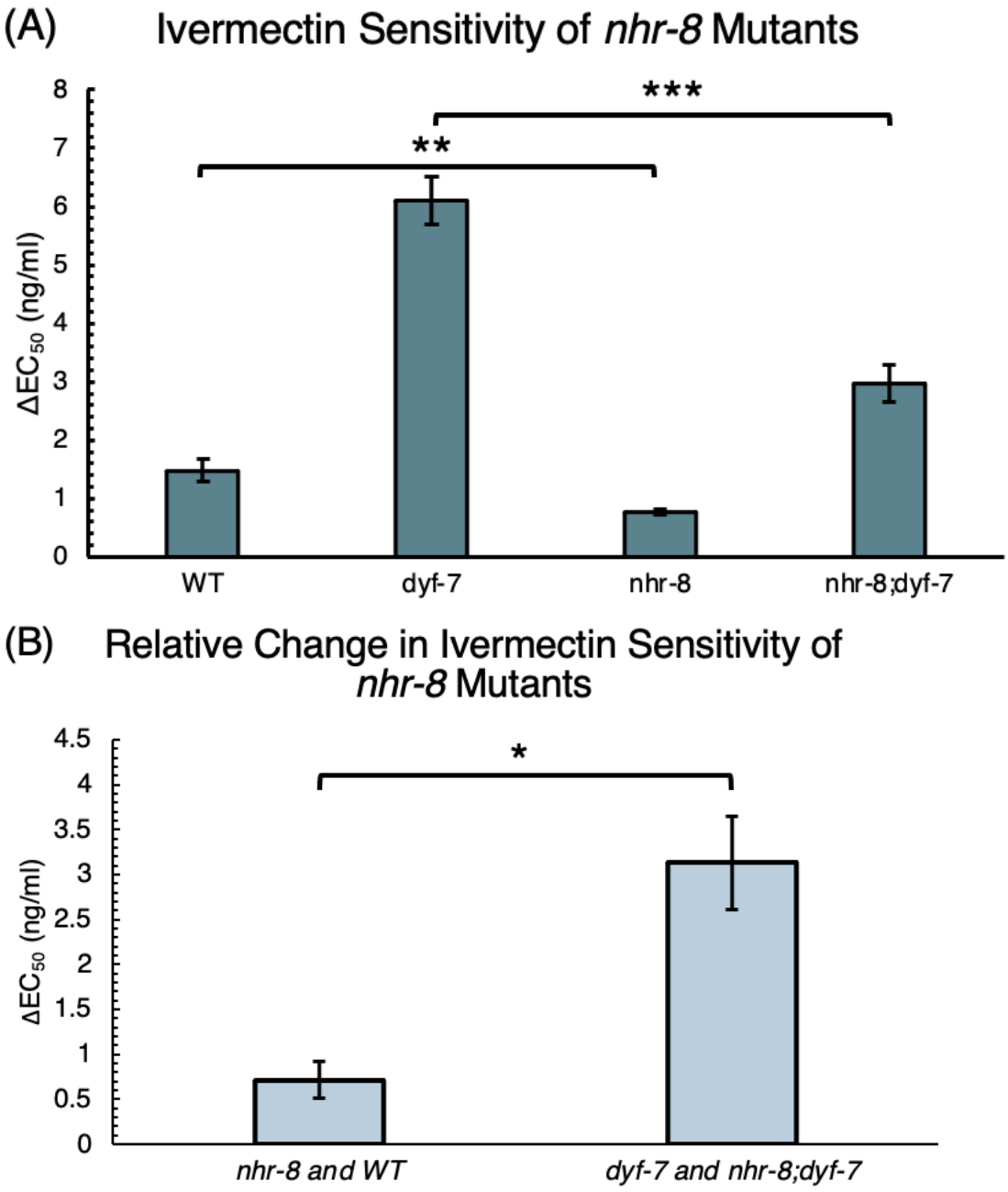

Figure 4 *Disruption of nhr-8 increases ivermectin sensitivity in dyf-7 worms. (A) EC_{50} of WT, dyf-7, nhr-8, and nhr-8;dyf-7 worms. EC50 is the concentration of ivermectin at which half the worms fail to develop into gravid adults. (B) Increase in ivermectin sensitivity between* nhr-8 *and* WT *relative to the increase from* Dyf-7 *to* nhr-8;Dyf-7.

3.2 PMK-1 is not required for dyf-mediated ivermectin resistance

The PMK-1/SKN-1 pathway is involved in xenobiotic detoxification and detoxification of harmful compounds resulting from oxidative stress. As Dyf mutants are also resistant to paraquat-induced oxidative stress [100], I was interested in whether the PMK-1/SKN-1 pathway may play a role in the mechanism underlying Dyf-mediated ivermectin resistance. Similar to 3.1, I constructed a *pmk-1;dyf-7* double mutant and assessed its level of resistance compared to *dyf-7* worms.

Disruption of *pmk-1* in *dyf-7* mutants had no significant effect on the level of ivermectin resistance in *dyf-7* mutants (Fig 5A). Interestingly, *pmk-1* single mutants showed a slight but significant increase in sensitivity compared to WT worms (Fig 5A); qualitatively, a similar increase can be seen when comparing the EC_{50} of *pmk-1;dyf-7* and *pmk-1.* However, the magnitude of the increase in sensitivity from WT to *pmk-1* is not significantly different from the increase from *dyf-7* to *dyf-7;pmk-1* (Fig 5B).

Unfortunately, ivermectin sensitivity assays with *skn-1;dyf-7* mutants could not be performed as the available strain was not constructed properly and lacked the Dyf phenotype. However, results from ivermectin sensitivity assays with the *skn-1* single mutant (results not shown here) are consistent with past findings and provide evidence that mutations in *skn-1* increase sensitivity to ivermectin in a wild-type background.

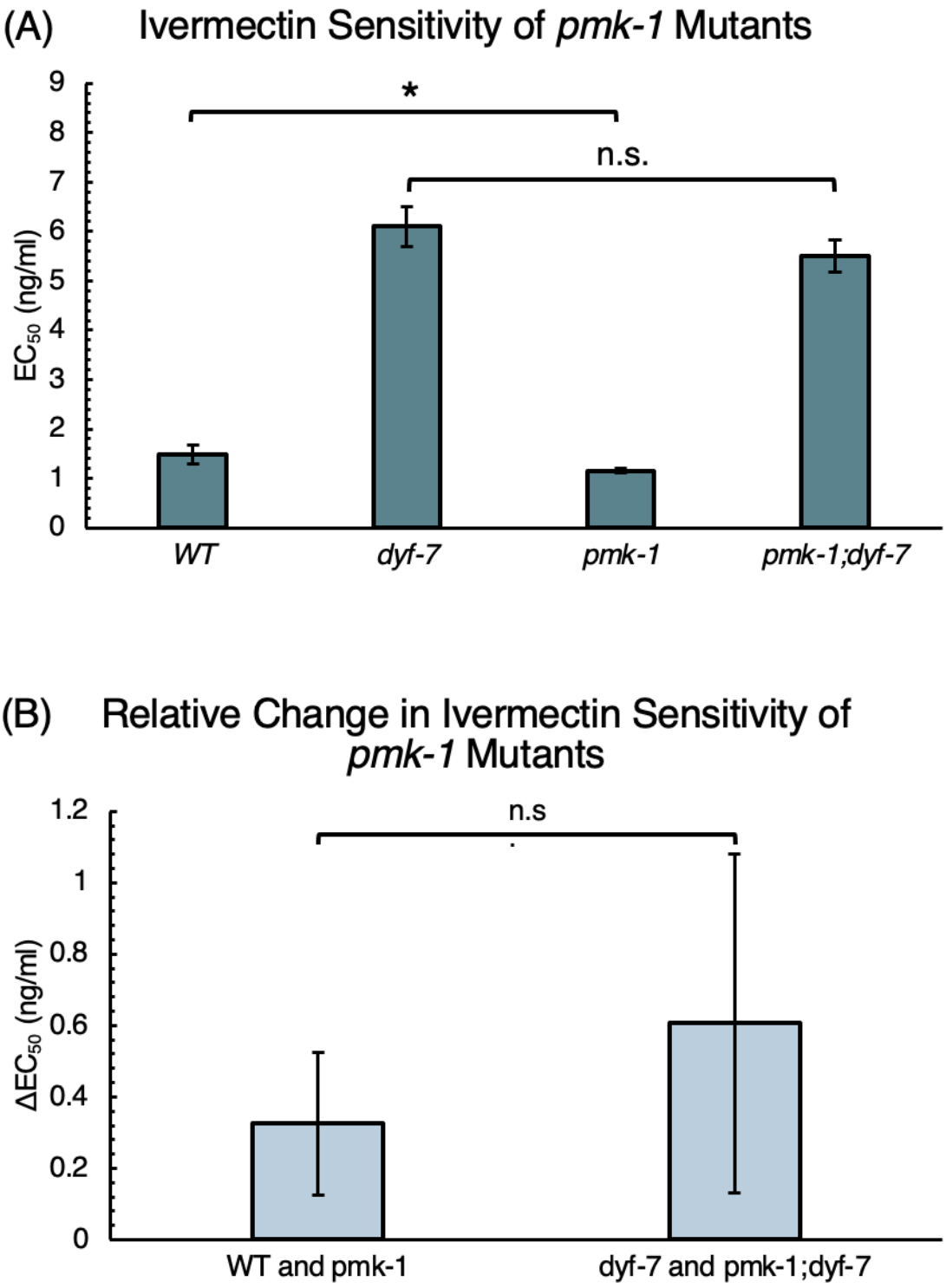

Figure 5 *Disruption of pmk-1 does not significantly affect ivermectin sensitivity in dyf-7 worms. (A) EC_{50} of WT, dyf-7, pmk-1, and pmk-1;dyf-7 worms. EC_{50} is the concentration of ivermectin at which half the worms fail to develop into gravid adults. (B) Change in ivermectin sensitivity between* pmk-1 *and* WT *relative to change between* Dyf-7 *and pmk-1;dyf-7.*

3.3 TBC-2 is required for dyf-mediated ivermectin resistance

TBC-2 is an endosomal trafficking protein that has been implicated in the regulation of sensory neuron cilium length [95], and of the longevity and stress resistance of long-lived mitochondrial mutants [96]. Because of its role in regulating sensory neuron structure and stress resistance, *tbc-2* was of interest in the study of Dyf-mediated ivermectin resistance. To assess the role of *tbc-2* in Dyf-mediated ivermectin resistance, I constructed a *tbc-2;dyf-7* double mutant and assessed its relative level of resistance compared to *dyf-7.*

My results suggest that *tbc-2* isbe required for Dyf-mediated ivermectin resistance. Disruption of *tbc-2* activity in *dyf-7* mutants completely rescues ivermectin sensitivity (Fig 6A). I observed no significant difference between the *tbc-2;dyf-7* double mutant compared to the *tbc-2* single mutant or wild-type (Fig 6A). In addition to the increased sensitivity of *tbc-2;dyf-7* mutants compared to *dyf-7*, the *tbc-2* single mutant showed a slight increase in sensitivity compared to WT. However, mutating *tbc-2* increased sensitivity significantly more in the *dyf-7* background compared to the WT background (Fig 6B), suggesting that *tbc-2* mediates ivermectin sensitivity along a *dyf*-specific pathway.

Qualitatively, the results from ivermectin sensitivity assays performed on the *tbc-2* mutants differed from others discussed. Normally, when I count the number of worms on each ivermectin plate, I observe mostly gravid worms and a minority that are L4s, which are excluded. However, with both *tbc-2* and *tbc-2;dyf-7* there was a wide range of developmental stages present on the plates. Interestingly, the staggered developmental phenotype was exacerbated in the *tbc-2;dyf-7* mutant compared to *tbc-2.*

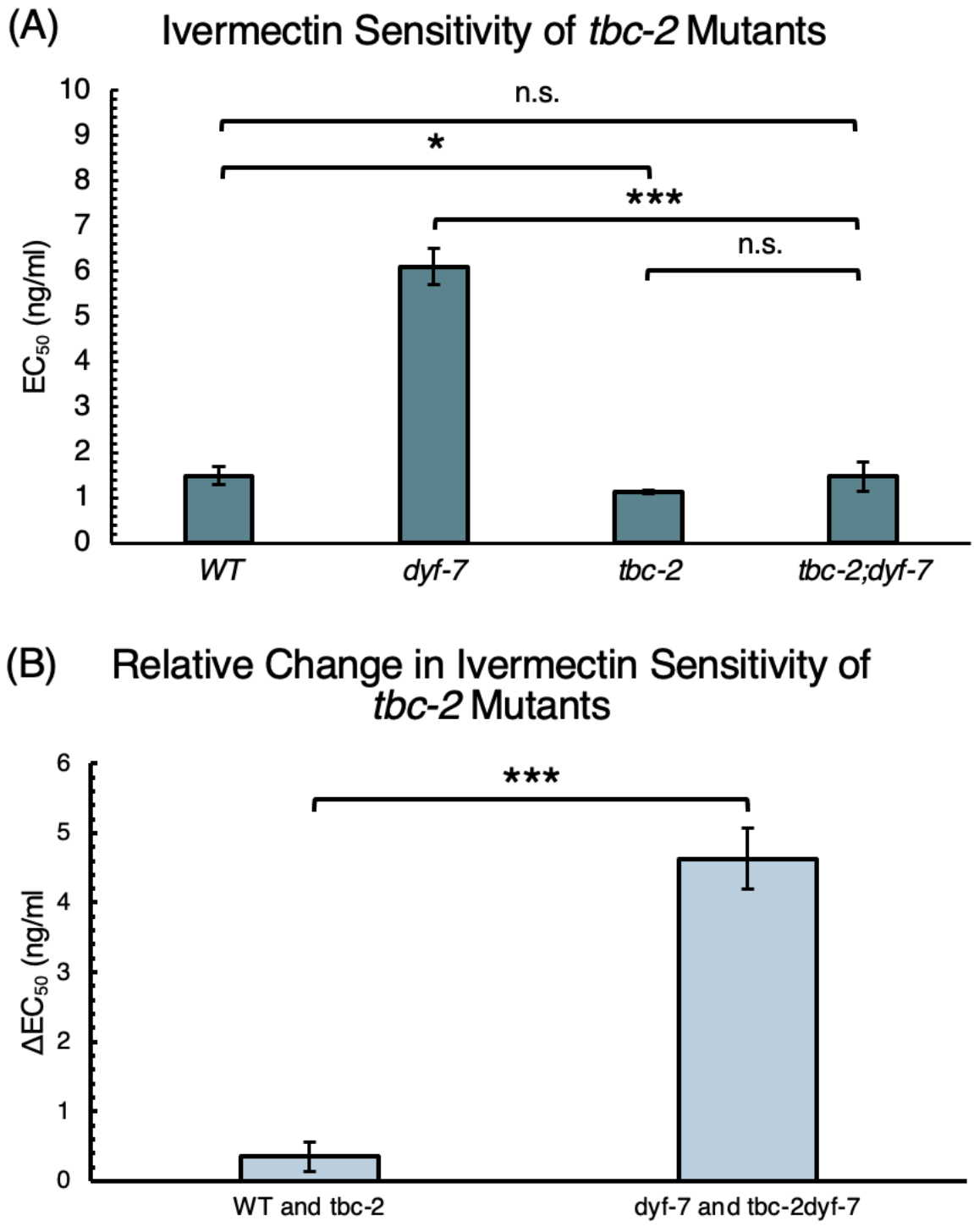

Figure 6 *Disruption of* tbc-2 *increases ivermectin sensitivity in* Dyf-7 *worms. (A)* EC_{50} *of WT,* Dyf-7, tbc-2, *and* tbc-2;Dyf-7 *worms.* EC_{50} *is the concentration of ivermectin at which half the worms fail to develop into gravid adults. (B) Change in ivermectin sensitivity between tbc-2 and* WT *relative to change between dyf-7 and tbc-2;dyf-7.*

3.4 Dyf mutants are resistant to osmotic stress

Originally, I had planned to perform RNA sequencing on WT, *dyf-3,* and *dyf-7* to help inform my selection of the stress response genes I wanted to investigate. Unfortunately, because of troubleshooting and time-constraints, I was unable to do so. To supplement candidate gene selection, I performed an osmotic stress assay to assess whether genes involved in osmotic stress resistance may be involved in Dyf-mediated ivermectin resistance. Osmotic stress induces a change in the movement of water across the cell membrane; in hyperosmotic conditions, such as in the assays I performed, water is pulled out of the cells leading to collapse of the *C. elegans* hydrostatic skeleton and paralysis of the worm [101]. Although the mechanism of osmotic stress-induced death is not analogous to that of ivermectin-induced death, many osmotic stress response genes are involved in xenobiotic detoxification [102].

An acute osmotic stress assay revealed that both *dyf-3* and *dyf-7* mutants are resistant to osmotic stress (Fig 7). Although I do not have time to investigate the role of known osmotic stress response genes in Dyf-mediated resistance, I am hopeful that these results can add to our understanding of Dyf mutants and guide future research.

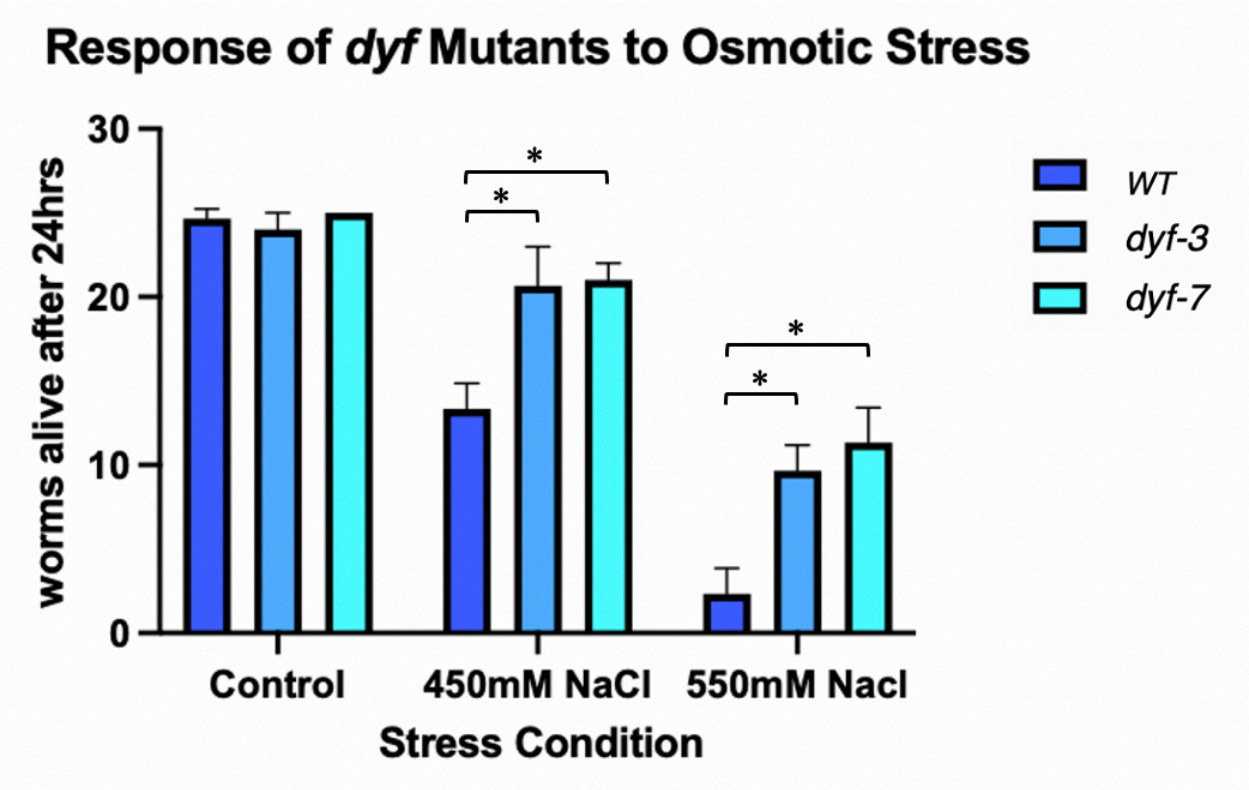

Figure 7 *Dyf mutants are resistant to osmotic stress. Comparison of survival after 24 hrs on 450mM NaCl or 550mM NaCl between WT, dyf-3, and dyf-7 worms.*

3.5 Internal ivermectin concentration is not significantly affected by Dyf mutations

Since Dyf-mediated ivermectin resistance was first discovered, researchers have been quick to assume that it is a result of defective drug entry. In theory the effect of Dyf mutations on ivermectin entry should be easy to test with the use of fluorescent markers; in practice, the chemical structure of ivermectin has made it difficult to fluorescently tag the drug without significantly hindering its efficacy. As such, I decided to use tritium labelled ivermectin (^{3}H-IVM) to assess whether drug entry is affected in Dyf mutants. Initially, I planned to use autoradiography to visualize the localization of ^{3}H-IVM in the worms; unfortunately, the level of radioactivity in our samples was too low to expose our visualization medium. Instead, we

designed a novel technique using a scintillation counter to assess the internal concentration of ^{3}H-IVM.

Scintillation counters detect and measure ionizing radiation by counting the number of light pulses resulting from excitation of the scintillation fluid by incident radiation. A known number of worms exposed to ^{3}H-IVM were added to scintillation fluid and placed in a scintillation counter to measure the radioactivity by counting the number of beta particles emitted per minute (CPMB). To account for the efficiency of the counter, the CPMB measurements were converted to DPM, the absolute activity of the sample. The total DPM per sample was divided by the number of worms in the sample to get the activity per worm in DPM.

Because ivermectin and the *C. elegans* cuticle are both lipophilic, the drug is prone to adhering to the outside of the worm. To ensure that the assay assessed only the internal ivermectin concentration, external presence of the drug had to be removed. After initial exposure, the worms are washed three times and transferred to a seeded plate for 24 hours to molt, thus shedding any residual ^{3}H-IVM stuck to the cuticle. After 24 hours on food the worms are washed again before being added to scintillation fluid. The supernatant from each wash was retained for analysis to ensure that the drug was being effectively removed from the exterior of the worm.

Analysis of the washes revealed that the percent of total activity (sum of the activity in sample, wash 1, wash 2, wash 3, and wash 4) absorbed by the worms is not significantly correlated with the percent of activity found on the plate after molting (wash 4, Fig 8A). Furthermore, the percent of total activity in wash 4 is significantly greater than that in wash 3 ($P < 0.05$) (Fig 8B). If inconsistencies in the amount of drug being removed from the cuticle during molting were affecting measurements of the internal concentration, I would expect to observe a

negative correlation between the percent of total activity in the sample and the percent of total activity in wash 4. Additionally, if ivermectin was not being removed from the cuticle during molting, I would expect that the percent of total activity in wash 4 would be less than in wash 3, directly prior to being transferred to the seeded plate. Taken together, my results suggest that excess ivermectin is being successfully removed from the cuticle during molting. Furthermore, percent of total activity in each wash remained constant across replicates (Fig 8B), supporting the conclusion that results are not being influenced by inconsistencies in the removal of drug from the exterior of the worm.

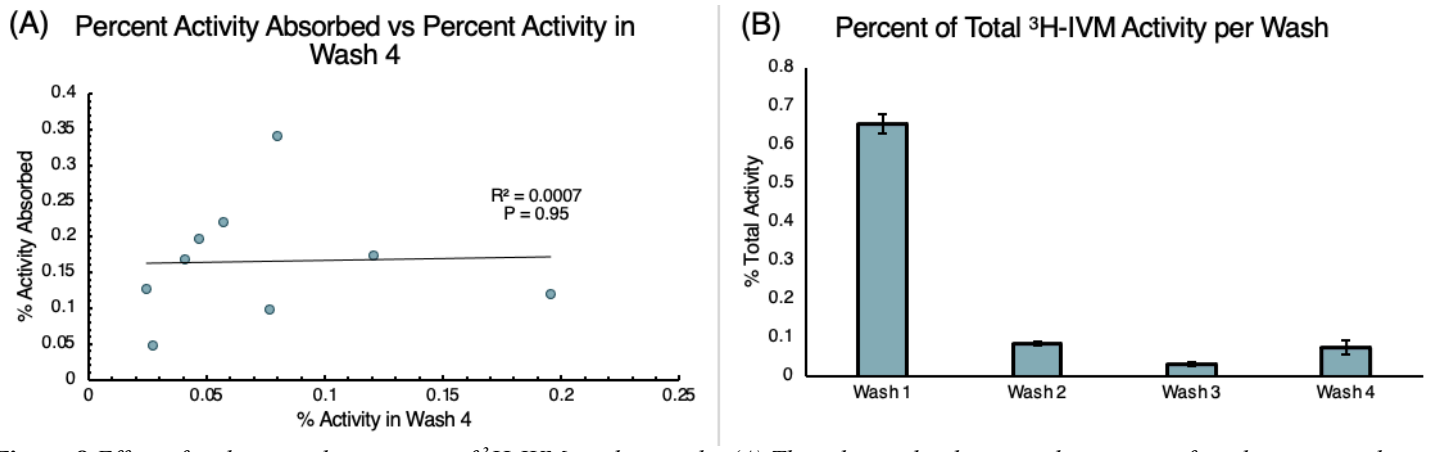

Figure 8 *Effect of molting on the presence of ^{3}H-IVM on the cuticle. (A) The relationship between the percent of total activity in the sample vs the percent of total activity in wash 4. Wash 4 contains activity from ^{3}H-IVM left on the plate after worms spend 24hrs on food. (B) Percent of total activity in each wash. Total activity is the sum of activity in the sample, wash 1, wash 2, wash 3, and wash 4.*

In addition to adhering to the cuticle, ivermectin also adheres to the pipette tips during transfer of the drug, potentially affecting the concentration of drug that the worms are exposed to. Using the sum of the activity in the washes as an indicator of external concentration, analysis revealed inconsistencies in the concentration of drug that worms were exposed to across replicates (Fig 9A). Furthermore, external concentration of ^{3}H-IVM negatively correlated with DPM/worm (Fig 9A), suggesting that the concentration of drug that worms are exposed to

affects the concentration inside the worm. To allow for comparisons of internal concentration across replicates, DPM/worm was adjusted for the external concentration (Fig 9B). The relationship between external concentration and DPM/worm did not significantly change when analyzed for the different strains, thus all samples were adjusted uniformly.

The effect of external ivermectin concentration on measurements of the internal activity was also observed when analyzing the relationship between the percent of total activity in the sample and the number of worms. As the number of worms in the sample increases, one would predict that the percent of the total activity in the sample would increase linearly. However, no significant relationship between the percent of total activity absorbed and the number of worms was observed in the raw data (Fig 9C). A multiple linear regression revealed a significant relationship between the external concentration of ivermectin and the percent of total activity absorbed by the worms ($P < 0.05$). Adjusting the percent activity in the sample for the external concentration rescued the relationship between the percent of total activity absorbed and the number of worms (Fig 9D).

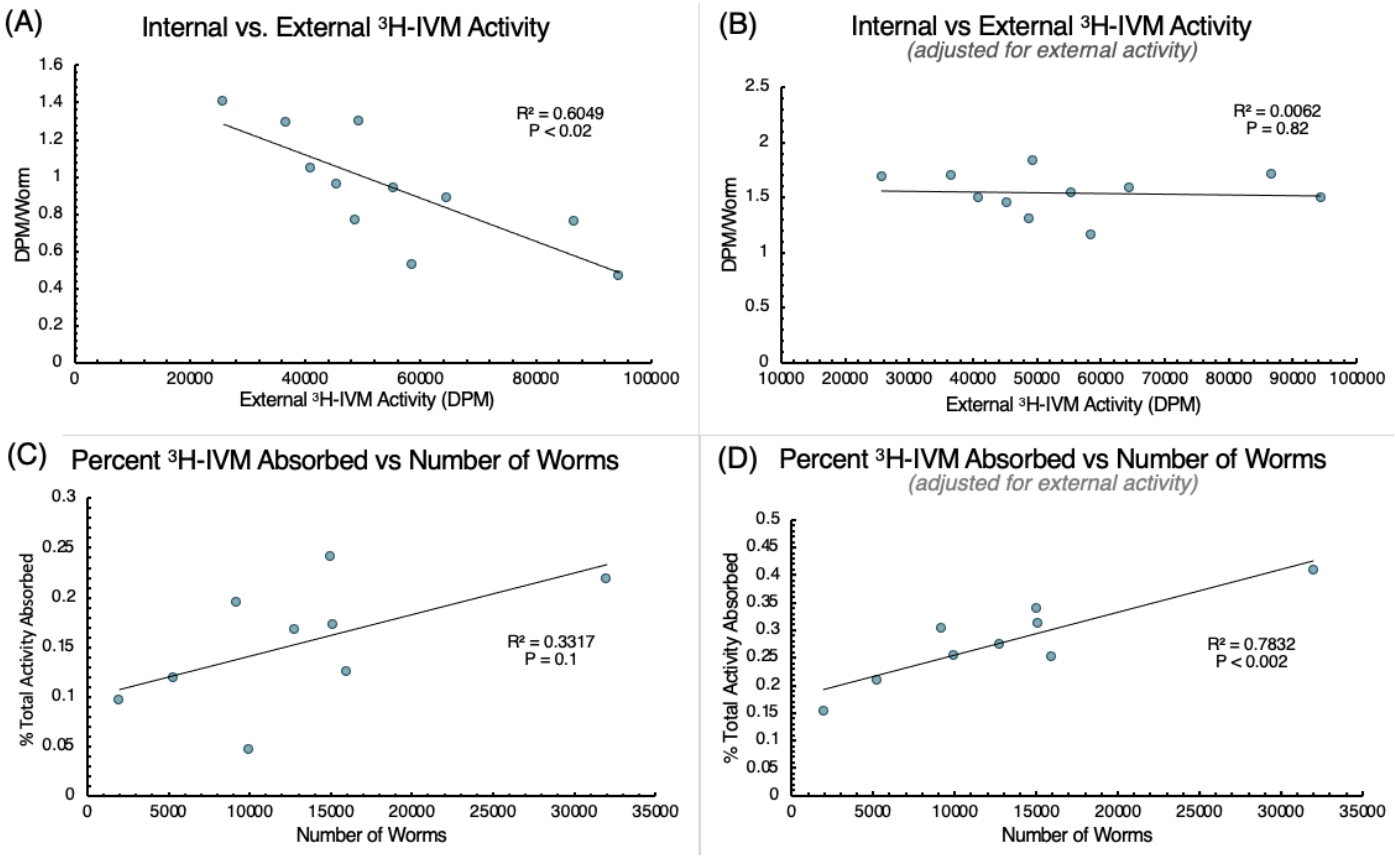

Figure 9 *External [^{3}H-IVM] negatively affects the concentration of ivermectin inside the worms. (A) The relationship between DPM/worm and the external ^{3}H-IVM concentration. The external concentration is indicated by the sum of the activity in each wash. (B) The relationship between internal vs external ^{3}H-IVM activity after correcting for differences in external ^{3}H-IVM activity. (C) The relationship between the percent of total activity in the sample and the number of worms. (D) The relationship between the percent of total activity in the sample and the number of worms corrected for external ^{3}H-IVM activity.*

After correcting for the effect of the concentration of ivermectin that worms were exposed to, I converted measurements of DPM/worm to ng/ml to assess differences in the internal concentration of ivermectin between strains. The internal concentration of ivermectin in WT worms is not significantly different from either *dyf-3* or *dyf-7* (Fig 10), suggesting that drug-entry is not significantly affected in Dyf worms.

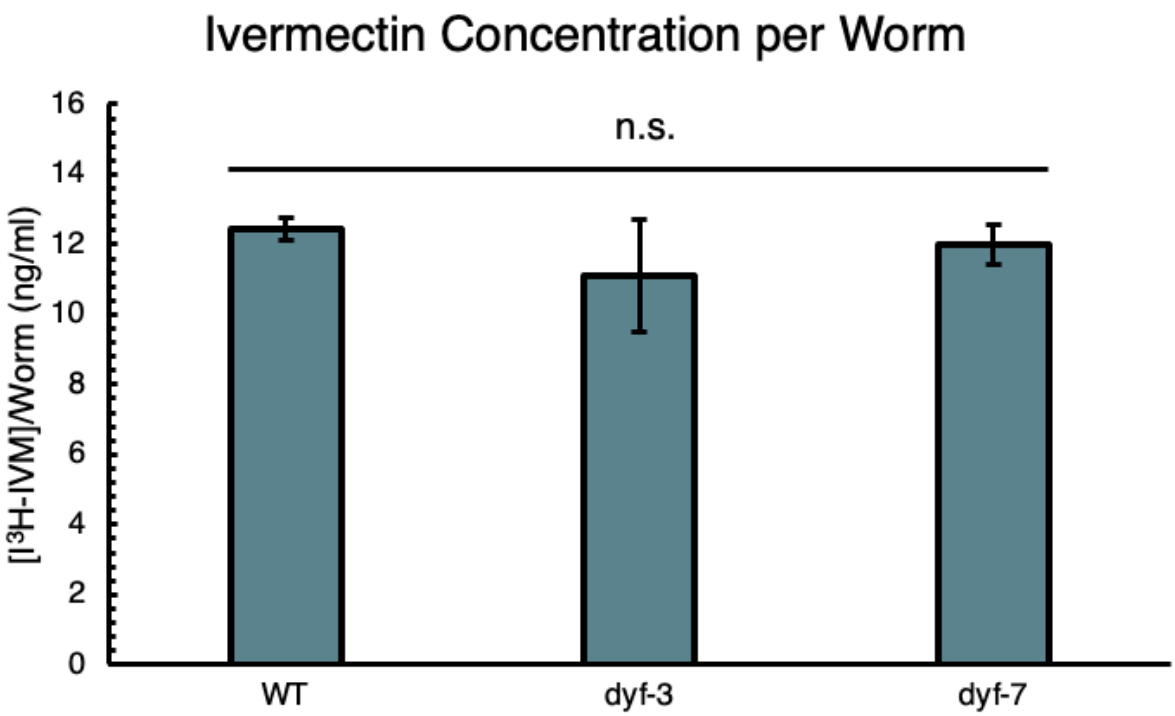

Figure 10 *Drug entry is not significantly affected by dyf mutations. Mean [^{3}H-IVM] concentration per worm for wild-type worms is not significantly different than to dyf-3 worms or dyf-7 worms. Additionally, there is no significant difference in the mean [^{3}H-IVM] concentration per worm between dyf-3 and dyf-7 worms.*

4 Discussion and conclusion

4.1 Discussion of results

The role of stress response genes in ivermectin resistance

One of the two hypotheses explaining Dyf-mediated ivermectin resistance is that defective sensory neuron morphology activates an inappropriate stress response that counteracts drug effects. In the first half of my project, I wanted to test the stress response hypothesis by looking for epistasis between mutants defective in candidate stress response pathways and Dyf mutants. My results suggest that NHR-8, a nuclear hormone receptor that upregulates expression of phase III xenobiotic detoxification enzymes, is required for Dyf-mediated ivermectin resistance. *nhr-8;dyf-7* worms were significantly more sensitive to ivermectin than *dyf-7* worms. Consistent with past results [88], disruption of *nhr-8* in WT worms also significantly increased sensitivity. However, the increase from *dyf-7* to *nhr-8;dyf-7* was significantly greater than from WT to *nhr-8,* indicating that *nhr-8* is involved in the mechanism underlying Dyf-mediated ivermectin resistance, and does not regulate ivermectin sensitivity through a parallel pathway.

Disruption of *nhr-8* in *dyf-7* worms did not completely rescue ivermectin sensitivity. One possibility is that in *dyf-7* worms certain phase III genes, namely Pgps and mrps, are upregulated independent from *nhr-8*. Guerrero *et al.* [93] performed a similar experiment in *osm-3* mutants; another strain of ivermectin resistant Dyf mutants. In addition to assessing resistance of *osm-3* and *nhr-8;osm-3* double mutants, they compared expression of Pgps [93]. Multiple Pgps were upregulated in *osm-3* mutants, and were downregulated in *nhr-8;osm-3* worms. However, upregulation of certain *pgp* genes, namely *pgp-3* and *pgp-5,* was not rescued by disruption of *nhr-8* [93]. Like my results, disruption of *nhr-8* in *osm-3* worms did not completely rescue ivermectin sensitivity [93], possibly as a result of residual overexpression of Pgps.

In addition to NHR-8, I assessed the role of the p38 MAP kinase, PMK-1, in Dyf-mediated ivermectin resistance. PMK-1 is involved in the longevity of long-lived mitochondrial mutants, regulates various stress response pathways, and activates the SKN-1 transcription factor in response to xenobiotic compounds [89]. Disruption of *pmk-1* in *dyf-7* mutants resulted in a slight, but insignificant increase in sensitivity, while disruption of *pmk-1* in WT worms resulted in a slight, but significant increase in sensitivity. However, the effect of *pmk-1* in WT worms and *dyf-7* mutants appears to be additive, suggesting that its effect on ivermectin sensitivity occurs via a parallel mechanism to that underlying Dyf-mediated resistance.

Aside from resistance to ivermectin, Dyf mutants are also resistant to other forms of xenobiotic stress. Guerrero *et al.* found that *osm-3* mutants were resistant to tunicamycin (TM), a xenobiotic compound that induces ER stress. Disruption of *nhr-8* in *osm-3* mutants significantly increased sensitivity to TM [93]; on the other hand, disruption of *pmk-1* had little to no effect on TM resistance. Pairing my results and those from Guerrero *et al.* [93] suggests that Dyf-mediated ivermectin resistance is regulated by a general xenobiotic stress mechanism conserved across Dyf mutants. Far more is known about xenobiotic stress resistance in *C. elegans* than is known about Dyf-mediated ivermectin resistance; should the two be regulated by a conserved mechanism, we would be far closer to understanding ivermectin resistance in both *C. elegans,* and nematode parasites.

In addition to regulating expressing of phase III xenobiotic detoxification enzymes, *nhr-8* regulates cholesterol balance, fatty acid desaturation, apolipoprotein production, and bile acid metabolism [103]. Through regulating bile-acid like steroid and cholesterol availability, NHR-8 regulates DAF-12/NR, a nuclear receptor that involved in determining entry into the stress resistant developmental stage, dauer [104]. Dyf mutants are dauer defective – one of the main

reasons stress response genes were implicated in Dyf-mediated ivermectin resistance. Interestingly, *nhr-8* mutants exhibit enhanced entry into dauer compared to WT worms, along with other phenotypes such as developmental arrest, unsaturated fatty acid deficiency, reduced fertility, and shortened life span [103].

Another piece of evidence suggesting that Dyf-mediated ivermectin resistance may be mediated by metabolic changes comes from my initial ivermectin sensitivity assays with *nhr-8* and *nhr-8;dyf-7*. Normally, before worms are transferred to ivermectin plates their developmental stage is synchronized by performing an egg preparation and leaving eggs to hatch on unseeded plates so worms arrest at L1. However, ivermectin assays performed with *nhr-8* and *nhr-8;dyf-7* following L1 synchronization produced qualitatively similar, but quantitatively inconsistent results. In contrast, when eggs were placed directly onto seeded ivermectin plates, results were stable throughout multiple replicates, suggesting that starvation during L1 arrest may have been affecting ivermectin sensitivity.

Interestingly, when eggs were placed directly onto seeded plates, the EC_{50} of *nhr-8* worms decreased, whereas that of *nhr-8;dyf-7* and *dyf-7* increased, compared to trials that involved L1 arrest. Potentially, starvation during L1 arrest is protective in *nhr-8* worms by inducing entry into dauer, whereas starvation negatively impacts dauer defective *dyf-7* worms. The increase in the EC_{50} of *nhr-8;dyf-7* worms from trials including L1 arrest and trials where eggs were placed directly onto seeded plates, could suggest that disruption of *nhr-8* in *dyf-7* worms does not completely rescue entry into dauer. However, it's worth noting that the EC_{50} of *dyf-7* worms increased far more between trial conditions than that of *nhr-8;dyf-7* worms.

Also relevant to the discussion of the role of metabolic changes in Dyf-mediated ivermectin resistance, are results from resistance assays with *tbc-2* and *tbc-2;dyf-7.* TBC-2 is an

endosomal trafficking protein that has been implicated in positively regulating the stress resistance and increased life span of dauer constitutive mitochondrial mutants [96]. TBC-2 controls localization of DAF-16 to the nucleus in response to various stressors; disrupting *tbc-2* in stress resistant mitochondrial mutants suppresses nuclear localization of DAF-16 and eliminates certain stress resistant phenotypes [96]. DAF-16 is a FOXO transcription factor that upregulates expression of genes involved in stress protection and metabolism [105], and is required for entry into dauer [106].

Although DAF-16 has not been implicated in Dyf-mediated ivermectin resistance, disruption of *tbc-2* in *dyf-7* mutants rescues ivermectin sensitivity. Interestingly, rescue of ivermectin sensitivity in *tbc-2;dyf-7* worms appears to be a developmental deficit, with worms surviving at higher concentrations, but not developing into gravid adults (H. J, Shields, unpublished observation). Furthermore, in both *tbc-2* single mutants and *tbc-2;dyf-7* double mutants, development is asynchronous at every dosage; however, developmental asynchrony is exacerbated in *tbc-2;dyf-7* worms compared to *tbc-2* worms. Magner *et al.* [103] found that *nhr-8* interacts with dauer formation network either in parallel or upstream from *daf-16*; it's possible that disruption of *tbc-2* in *dyf-7* worms eliminates the protection provided by *nhr-8* dysregulation through a mechanism involving *daf-16.* However, further research is required to determine whether TBC-2 regulates Dyf-mediated ivermectin resistance through *daf-16* and *nhr-8.*

Moving forward, another stress pathway of interest in understanding Dyf-mediated ivermectin resistance could be the osmotic stress response pathway. Results from osmotic stress assays show that *dyf-3* and *dyf-7* worms are resistant to osmotic stress compared to WT. Xenobiotic stress response genes have been linked to the osmotic stress response, particularly

genes downstream of SKN-1 [107]. In future studies, genes upregulated by SKN-1 in response to osmotic stress may be of interest in the context of Dyf-mediated ivermectin resistance.

Drug entry in dyf mutants and a novel technique for assessing internal ivermectin concentration

Since Dyf-mediated ivermectin resistance was discovered, the most prevalent mechanism proposed was that Dyf mutations affect drug entry into the worm. Unfortunately, attempts to fluorescently label ivermectin have failed to retain the efficacy of the drug, making it challenging to reliably conclude that drug entry is affected in Dyf mutants. As such, we still don't know how ivermectin enters *C. elegans* or nematode parasites, or whether Dyf mutations affect drug entry.

I developed a novel technique using tritium labelled ivermectin to assess whether drug entry is compromised Dyf mutants. My results show no significant difference in the internal concentration of ivermectin between WT, *dyf-3,* and *dyf-7* worms. Unfortunately, the ^{3}H-IVM assay is extremely sensitive and is easily affected by issues such as ineffective washing of ^{3}H-IVM off the worms, oral consumption of ^{3}H-IVM, and inconsistent dosage due to ^{3}H-IVM adhering to pipette tips. However, measuring radioactivity in wash solutions, allowing worms to mold and shed ^{3}H-IVM stuck to the cuticle, and assessing the presence of DiI in the intestine of the worms before and after being on food provide reliable checkpoints to assess whether results are indicative of the internal ^{3}H-IVM concentration.

Analysis of the washes revealed that the percent of total activity removed in each wash is consistent across trials for all strains. Thus, if there is residual ivermectin stuck to the cuticle when worms are added to the scintillation fluid, it does not explain any variance between trials. Interestingly, variance between trials was largely due to the concentration of drug that worms were exposed to. Comparing DPM/worm to the sum of the activity in all the washes revealed a

negative correlation between the internal and external concentrations of ivermectin. If the concentration of ivermectin administered were affecting the pharmacokinetics by increasing the concentration gradient, one would expect to observe a positive relationship between external and internal concentrations. One explanation for the negative relationship observed is that the increased activity in the washes indicates improved removal of excess ivermectin from the cuticle. However, the percent of total activity removed in each wash remains consistent across trials and the percent of total activity in the washes is not significantly correlated with DPM/worm. Another theory is that the negative relationship observed is indicative of the worms eating the drug. Because exposure to ivermectin results in pharyngeal paralysis of the worms, increased concentrations could reduce pumping thus reducing consumption of the drug. Although the presence of DiI in the intestine is used as an indicator of consumed ivermectin, there is no evidence to support the assumption that DiI and ivermectin are metabolized and excreted along the same timeline. Thus, the absence of DiI in the intestine may not indicate the absence of ivermectin. Fortunately, the relationship between external and internal concentrations of ivermectin could be corrected for in my analysis.

Interestingly, the effect of increased external ivermectin concentrations on internal concentrations was not significantly different between wild-type and Dyf worms, suggesting that acute effects of ivermectin are similar between wild-type and Dyf worms. Performing the scintillation assay after different exposure times may provide insight into temporal differences in the response to ivermectin. However, it is worth noting that the external concentrations varied more between trials with wild-type worms, thus further investigations where external concentration is experimentally manipulated would be required to conclude that acute effects of ivermectin are similar between wild-type and Dyf worms.

4.2 Concluding remarks

The development of ivermectin resistance among nematode parasites has deleterious ramifications for both veterinary medicine globally and public health programs in underdeveloped nations. Not only will the findings from this project help combat the spread of resistance by informing the development of new antiparasitic agents, but the mutant strains I've constructed can be used to assess the susceptibility of new antiparasitic drugs to known mechanisms of resistance. My findings suggest that *nhr-8,* a nuclear hormone receptor involved in xenobiotic detoxification, is required for ivermectin resistance in *dyf-7* worms, potentially through upregulation of Pgps, and/or alterations in metabolism. Additionally, I developed a novel technique using tritium labelled ivermectin to assess the effect of Dyf mutations on drug entry into the worm. In contrast to previous assumptions, Dyf mutations do not appear to significantly impair drug entry. Further research into the effect of Dyf mutations on *nhr-8* expression, and the interaction between *nhr-8* and genes involved in regulating metabolism are necessary to fully unravel the mechanism underlying Dyf-mediated ivermectin resistance.

References

1. Õmura, S. and A. Crump, *The life and times of ivermectin—a success story.* Nature Reviews Microbiology, 2004. **2**(12): p. 984-989.
2. Macrelli, M., et al., *First detection of ivermectin resistance in Oesophagostomum dentatum in pigs.* Veterinary parasitology, 2019. **270**: p. 1-6.
3. Currie, B.J., et al., *First documentation of in vivo and in vitro ivermectin resistance in Sarcoptes scabiei.* Clinical Infectious Diseases, 2004. **39**(1): p. e8-e12.
4. Osei-Atweneboana, M.Y., et al., *Phenotypic evidence of emerging ivermectin resistance in Onchocerca volvulus.* PLoS neglected tropical diseases, 2011. **5**(3): p. e998.
5. Craig, T., et al., *Evidence of ivermectin resistance by Parascaris equorum on a Texas horse farm.* Journal of Equine Veterinary Science, 2007. **27**(2): p. 67-71.
6. Schougaard, H. and M. Nielsen, *Apparent ivermectin resistance of Parascaris equorum in foals in Denmark.* Veterinary Record-English Edition, 2007. **160**(13): p. 439-440.
7. Coles, G., A. Rhodes, and A. Wolstenholme, *Rapid selection for ivermectin resistance in Haemonchus contortus.* Veterinary parasitology, 2005. **129**(3-4): p. 345-347.
8. Waghorn, T.S., C.M. Miller, and D.M. Leathwick, *Confirmation of ivermectin resistance in Ostertagia ostertagi in cattle in New Zealand.* Veterinary parasitology, 2016. **229**: p. 139-143.
9. Dent, J.A., et al., *The genetics of ivermectin resistance in Caenorhabditis elegans.* Proceedings of the National Academy of Sciences, 2000. **97**(6): p. 2674-2679.
10. Lipari, V., *The Relation between Dye-Filling and Ivermectin Resistance in Caenorhabditis elegans*. 2018, McGill University (Canada): Ann Arbor. p. 59.
11. Urdaneta-Marquez, L., et al., *A dyf-7 haplotype causes sensory neuron defects and is associated with macrocyclic lactone resistance worldwide in the nematode parasite Haemonchus contortus.* International journal for parasitology, 2014. **44**(14): p. 1063-1071.
12. Crump, A., *Ivermectin: enigmatic multifaceted 'wonder'drug continues to surprise and exceed expectations.* The Journal of antibiotics, 2017. **70**(5): p. 495-505.
13. Campbell, W., et al., *Ivermectin: a potent new antiparasitic agent.* Science, 1983. **221**(4613): p. 823-828.
14. Ōmura, S. and A. Crump, *Ivermectin: panacea for resource-poor communities?* Trends in parasitology, 2014. **30**(9): p. 445-455.
15. Enk, C.D., *Onchocerciasis—river blindness.* Clinics in dermatology, 2006. **24**(3): p. 176-180.
16. Anderson, J. and H. Fuglsang, *Further studies on the treatment of ocular onchocerciasis with diethylcarbamazine and suramin.* Br J Ophthalmol, 1978. **62**(7): p. 450-7.
17. Taylor, H.R., *Ivermectin treatment of onchocerciasis.* Aust N Z J Ophthalmol, 1989. **17**(4): p. 435-8.
18. Goa, K.L., D. McTavish, and S.P. Clissold, *Ivermectin.* Drugs, 1991. **42**(4): p. 640-658.
19. Colatrella, B., *The Mectizan Donation Program: 20 years of successful collaboration - a retrospective.* Ann Trop Med Parasitol, 2008. **102 Suppl 1**: p. 7-11.

20. Meeting on Strategies for Ivermectin Distribution through Primary Health Care, S. and W.H.O.P.f.t.P.o. Blindness, *Strategies for ivermectin distribution through primary health care systems : report of the Meeting on Strategies for Ivermectin Distribution through Primary Health Care Systems, Geneva, 22-25 April 1991*. 1991, World Health Organization: Geneva.
21. Vinkeles Melchers, N.V.S., et al., *The burden of skin disease and eye disease due to onchocerciasis in countries formerly under the African Programme for Onchocerciasis Control mandate for 1990, 2020, and 2030.* PLOS Neglected Tropical Diseases, 2021. **15**(7): p. e0009604.
22. Tekle, A.H., et al., *Progress towards onchocerciasis elimination in the participating countries of the African Programme for Onchocerciasis Control: epidemiological evaluation results.* Infectious Diseases of Poverty, 2016. **5**(1): p. 1-25.
23. Laing, R., V. Gillan, and E. Devaney, *Ivermectin – Old Drug, New Tricks?* Trends in Parasitology, 2017. **33**(6): p. 463-472.
24. Butler, A.J., et al., *Prevalence of anthelmintic resistant cyathostomins in Prince Edward Island, Canada.* Veterinary Parasitology: Regional Studies and Reports, 2021. **26**: p. 100629.
25. Howell, S.B., et al., *Prevalence of anthelmintic resistance on sheep and goat farms in the southeastern United States.* Journal of the American Veterinary Medical Association, 2008. **233**(12): p. 1913-1919.
26. Kaplan, R.M. and A.N. Vidyashankar, *An inconvenient truth: global worming and anthelmintic resistance.* Veterinary parasitology, 2012. **186**(1-2): p. 70-78.
27. Waghorn, T., et al., *Prevalence of anthelmintic resistance on 62 beef cattle farms in the North Island of New Zealand.* New Zealand Veterinary Journal, 2006. **54**(6): p. 278-282.
28. Osei-Atweneboana, M.Y., et al., *Prevalence and intensity of Onchocerca volvulus infection and efficacy of ivermectin in endemic communities in Ghana: a two-phase epidemiological study.* The Lancet, 2007. **369**(9578): p. 2021-2029.
29. Nana-Djeunga, H.C., et al., *Reproductive status of Onchocerca volvulus after ivermectin treatment in an ivermectin-naive and a frequently treated population from Cameroon.* PLoS neglected tropical diseases, 2014. **8**(4): p. e2824.
30. Remme, J.H., et al., *Efficacy of ivermectin against Onchocerca volvulus in Ghana.* The Lancet, 2007. **370**(9593): p. 1123-1124.
31. Reddy, P.P., *Avermectins*, in *Recent advances in crop protection*. 2012, Springer. p. 13-24.
32. Albers-Schoenberg, G., et al., *Avermectins. structure determination.* Journal of the American Chemical Society, 1981. **103**(14): p. 4216-4221.
33. Dent, J.A., M.W. Davis, and L. Avery, *avr-15 encodes a chloride channel subunit that mediates inhibitory glutamatergic neurotransmission and ivermectin sensitivity in Caenorhabditis elegans.* Embo j, 1997. **16**(19): p. 5867-79.
34. Johnson-Arbor, K., *Ivermectin: a mini-review.* Clinical Toxicology, 2022. **60**(5): p. 571-575.
35. Wolstenholme, A.J. and A. Rogers, *Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics.* Parasitology, 2005. **131**(S1): p. S85-S95.

36. Estrada-Mondragon, A. and J.W. Lynch, *Functional characterization of ivermectin binding sites in α1β2γ2L GABA (A) receptors.* Frontiers in molecular neuroscience, 2015. **8**: p. 55.
37. Shan, Q., J.L. Haddrill, and J.W. Lynch, *Ivermectin, an unconventional agonist of the glycine receptor chloride channel.* Journal of Biological Chemistry, 2001. **276**(16): p. 12556-12564.
38. Cheeseman, C.L., et al., *High-affinity ivermectin binding to recombinant subunits of the Haemonchus contortus glutamate-gated chloride channel.* Molecular and biochemical parasitology, 2001. **114**(2): p. 161-168.
39. Prichard, R.K., *Ivermectin resistance and overview of the Consortium for Anthelmintic Resistance SNPs.* Expert Opinion on Drug Discovery, 2007. **2**(sup1): p. S41-S52.
40. Alfarouk, K.O., et al., *Resistance to cancer chemotherapy: failure in drug response from ADME to P-gp.* Cancer cell international, 2015. **15**(1): p. 1-13.
41. Wang, X., H. Zhang, and X. Chen, *Drug resistance and combating drug resistance in cancer.* Cancer Drug Resist, 2019. **2**(2): p. 141-160.
42. Vasiliou, V., K. Vasiliou, and D.W. Nebert, *Human ATP-binding cassette (ABC) transporter family.* Human genomics, 2009. **3**(3): p. 1-10.
43. Fojo, A.T., et al., *Expression of a multidrug-resistance gene in human tumors and tissues.* Proceedings of the National Academy of Sciences, 1987. **84**(1): p. 265-269.
44. MacGowan, A. and E. Macnaughton, *Antibiotic resistance.* Medicine, 2017. **45**(10): p. 622-628.
45. Prevention, E.C.f.D. and Control, *Antimicrobial resistance surveillance in Europe 2015. annual report of the European antimicrobial resistance surveillance network (EARS-Net).* ECDC, 2015.
46. Šašková, K.G., et al., *Enzymatic and structural analysis of the I47A mutation contributing to the reduced susceptibility to HIV protease inhibitor lopinavir.* Protein Science, 2008. **17**(9): p. 1555-1564.
47. Matthew, A.N., et al., *Drug Design Strategies to Avoid Resistance in Direct-Acting Antivirals and Beyond.* Chem Rev, 2021. **121**(6): p. 3238-3270.
48. Romano, K.P., et al., *Molecular mechanisms of viral and host cell substrate recognition by hepatitis C virus NS3/4A protease.* Journal of virology, 2011. **85**(13): p. 6106-6116.
49. Kotze, A.C. and R.K. Prichard, *Chapter Nine - Anthelmintic Resistance in Haemonchus contortus: History, Mechanisms and Diagnosis*, in *Advances in Parasitology*, R.B. Gasser and G.V. Samson-Himmelstjerna, Editors. 2016, Academic Press. p. 397-428.
50. Sangster, N., C. Davis, and G. Collins, *Effects of cholinergic drugs on longitudinal contraction in levamisole-susceptible and-resistant Haemonchus contortus.* International journal for parasitology, 1991. **21**(6): p. 689-695.
51. Fauvin, A., et al., *cDNA-AFLP analysis in levamisole-resistant Haemonchus contortus reveals alternative splicing in a nicotinic acetylcholine receptor subunit.* Molecular and biochemical parasitology, 2010. **170**(2): p. 105-107.
52. Williamson, S.M., et al., *Candidate anthelmintic resistance-associated gene expression and sequence polymorphisms in a triple-resistant field isolate of Haemonchus contortus.* Molecular and biochemical parasitology, 2011. **180**(2): p. 99-105.

53. Blackhall, W.J., et al., *Selection at a P-glycoprotein gene in ivermectin-and moxidectin-selected strains of Haemonchus contortus.* Molecular and biochemical parasitology, 1998. **95**(2): p. 193-201.
54. Blackhall, W.J., et al., *Haemonchus contortus: selection at a glutamate-gated chloride channel gene in ivermectin-and moxidectin-selected strains.* Experimental parasitology, 1998. **90**(1): p. 42-48.
55. McCavera, S., et al., *An ivermectin-sensitive glutamate-gated chloride channel from the parasitic nematode Haemonchus contortus.* Molecular pharmacology, 2009. **75**(6): p. 1347-1355.
56. Lynagh, T. and J.W. Lynch, *Ivermectin binding sites in human and invertebrate Cys-loop receptors.* Trends in pharmacological sciences, 2012. **33**(8): p. 432-441.
57. Lynagh, T. and J.W. Lynch, *A glycine residue essential for high ivermectin sensitivity in Cys-loop ion channel receptors.* International journal for parasitology, 2010. **40**(13): p. 1477-1481.
58. Xu, M., et al., *Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog.* Molecular and biochemical parasitology, 1998. **91**(2): p. 327-335.
59. Sangster, N.C., et al., *Haemonchus contortus: Sequence heterogeneity of internucleotide binding domains from p-glycoproteinsand an association with avermectin/milbemycin resistance.* Experimental parasitology, 1999. **91**(3): p. 250-257.
60. Bourguinat, C., et al., *P-glycoprotein-like protein, a possible genetic marker for ivermectin resistance selection in Onchocerca volvulus.* Mol Biochem Parasitol, 2008. **158**(2): p. 101-11.
61. Roulet, A. and R. Prichard. *Ivermectin and moxidectin cause constitutive and induced over expression of different P-glycoproteins in resistant Haemonchus contortus*. in *Annual Meeting of the American Association of Veterinary Parasitologists, Honolulu, USA*. 2006.
62. Lloberas, M., et al., *Comparative tissue pharmacokinetics and efficacy of moxidectin, abamectin and ivermectin in lambs infected with resistant nematodes: Impact of drug treatments on parasite P-glycoprotein expression.* International Journal for Parasitology: Drugs and Drug Resistance, 2013. **3**: p. 20-27.
63. Lespine, A., et al., *P-glycoproteins and other multidrug resistance transporters in the pharmacology of anthelmintics: prospects for reversing transport-dependent anthelmintic resistance.* International Journal for Parasitology: Drugs and Drug Resistance, 2012. **2**: p. 58-75.
64. Lifschitz, A., et al., *Interference with P-glycoprotein improves ivermectin activity against adult resistant nematodes in sheep.* Veterinary parasitology, 2010. **172**(3-4): p. 291-298.
65. Molento, M.B. and R.K. Prichard, *Effects of the multidrug-resistance-reversing agents verapamil and CL 347,099 on the efficacy of ivermectin or moxidectin against unselected and drug-selected strains of Haemonchus contortus in jirds (Meriones unguiculatus).* Parasitology research, 1999. **85**: p. 1007-1011.
66. Kaletta, T. and M.O. Hengartner, *Finding function in novel targets: C. elegans as a model organism.* Nature Reviews Drug Discovery, 2006. **5**(5): p. 387-399.
67. Riddle, D.L., et al., *C. Elegans Ii.* 1997.
68. Brenner, S., *The genetics of Caenorhabditis elegans.* Genetics, 1974. **77**(1): p. 71-94.

69. Boulin, T. and O. Hobert, *From genes to function: the C. elegans genetic toolbox.* Wiley Interdisciplinary Reviews: Developmental Biology, 2012. **1**(1): p. 114-137.
70. Geary, T.G. and D.P. Thompson, *Caenorhabditis elegans: how good a model for veterinary parasites?* Veterinary Parasitology, 2001. **101**(3): p. 371-386.
71. Kaul, A., *The mechanism of Ivermectin-induced cytotoxicity in C. elegans.* 2004.
72. Zemkova, H., et al., *Allosteric modulation of ligand gated ion channels by ivermectin.* Physiological research, 2014. **63**.
73. Glendinning, S.K., et al., *Glutamate-gated chloride channels of Haemonchus contortus restore drug sensitivity to ivermectin resistant Caenorhabditis elegans.* PloS one, 2011. **6**(7): p. e22390.
74. Dent, J.A., *What canCaenorhabditis elegans tell us about nematocides and parasites?* Biotechnology and Bioprocess Engineering, 2001. **6**(4): p. 252-263.
75. Page, A.P., *The sensory amphidial structures of Caenorhabditis elegans are involved in macrocyclic lactone uptake and anthelmintic resistance.* International Journal for Parasitology, 2018. **48**(13): p. 1035-1042.
76. Janukavicius, P., *dyf-7 is responsible for the low levels of ivermectin resistance in Caenorhabditis elegans strains IVR6 and IVR10.* 2013.
77. Bargmann, C.I., *Chemosensation in C. elegans.* WormBook: The online review of C. elegans biology [Internet], 2006.
78. Hilliard, M.A., C.I. Bargmann, and P. Bazzicalupo, *C. elegans Responds to Chemical Repellents by Integrating Sensory Inputs from the Head and the Tail.* Current Biology, 2002. **12**(9): p. 730-734.
79. Zou, W., et al., *Polymodal Responses in C. elegans Phasmid Neurons Rely on Multiple Intracellular and Intercellular Signaling Pathways.* Scientific Reports, 2017. **7**(1): p. 42295.
80. Fielenbach, N. and A. Antebi, *C. elegans dauer formation and the molecular basis of plasticity.* Genes & development, 2008. **22**(16): p. 2149-2165.
81. Razzauti, A. and P. Laurent, *Ectocytosis prevents accumulation of ciliary cargo in C. elegans sensory neurons.* eLife, 2021. **10**: p. e67670.
82. Heiman, M.G. and S. Shaham, *DEX-1 and DYF-7 establish sensory dendrite length by anchoring dendritic tips during cell migration.* Cell, 2009. **137**(2): p. 344-355.
83. Murayama, T., et al., *The dyf-3 Gene Encodes a Novel Protein Required for Sensory Cilium Formation in Caenorhabditis elegans.* Journal of Molecular Biology, 2005. **346**(3): p. 677-687.
84. Inglis, P., et al., *The sensory cilia of Caenorhabditis elegans. WormBook, ed. The C. elegans Research Community, WormBook, doi: 10.1895/wormbook. 1.126. 1*. 2006.
85. Xu, C., C.Y.-T. Li, and A.-N.T. Kong, *Induction of phase I, II and III drug metabolism/transport by xenobiotics.* Archives of pharmacal research, 2005. **28**(3): p. 249-268.
86. Larigot, L., et al., *Cytochromes P450 of Caenorhabditis elegans: Implication in Biological Functions and Metabolism of Xenobiotics.* Biomolecules, 2022. **12**(3): p. 342.
87. Fontaine, P. and K. Choe, *The transcription factor SKN-1 and detoxification gene ugt-22 alter albendazole efficacy in Caenorhabditis elegans.* Int J Parasitol Drugs Drug Resist, 2018. **8**(2): p. 312-319.

88. Ménez, C., et al., *The transcription factor NHR-8: A new target to increase ivermectin efficacy in nematodes.* PLoS Pathog, 2019. **15**(2): p. e1007598.
89. Keshet, A., et al., *PMK-1 p38 MAPK promotes cadmium stress resistance, the expression of SKN-1/Nrf and DAF-16 target genes, and protein biosynthesis in Caenorhabditis elegans.* Molecular Genetics and Genomics, 2017. **292**: p. 1341-1361.
90. Tullet, J.M.A., et al., *The SKN-1/Nrf2 transcription factor can protect against oxidative stress and increase lifespan in C. elegans by distinct mechanisms.* Aging Cell, 2017. **16**(5): p. 1191-1194.
91. Ewald, C.Y., et al., *Dauer-independent insulin/IGF-1-signalling implicates collagen remodelling in longevity.* Nature, 2015. **519**(7541): p. 97-101.
92. Peddibhotla, S., et al., *Discovery of ML358, a selective small molecule inhibitor of the SKN-1 pathway involved in drug detoxification and resistance in nematodes.* ACS Chemical Biology, 2015. **10**(8): p. 1871-1879.
93. Guerrero, G.A., et al., *NHR-8 and P-glycoproteins uncouple xenobiotic resistance from longevity in chemosensory C. elegans mutants.* eLife, 2021. **10**: p. e53174.
94. Yan, R., et al., *The role of several ABC transporter genes in ivermectin resistance in Caenorhabditis elegans.* Veterinary parasitology, 2012. **190**(3-4): p. 519-529.
95. van der Vaart, A., S. Rademakers, and G. Jansen, *DLK-1/p38 MAP Kinase Signaling Controls Cilium Length by Regulating RAB-5 Mediated Endocytosis in Caenorhabditis elegans.* PLOS Genetics, 2015. **11**(12): p. e1005733.
96. Traa, A., et al., *Endosomal trafficking protein TBC-2 modulates stress resistance and lifespan through DAF-16-dependent and independent mechanisms.* Aging Cell, 2023: p. e13762.
97. Sanchez, A.D. and J.L. Feldman, *A proximity labeling protocol to probe proximity interactions in C. elegans.* STAR Protoc, 2021. **2**(4): p. 100986.
98. Stiernagle, T., *Maintenance of C. elegans.* 1999.
99. Knight, C.G., et al., *A novel mode of ecdysozoan growth in Caenorhabditis elegans.* Evolution & Development, 2002. **4**(1): p. 16-27.
100. Fujii, M., et al., *Mutations in chemosensory cilia cause resistance to paraquat in nematode Caenorhabditis elegans.* Journal of Biological Chemistry, 2004. **279**(19): p. 20277-20282.
101. Wheeler, J.M. and J.H. Thomas, *Identification of a novel gene family involved in osmotic stress response in Caenorhabditis elegans.* Genetics, 2006. **174**(3): p. 1327-36.
102. Melo, Justine A. and G. Ruvkun, *Inactivation of Conserved C. elegans Genes Engages Pathogen- and Xenobiotic-Associated Defenses.* Cell, 2012. **149**(2): p. 452-466.
103. Magner, D.B., et al., *The NHR-8 nuclear receptor regulates cholesterol and bile acid homeostasis in C. elegans.* Cell metabolism, 2013. **18**(2): p. 212-224.
104. Antebi, A., et al., *daf-12 encodes a nuclear receptor that regulates the dauer diapause and developmental age in C. elegans.* Genes & development, 2000. **14**(12): p. 1512-1527.
105. Murphy, C.T., *The search for DAF-16/FOXO transcriptional targets: approaches and discoveries.* Experimental gerontology, 2006. **41**(10): p. 910-921.

106. Jensen, V.L., M. Gallo, and D.L. Riddle, *Targets of DAF-16 involved in Caenorhabditis elegans adult longevity and dauer formation.* Experimental gerontology, 2006. **41**(10): p. 922-927.
107. Dodd, W., et al., *A damage sensor associated with the cuticle coordinates three core environmental stress responses in Caenorhabditis elegans.* Genetics, 2018. **208**(4): p. 1467-1482.