INVESTIGATING THE MODE OF ACTION OF IVERMECTIN AGAINST THE FILARIAL NEMATODE *BRUGIA MALAYI* INCLUDING THE CONTRIBUTIONS OF HOST IMMUNITY AND PARASITE GENES INFLUENCING RESISTANCE AND HYPERSENSITIVITY

by

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(Under the Direction of Ray Kaplan)

ABSTRACT

Ivermectin is a drug of incredible public health importance that's used in mass drug administration programs to eliminate lymphatic filariasis and onchocerciasis. The recent WHO recommendation of triple drug therapy for lymphatic filariasis MDA and the adoption of the 2021-2030 Road Map for Neglected Tropical Diseases likely mean use of ivermectin in elimination programs for lymphatic filariasis will expand worldwide. In lymphatic filariasis, caused by *Wuchereria bancrofti, Brugia malayi*, and *B. timori,* ivermectin causes long lasting suppression of the production of the transmittable blood-stage form called microfilariae (Mf) and rapidly clears Mf from the bloodstream. Despite ivermectin's decades long global use, we still don't have a full understanding of how it clears filarial parasites, and the recent expansion and challenges of ivermectin use make this understanding even more critical. Ivermectin's potency *in vivo*, but not *in vitro*, suggests that a host component may be involved, likely the immune system. To examine this, parasite naïve patients were given the label dose of ivermectin used in mass drug

administration campaigns, and we compared their immune functioning to a placebo treated group. We found that there was no difference between the groups in their complete blood counts, cytokine levels, expression of a panel of innate immunity genes, or the ability of their immune cells the kill microfilariae.

We previously performed a transcriptomics study in which gerbils infected with *B. malayi* were treated with ivermectin and differently expressed candidate genes were then investigated in phenotypic assays of ivermectin response using mutant strains of *C. elegans.* Through this approach we have identified several genes that influence resistance and hypersensitivity to ivermectin and found that the processes of development and fertility are affected by ivermectin independently of each other. This research has contributed to our understanding of ivermectin's mechanism of action against filarial worms, including a lack of evidence for the involvement of host immunity and a selection of new candidate genes and pathways involved in ivermectin resistance and hypersensitivity.

INDEX WORDS: Ivermectin, *Brugia malayi,* lymphatic filariasis, *Caenorhabditis elegans*, innate immunity, resistance

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iv

CHAPTERS

1 INTRODUCTION AND LITERATURE REVIEW 1

Introduction 1

Lymphatic Filariasis 2

Ivermectin 8

Ivermectin and Filarial Parasites 10

Ivermectin Resistance 12

Investigation of Ivermectin Resistance and *C. elegans* 14

Conclusions 19

References 20

2 LACK OF DETECTABLE SHORT-TERM EFFECTS OF A SINGLE DOSE OF IVERMECTIN ON THE HUMAN IMMUNE SYSTEM 38

Abstract 39

Introduction 41

Materials and Methods 43

Results 47

Discussion 54

Conclusions 57

Acknowledgements 58

Ethics Approval and Consent to Participate 59

References 60

3 IDENTIFICATION OF NEW GENES INFLUENCING THE RESPONSE TO IVERMECTIN IN C. ELEGANS 65

Abstract 66

Introduction 68

Materials and Methods 74

Results 82

Discussion 115

References 124

4 CONCLUSIONS 135

References 140

APPENDICES

A Chapter 2 Supplementary Information 142

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Ivermectin is a widely used drug of incredible global health and veterinary importance, used to treat and/or prevent variety of nematode infections and ectoparasite infestations. Ivermectin is one of the key components of global mass drug administration programs targeting filarial diseases such as lymphatic filariasis for elimination as a public health concern. It removes the larval stages of filarial parasites from the circulation within hours, yet concentrations similar to peak plasma concentrations in treated individuals have no direct effect on the larvae *in vitro*. Despite over 30 years of use in human and animal medicine, the means by which it clears filarial parasites is still unclear. The work presented in this dissertation has attempted to investigate factors that affect ivermectin's efficacy against filarial worms, including both host immune factors and parasite genes contributing to resistance and hypersensitivity.

The following specific aims are addressed in this dissertation:

Specific Aim 1: Investigate the effects of ivermectin on the human immune system to identify host factors contributing to the efficacy of ivermectin. To accomplish this, we administered the normal human dose of ivermectin (150µg/kg) to healthy, parasite-naïve human volunteers and measured changes in: cytokine and chemokine levels, gene expression in polymorphonuclear cells and peripheral blood mononuclear cells, complete

blood counts, and the ability of PMN and PBMC populations to attach to and kill *Brugia malayi* microfilariae.

Specific Aim 2: Identify new nematode genes that affect to the parasite's response to drug treatment by performing a phenotypic screen on model nematode *Caenorhabditis elegans.* We utilized strains of *C. elegans* with mutations in genes known to be affected in *B. malayi* after ivermectin treatment of hosts.

Lymphatic Filariasis

January 30th, 2020 marked the first observance of World Neglected Tropical Diseases Day with the World Health Organization officially recognizing the date in May of 2021 (Hotez et al., 2020; *World Health Assembly Adopts Decision to Recognize 30 January as World NTD Day*, 2021; *World NTD Day Officially Recognized at WHA 74*, 2021). The date commemorates the anniversary of the London Declaration on Neglected Tropical Diseases, the public-private partnership launched in 2012 to support the WHO's 2020 Roadmap on NTD's, which set targets for the control, elimination, and eradication of 17 diseases which primarily affect the world's poorest populations. Despite successes, many goals of the 2020 Roadmap were not met, and in November 2020 the WHO produced a roadmap for 2021-2030, setting targets for 20 diseases and groups founded on three fundamental pillars: accelerate programmatic action, intensify cross-cutting approaches, and change operating models and culture to facilitate country ownership (World Health Organization, 2020).

Of the neglected tropical diseases, lymphatic filariasis is one of the most widely distributed. Worldwide, approximately 859 million people required mass drug administration (MDA) for lymphatic filariasis as of 2019 (World Health Organization,

2020). Lymphatic filariasis (LF) is caused by three species of filarial (thread-like) nematode, *Wuchereria bancrofti, Brugia malayi,* and *Brugia timori* (Keating et al., 2014; Ottesen, 2006; Small et al., 2014). They are transmitted as infectious third-stage larvae by various species of mosquitos including members of the *Anopheles* and *Culex* genera. Adults in the lymphatics produce microfilariae (MF) that are taken up by new mosquitos, in which they develop into third stage larvae to continue the transmission cycle (Figure 1.1). Adult worms live between six and eight years; the damage they cause to the lymphatics prevents fluid from draining, resulting in pathology (Dreyer et al., 2000; Figueredo-Silva et al., 2002; Ottesen, 2006). This includes lymphoedema and elephantiasis, hydrocele, and acute dermatolymphangioadenitis (ADLA), acute inflammatory attacks usually resulting from secondary bacterial infections. As of 2013, approximately 68 million people were infected with LF (primarily *W. bancrofti*), including 36 million with severe pathology (hydrocele and lymphedema) (Ramaiah & Ottesen, 2014). In 2010, LF caused a direct loss of 2.78 million disability-adjusted life years (DALYs) per year (Hotez et al., 2014), and an estimated indirect loss of 5.32 million DALYs per year to infected individuals and their caretakers due to depressive illness (Ton et al., 2015). This total (8.1 million DALYs per year) ranked first among neglected tropical diseases (Hotez et al., 2014).

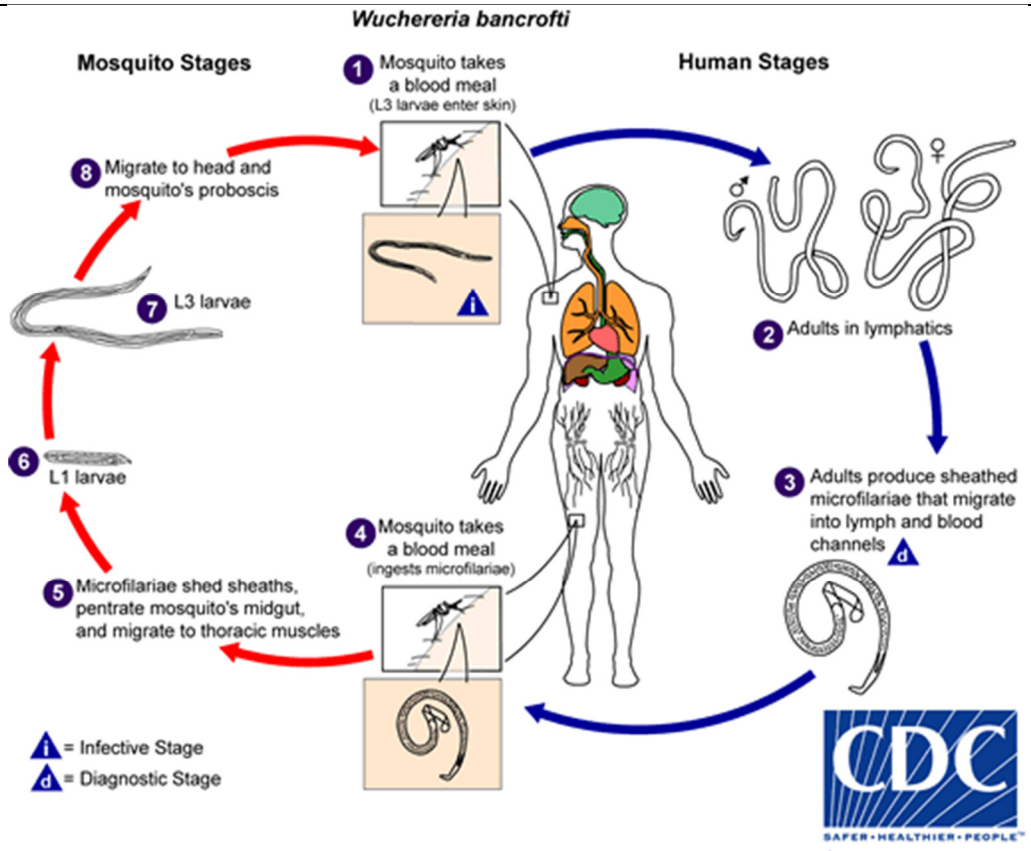

Figure 1.1: Life cycle of *W. bancrofti*. From (CDC, 2018)**.**

The primary intervention for lymphatic filariasis is mass drug administration with one of two regimens annually: ivermectin (IVM)/albendazole (ALB), also known as IA, or diethylcarbamazine (DEC)/ALB, also known as DA (World Health Organization, 2017). IA is used in regions where onchocerciasis is co-endemic, as DEC can cause severe adverse events (SEAs) in people infected with onchocerciasis. DA is used in all other communities. IVM and DEC work by clearing MF from the circulation and suppressing MF production by adults long-term, thereby preventing transmission (Brown et al., 2000; Gyapong et al., 2005). They have limited macrofilaricidal effects (ability to kill adult worms) and so MDA is required throughout the life of the worm.

WHO region – Région OMS	I. MDA not started – I. AMM non commencée	II. MDA started and not scaled to all endemic districts – II. AMM commencée et qui n'a pas été étendue à tous les districts d'endémie	III. MDA scaled to all endemic districts – III. AMM étendue à tous les districts d'endémie	IV. MDA stopped in all endemic districts and under surveillance – IV. AMM arrêtée dans tous les districts d'endémie et sous surveillance	V. Validated as having eliminated LF as a public health problem and under surveillance – V. Pays ayant obtenu la validation de leur conformité aux critères d'élimination de la FL en tant que problème de santé publique et restant en phase de surveillance
African – Afrique	Equatorial Guinea, Gabon – Guinée équatoriale, Gabon	Angola, Central African Republic, Madagascar, Nigeria, South Sudan – Angola, Madagascar, Nigéria, République centrafricaine, Soudan du Sud	Burkina Faso, Cameroon, Chad, Comoros, Congo, Côte d'Ivoire, Democratic Republic of the Congo, Eritrea, Ethiopia, Ghana, Guinea, Guinea-Bissau, Kenya, Liberia, Mali, Mozambique, Niger, Senegal, Sao Tome and Principe, Sierra Leone, Uganda, United Republic of Tanzania, Zambia, Zimbabwe – Burkina Faso, Cameroun, Comores, Congo, Côte d'Ivoire, Érythrée, Éthiopie, Ghana, Guinée, Guinée-Bissau, Kenya, Libéria, Mali, Mozambique, Niger, Ouganda, République démocratique du Congo, République-Unie de Tanzanie, Sao Tomé-et-Principe, Sénégal, Sierra Leone, Tchad, Zambie, Zimbabwe	Benin,[a,b] Mali,[a] Uganda[a] – Bénin, [a,b] Mali, [a] Ouganda [a]	Malawi, Togo – Malawi, Togo
Americas – Amériques			Guyana, Haiti – Guyane, Haïti	Brazil, Dominican Republic – Brésil, République dominicaine	
Eastern Mediterranean – Méditerranée orientale		Sudan – Soudan			Egypt, Yemen – Égypte, Yémen
South-East Asia – Asie du Sud-Est			India, Indonesia, Myanmar, Nepal, Timor-Leste – Inde, Indonésie, Myanmar, Népal, Timor-Leste	Bangladesh	Maldives, Sri Lanka, Thailand – Maldives, Sri Lanka, Thaïlande
Western Pacific – Pacifique occidental	New Caledonia – Nouvelle-Calédonie	Papua New Guinea – Papouasie-Nouvelle-Guinée	American Samoa, Fiji, French Polynesia, Federated States of Micronesia, Malaysia, Philippines, Samoa, Tuvalu – Fidji, États fédérés de Micronésie, Malaisie, Philippines, Polynésie française, Samoa américaines, Tuvalu	Brunei Darussalam, Lao People's Democratic Republic – Brunei Darussalam, République démocratique populaire Lao	Cambodia, Cook Islands, Kiribati, Marshall Islands, Niue, Palau, Tonga, Vanuatu, Vietnam, Wallis and Futuna – Cambodge, Îles Cook, Îles Marshall, Kiribati, Nioué, Palaos, Tonga, Vanuatu, Viet Nam, Wallis et Futuna
Total	**3**	**7**	**37**	**8**	**17**

[a] These countries postponed surveys intended for 2020 to 2021 and TAS1 was passed in all implementation units. – Ces pays ont repoussé les études qui étaient prévues pour 2020 et 2021 et la première enquête (TAS1) a été effectuée dans toutes les unités de mise en œuvre.
[b] Also implemented MDA in 2020. – Pays ayant mis en œuvre une AMM en 2020.

Figure 1.2: Country status in implementing mass drug administration (MDA) for lymphatic filariasis (LF) elimination as of 2020. From (World Health Organization, 2021).

The WHO has recently endorsed triple drug therapy (IVM/DEC/ALB, also known as IDA) in DA regions (World Health Organization, 2017). Recent evidence has shown that in comparison to DA, IDA is better at clearing parasites and suppressing MF production, which suggests that IDA may permanently sterilize or destroy adult worms (Weil et al., 2019). The combination also did not result in additional SAEs among those treated. On August 14th, 2018, Samoa, in the WHO Western Pacific Region, became the first country to begin MDA for LF with IDA (Moloo, 2018). The goal of IDA is to improve the efficacy of MDA at stopping transmission and to decrease the number of rounds of MDA needed to reach elimination as a public health concern (World Health Organization, 2017). Currently this requires *at least* five rounds of MDA with at least 65% coverage, with more rounds possibly being necessary in regions with high levels of transmission. Morbidity management is the second major component of interventions for LF. This is achieved through the development of packages of care for morbidity management and disability prevention (MMDP) (Addiss & Brady, 2007; World Health Organization, 2011). These are provided by the health systems and national elimination programs of member states and should include the four main treatments recommended by the WHO: MDA or other antifilarials, hydrocele surgery, ADLA treatment, and lymphedema management.

Intervention strategies for lymphatic filariasis are implemented by the Global Programme to Eliminate Lymphatic Filariasis (GPELF), a program organized by the WHO (Ramaiah & Ottesen, 2014; World Health Organization, 2021). The GPELF was founded in 2000 with the goal of eliminating LF as a public health concern by 2020 (a goal which has not been met). A major driver of the successes of the program are the

partnerships it has built not only with national governments, pharmaceutical company drug donors, and NGOs, but also with other disease control programs (Ichimori et al., 2014). For example, many national programs have begun integrating LF MDA with other MDA programs such as onchocerciasis (which also uses IA), schistosomiasis, and trachoma. A scale-up of integrated approaches targeting multiple NTD's has been identified by the WHO as a key component of the 2021-2030 roadmap for neglected tropical diseases (World Health Organization, 2020). They have identified four key themes: *integration* across NTDs to join the delivery of common interventions; *mainstreaming* into national health systems to improve program quality; *coordination* with other programs and stakeholders (such as vector management, which attempts to optimize the use of vector control resources, targeting both LF and malaria for example); and *strengthening country health systems,* both in terms of national system capacity and in global support, resources, and expertise. Lymphatic filariasis has been identified as one of several "skin NTDs" with opportunities for joint epidemiological surveillance, training and capacity building, social mobilization and community health education, and morbidity management and care. By integrating LF programs with other skin NTDs such as leishmaniasis, leprosy, buruli ulcer, and onchocerciasis, among others, resources can be used more cost effectively, cases can be detected earlier and with greater capacity for management, and national programs can have greater ownership leading to more long-term sustainability.

Despite the many challenges the GPELF has faced, there have been many successes in LF elimination. The first 15 years of the GPELF have averted an estimated 36 million clinical cases (Turner et al., 2016). This avoided the loss of an estimated 175

(116–250) million DALYs and provided a potential economic benefit of US$69.30–150.7 billion. More than 8.6 billion treatments have been delivered since 2000, and as a result 692 million people no longer require MDA for LF (World Health Organization, 2021). Additionally, 17 countries have been certified as having eliminated LF as a public health problem, with 8 more at the final surveillance stages. The WHO's Road map for neglected tropical diseases 2021-2030 renews the program's target of elimination of lymphatic filariasis as a public health concern by 2030, with several sub-targets and milestones indicating for tracking progress to elimination, shown in figure 1.3 (World Health Organization, 2020).

Indicator	2020 (provisional estimate)	2023	2025	2030
Number of countries validated for elimination as a public health problem (defined as: - infection sustained below transmission assessment survey thresholds for at least 4 years after stopping MDA; - availability of essential package of care in all areas of known patients)	17/72 (24%)	23/72 (32%)	34/72 (47%)	58/72 (81%)
Number of countries implementing post-MDA or post-validation surveillance	26 (36%)	37 (51%)	40 (56%)	72 (100%)
Population requiring MDA	TBC	330 million	180 million	0

Figure 1.3: WHO 2030 target, sub-targets, and milestones. From (World Health Organization, 2020)

Ivermectin

Ivermectin is a member of the avermectin/milbemycin class of the macrocyclic lactone family of compounds. The avermectins were identified as potent anthelmintic agents after screening the fermentation products of the filamentous bacterium *Streptomyces avermitilis* in an *in vivo* assay (Campbell, 2016). The safer, more effective semi-synthetic derivative ivermectin was quickly commercialized and made available for veterinary, agricultural, and aquaculture use in 1981 (Campbell, 2016; Crump, 2017). Several years later, ivermectin was registered for human use and provided free of charge for the control of onchocerciasis (and later lymphatic filariasis) by Merk & Co., Inc. (MSD outside the

US and Canada) (Crump, 2017; Gustavsen et al., 2018; Williams et al., 2020). This first-of-its-kind public-private partnership has provided "as much as is needed, for as long as needed" for over 30 years and expanded its commitment to medication donation after the WHO's recommendation of IDA (Williams et al., 2020). Ivermectin is also an important component of heartworm prevention; the macrocyclic lactones are the only drug class approved for this purpose.

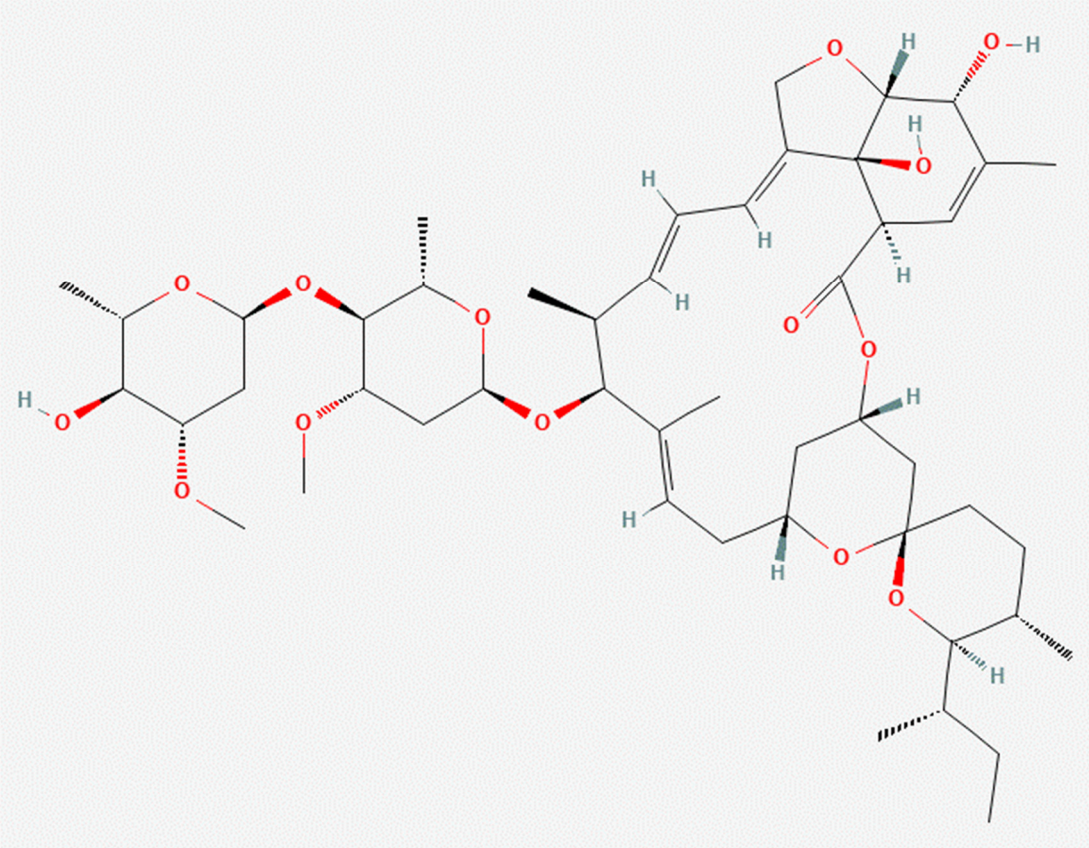

Figure 1.4: Chemical structure of ivermectin (ivermectin B1A). From (National Center for Biotechnology Information, 2022).

The primary target of ivermectin in nematodes is glutamate-gated chloride channels (GluCls) (Cully et al., 1994; Wolstenholme, 2012; Wolstenholme & Rogers, 2005). Ivermectin acts as an unconventional agonist, opening the channels slowly and effectively irreversibly, causing a long-lasting hyperpolarization of the neurons and

muscles expressing those channels (J. P. Arena et al., 1995; Atif et al., 2019; McCavera et al., 2009). This causes paralysis of body wall muscle, inhibition of pharyngeal pumping and loss of fertility (Wolstenholme, 2012; Wolstenholme & Rogers, 2005). When infected individuals are given a single dose of ivermectin, it typically causes a rapid and long-lasting reduction in the numbers of MF with the latter effect caused by a long-term sterilization of the adult female parasites (Basanez et al., 2008; Brown et al., 2000; Ottesen, 2006). As ivermectin does not kill adult worms, MDA programs using ivermectin rely instead on suppressing transmission in communities through this effect. In heartworm, caused by *Dirofilaria immitis,* ivermectin given as a monthly preventative kills third- and fourth-stage larvae that have infected over the previous month (Bowman & Atkins, 2009).

Ivermectin and Filarial Parasites

Despite ivermectin's decades long use we still do not completely understand how ivermectin works against filarial nematodes. The concentrations of ivermectin required to paralyze *B. malayi* and *D. immitis* MF are much higher than peak plasma concentrations (Storey et al., 2014; Wolstenholme et al., 2016). Additionally, ivermectin is ineffective at killing *B. malayi* MF *in vitro* and the killing of MF by human immune cells is not affected by ivermectin incubation or pre-treatment (Reaves, Wallis, Mccoy, et al., 2018). In *B. malayi* GluCls are expressed surrounding the excretory-secretory pore of MF and ivermectin treatment suppresses protein secretion (Moreno et al., 2010). This has led to the hypothesis that ivermectin clears MF by suppressing the secretion of immunomodulators. Potential immunomodulatory proteins present in the excretory/secretory products of *B. malayi* MF include Bm-MIF-1, a homolog of

macrophage inhibitory factor, superoxide dismutase, an anti-oxidant, and serine protease inhibitors (Bennuru et al., 2009). Additionally, the secretion of extracellular vesicles (EVs) by *B. malayi* and *D. immitis* is also inhibited by ivermectin, however resistant strains of *D. immitis* were not inhibited (Harischandra et al., 2018). EVs from *B. malayi* contained sex- and stage-specific cargo including immunomodulators like BmMIF-1 and the mi-RNA bma-let-7, which acts on toll-like receptors (Zamanian et al., 2015). These EVs are taken up by murine macrophages and induce classical macrophage activation, supporting their immunomodulatory effect (Zamanian et al., 2015). However, the specifics of these interactions and how they may lead to MF clearance are unknown. Similarly, GluCls are also expressed in the gonads of adult female *B. malayi* (Li et al., 2014), and yet how this results in the long-term suppression of fertility is also unclear.

There is also some evidence that ivermectin's action against filarial nematodes requires the direct involvement of the host immune system. In the model filarial parasite *Litomosoides sigmodontis,* killing of MF *in vitro* by ivermectin was dependent on the presence of rat spleen cells (Zahner & Schmidtchen, 1994). This was also observed to occur with neutrophils, with ivermectin-dependent killing likely requiring nitric oxide but not attachment (Zahner et al., 1997). Ivermectin enhances the binding of canine Polymorphonuclear Cells (PMNs) and Peripheral Blood Mononuclear Cells (PBMCs) to *Dirofilaria immitis* MF in vitro (Vatta et al., 2014) and the intensity of binding correlates with the drug-resistance status of the parasite (Berrafato et al., 2019). Human PMNs and PBMCs can also bind to and kill *Brugia malayi* MF (McCoy et al., 2017) in a batch dependent manner (Reaves, Wallis, McCoy, et al., 2018); the differences seem to be related to the physical condition of the parasites, an interesting observation that might

implicate a multifactorial mechanism of recognition and killing in the presence of ivermectin. While GluCls are exclusive to invertebrates, ivermectin sensitive channels are expressed in the mammalian nervous system, cells of the immune system, and other tissues. (Alam et al., 2006; Estrada-Mondragon & Lynch, 2015).

Ivermectin Resistance

Resistance to ivermectin or other A/M drugs by filarial worms has been reported in the field and conclusively demonstrated in the laboratory (Ballesteros et al., 2018; Bourguinat et al., 2011; C. C. Evans et al., 2017; Geary et al., 2011; Maclean et al., 2017). The molecular target of ivermectin was first identified in *C. elegans* through experiments with glutamate-gated chloride channels in Xenopus oocytes (P. Arena et al., 1992). Further work identified the subunits involved as well as binding properties and confirmation of the mechanism in other nematode species and for other members of the avermectin/milbemycins (J. P. Arena et al., 1995; Cully et al., 1994; Dent et al., 1997; Geary et al., 1993; Glendinning et al., 2011; Holden-Dye & Walker, 2006, 2006; Wolstenholme & Rogers, 2005). Ivermectin resistance could be obtained in *C. elegans* through mutations in genes encoding GluCl subunits and genes that affected dye filling of amphid neurons (Dent et al., 2000); mutations in *avr-14, avr-15,* and *glc-1* in combination were required for a strong resistant phenotype. However, mutations in GluCl subunits do not appear to be the mechanism of ivermectin resistance in parasitic nematodes, which may be polygenic in nature as there has not yet been as single gene that has been validated in causing resistance in wild isolates of ivermectin resistant parasites (Beech et al., 2011; Cobo, 2016; Doyle et al., 2019; McCAVERA et al., 2007)

Many studies have examined the genomes of resistant isolates in comparison to susceptible in an attempt to identify genes associated with resistance (Ballesteros et al., 2018; Bourguinat, Keller, et al., 2017; Bourguinat, Lefebvre, et al., 2017; Doyle et al., 2019; Lau et al., 2021; Redman et al., 2008, 2012; Rezansoff et al., 2016; Sanchez et al., 2020). These various studies have identified a range of associated genes including beta-tubulin and ATP-binding cassette (ABC) transporters such as p-glycoprotein. Ivermectin treatment selected for different alleles of the beta-tubulin isotype I gene in both *Onchocerca volvulus* and *Haemonchus contortus* (Bourguinat et al., 2007; Cobo, 2016; Eng et al., 2006; Prichard & Roulet, 2007). In ivermectin treated hosts, *O. volvulus* had increased frequencies of the "B" allele, which was later validated through genotyping samples collected before and after the initiation of ivermectin treatment (Eng et al., 2006). Genotyping adult female *O. volvulus* from pre- and post-ivermectin treatment samples was also able to link treatment, beta-tubulin allele, and fertility; after three years of repeated treatments, the frequency of worms homozygous for the A allele significantly decreased and the frequency of heterozygotes increased, in a dose-dependent manner (Bourguinat et al., 2007). A homozygous genotype was significantly associated with high fertility, which may mean that ivermectin could have different effects on embryostasis based on the genotype or that the heterozygote disadvantage may go away under ivermectin selection pressure. In *H. contortus,* comparing ivermectin naïve worms with those that had been passaged *in vivo* under strong ivermectin pressure for many generations found that ivermectin-pressured worms had a higher frequency of the F200Y polymorphism, which has previously been linked to benzimidazole resistance (Eng et al., 2006). Ivermectin also binds to *H. contortus* alpha- and beta-tubulin, increasing

polymerization and protecting it from trypsin-mediated proteolysis (Ashraf et al., 2015). Mutations (Ardelli, 2013; Ardelli & Prichard, 2007; Bourguinat et al., 2008; Prichard & Roulet, 2007) or changes in the expression (Ardelli, 2013; James & Davey, 2009; Peachey et al., 2017; Prichard & Roulet, 2007) of p-glycoprotein and ABC transporters has repeatedly been identified in screens of A/M resistant nematodes and this relationship has been functionally characterized (Prichard & Roulet, 2007). A relationship between p-glycoprotein/ABC family transporters and resistance has been shown in a range of nematode species including *H. contortus, Cooperia spp., O. volvulus, B. malayi, C. elegans,* and *D. immitis* (Ardelli & Prichard, 2007; James & Davey, 2009; Mate et al., 2022; Sheps et al., 2004; Stitt et al., 2011; Tompkins et al., 2011; Yan et al., 2012). There is also evidence that the host ABC transporters play a role in ivermectin efficacy as ivermectin affects the expression of ABC transporter genes in treated hosts.

Investigation of Ivermectin Resistance and *C. elegans*

Given the impact of filarial diseases to humans and animals, resistance to ivermectin is a concern that warrants further study (Kaplan, 2020; Kaplan & Vidyashankar, 2012; Lee et al., 2010; Ramaiah & Ottesen, 2014). However, the molecular tools for investigating resistance mechanisms in filarial nematodes are lacking (Kalinna & Brindley, 2007; Lok, 2012; Ward, 2015). While many filarial parasites have had their genomes sequenced and annotated, they are majorly annotated based on evidence from *C.elegans* orthologs (Ghedin et al., 2007; Godel et al., 2012; Scott & Ghedin, 2009; Tracey et al., 2020). Few filarial genes have direct evidence for their function and so understanding the impacts of mutations or differences in expression of those genes is difficult. Additionally, the culture and maintenance of parasite life cycles

is a challenge in most filarial nematode; of the major filarial parasites, only *B. malayi* has a small animal model (Ewert & Folse, 1984; Jackson-Thompson et al., 2018; Mutafchiev et al., 2014; Risch et al., 2021; Voronin et al., 2019). Access to parasite materials, generation time, and culture conditions are among the major limitations to studying drug resistance (and other aspects of biology) in filarial parasites. Molecular tools in *B. malayi* include RNAi with both dsRNA and siRNA, but results are inconsistent and there is no widely accepted protocol (Aboobaker & Blaxter, 2003; Dalzell et al., 2011, 2012; Landmann et al., 2012; Singh et al., 2012; Song et al., 2010; Verma et al., 2017). Efficacy may be influenced by the site of tissue expression as well as other unknown factors (Dalzell et al., 2011). *B. malayi* can also be transformed using biolistic particle bombardment and microinjection, though these have not observed to be heritable (Lok, 2012). The development of tools for stable, heritable transgenesis of filarial nematodes is ongoing, with successes in integrating a GFP and luciferase reporter into the *B. malayi* genome with the piggyBac transposon system (Liu et al., 2018; Xu et al., 2011). This system has also been reported to be successful in achieving CRISPR-mediated transfection of *B. malayi,* though this has not yet been repeated by other groups (Liu, De, et al., 2020; Liu, Grote, et al., 2020).

One widely used solution is to study the function of genes and mechanisms of resistance in the model nematode *C. elegans* (Hahnel et al., 2020). *C. elegans* is a free-living nematode found worldwide that lives in the soil and rotting vegetation (Corsi et al., 2015; W. Kim et al., 2018; Nigon & Félix, 2017). It can be quickly and easily grown in the lab at room temperature on a basic agar substrate and an *E. coli* auxotroph as food. Advantages of *C. elegans* as a model system include transparency (allowing structures to

be viewed easily), a quick generation time of only a few days from egg to adulthood (figure 1.5), the ability to grow large amounts of *C. elegans* in liquid culture (and the ability to developmentally synchronize them), and the extensive research community. *C. elegans* is a clade V nematode, making it more closely related to nematodes such as *H. contortus* then to filarial parasites (Blaxter, 2003; Haag et al., 2018). First developed as a model organism for neurology by Sydney Brenner, it has since been used in many major advancements in molecular biology (Haag et al., 2018; Nigon & Félix, 2017). It was the first multicellular organism to have its genome sequenced (and has best annotated genome of nematodes), it has had its entire cell linage mapped with a fixed number of adult somatic cells, and was used to discover RNAi (resulting in a Nobel Prize).

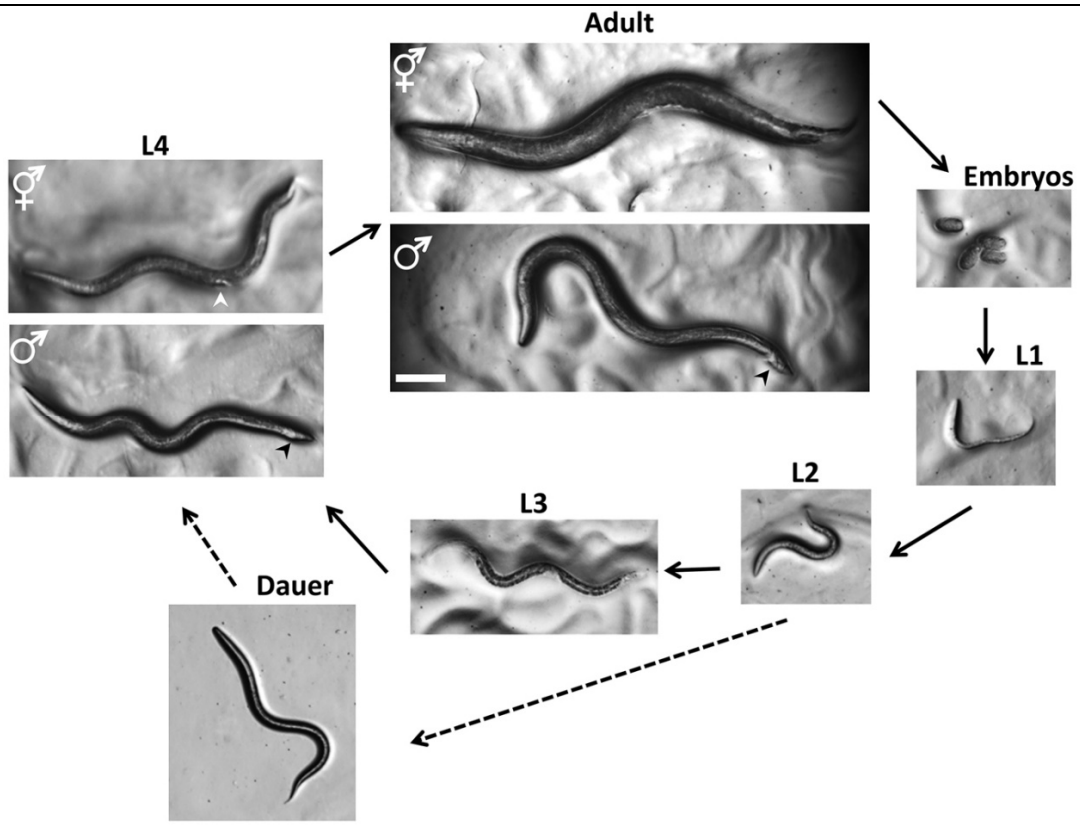

Figure 1.5: Life cycle of *C. elegans*. From (Corsi et al., 2015). Bar 0.1mm.

C. elegans has an extensive and constantly growing range of tools and resources available to use to study its biology. *C. elegans* is routinely transformed to express proteins tagged with fluorescent reporters or other useful tags through biolistic particle bombardment and microinjection of the gonads (Nance & Frøkjær-Jensen, 2019; Ward, 2015). CRISPR can be used to make precise mutations, deletions, and additions and the community is rapidly developing even more efficient methods of CRISPR/Cas9 based mutagenesis (Arribere et al., 2014; Dickinson & Goldstein, 2016; H.-M. Kim & Colaiácovo, 2016; Paix et al., 2015, 2017; Schwartz et al., 2021). Similarly, there are multiple different methods that can be used to produce conditional expression of genes including the Cre and Flp recombinases (Davis et al., 2008; Kage-Nakadai et al., 2014), expression under cell- and condition-specific promotors (Hamakawa & Hirotsu, 2017; Shen et al., 2014), and combinations of the two (Esposito et al., 2007). Many transgenic and other mutant strains generated by the community are available through the *C. elegans* Genetics Center (cgc.umn.edu). Additionally, the C. elegans Natural Diversity Resource CeNDR (https://elegansvariation.org/) is a collection of wild isolates collected from around the world. All of them have had their genomes sequenced, making them a powerful resource for studying natural diversity and identifying loci associated with traits of interest (Cook et al., 2017). For situations where transgenics aren't necessary or appropriate, RNAi in *C. elegans* can be accomplished with microinjection, soaking, and feeding with dsRNA expressing bacteria (Grishok, 2005; Kamath et al., n.d.; Zhuang & Hunter, 2012). Libraries of bacterial clones expressing dsRNA covering the majority of *C. elegans* genes are commercially available, allowing relatively quick genome-wide screens (Fraser et al., 2000; Kamath et al., 2003; Pothof et al., 2003; Rual et al., 2004).

Data from studies in *C. elegans* are compiled into the WormBase database including microarray results, transcriptomics, RNAi phenotypes, protein and gene interactions, tissue expression, sequence and ortholog information, and functional annotation (Howe et al., 2016). The WormBase website (wormbase.org) has built-in tools for datamining and bioinformatic analysis such as gene enrichment analysis (Angeles-Albores et al., 2018). The community also publishes the WormBook, a collection of peer-reviewed articles covering various aspects of *C. elegans* biology, molecular techniques, and even other nematode species (*Wormbook*, n.d.; wormbook.org).

Given the wealth of resources for *C. elegans,* it's not surprising that it is commonly used as a model for parasitic nematode biology and that this use has been frequently discussed (Geary & Thompson, 2001; Hahnel et al., 2020; Hoffmann et al., 2000; Holden-Dye & Walker, 2012; Nigon & Félix, 2017). *C. elegans* is commonly used as a first step in high-throughput screening, which has identified compounds that later show activity in parasitic nematode species of interest (Elfawal et al., 2019; Mathew et al., 2016; Weeks et al., 2018; Zamanian & Chan, 2021). Additionally, the various automated platforms for measuring various phenotypes in *C. elegans* can be repurposed for analyzing parasites (Gunderson et al., 2020; Hahnel et al., 2021; Lockery et al., 2012; Ward, 2015; Weeks et al., 2016). *C. elegans* has been used to identify targets of anthelmintics and mechanisms of resistance, such as ivermectin (as previously stated) and benzimidazoles. The identification of *ben-1* point mutations associated with benzimidazole resistance was the first time a direct causal link was made between a mutation and anthelmintic resistance, demonstrated in part by expressing *H. contortus* beta-tubulin genes in *C. elegans* (Kwa et al., 1993, p. 1, 1994, 1995; Roos et al., 1990;

Sangster et al., 2018). Screens of *C. elegans* natural and transgenic strains have identified additional loci associated with A/M, benzimidazole, and emodepside response (Avramenko et al., 2020; Cook et al., 2017; K. S. Evans et al., 2021; Hahnel et al., 2018; Wit et al., 2021). Similarly, expressing parasite genetic elements in *C. elegans* can help better understand aspects of nematode parasite genomics and molecular mechanisms of action (Britton & Murray, 2006; Brooks & Isaac, 2002). It has been used to identify the functions of parasite promotors (Britton et al., 1999), developmental biology (Couthier et al., 2004), and the importance of different receptor subunits to drug action (Sloan et al., 2015).

Conclusions

This dissertation aims to explore mechanisms of ivermectin action and resistance in filarial worm parasites. Ivermectin is highly effective *in vivo* but not *in vitro,* implying that ivermectin's effects on GluCls are not sufficient explanation for its action and that a host component may be involved (Wolstenholme et al., 2016). Human immune cells from parasite naïve patients can attach to and kill *Brugia malayi* microfilariae and ivermectin is able to bind to receptors present on human immune cells (Alam et al., 2006; McCoy et al., 2017; Reaves, Wallis, McCoy, et al., 2018). In chapter 2, I explore the hypothesis that ivermectin acts directly on the host immune system through a clinical study in collaboration with UGA's CTRU. Our lab previously performed a transcriptomics study in which gerbils infected with *B. malayi* were treated with ivermectin and we measured which genes were differently expressed in the parasite compared to worms from control hosts (Maclean et al., 2019). In chapter 3, I functionally characterize the effects these genes have on ivermectin sensitivity using *C. elegans* as a model nematode.

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CHAPTER 2

LACK OF DETECTABLE SHORT-TERM EFFECTS OF A SINGLE DOSE OF IVERMECTIN ON THE HUMAN IMMUNE SYSTEM[1]

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[1] Wilson, N.E., Reaves, B.J. & Wolstenholme, A.J. Lack of detectable short-term effects of a single dose of ivermectin on the human immune system. *Parasites Vectors* **14,** 304 (2021). https://doi.org/10.1186/s13071-021-04810-6

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Abstract

*Background***.** Ivermectin is widely used in human and animal medicine to treat and prevent parasite nematode infections. It has been suggested that its mode of action requires the host immune system, as it is difficult to reproduce its clinical efficacy in vitro. We therefore studied the effects of a single dose of ivermectin (Stromectol® – 0.15 mg/kg) on cytokine levels and immune cell gene expression in human volunteers. This dose reduces bloodstream microfilariae rapidly and for several months when given in mass drug administration programmes.

Methods. Healthy volunteers with no travel history to endemic regions were given 3-4 tablets, depending on their weight, of either ivermectin or a placebo. Blood samples were drawn immediately prior to administration, 4 hrs and 24 hrs afterwards, and complete blood counts performed. Serum levels of 41 cytokines and chemokines were measured using Luminex® and expression levels of 770 myeloid-cell related genes determined using the Nanostring nCounter®. Cytokine levels at 4 hrs and 24 hrs post-treatment were compared to the levels pre-treatment using simple t-tests to determine if any individual results required further investigation, taking $p = <0.05$ as the level of significance. Nanostring data were analysed on the proprietary software, nSolver™

Results. No significant differences were observed in complete blood counts or cytokine levels at either time point between people given ivermectin vs placebo. Only three genes showed a significant change in expression in Peripheral Blood Mononuclear Cells 4 hours after ivermectin was given; there were no significant changes 24 hrs after drug administration or in Polymorphonuclear cells at either time point. Leukocytes isolated

from those participants given ivermectin showed no difference in their ability to kill *Brugia malayi* microfilariae in vitro.

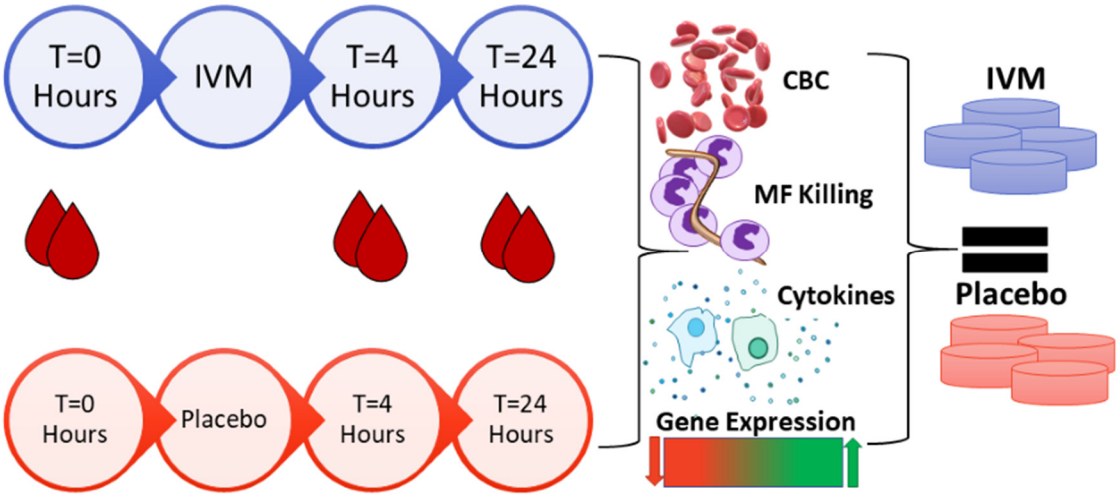
Conclusions. Overall, our data do not support a direct effect of ivermectin, when given at the dose used in current filarial elimination programmes, on the human immune system.

Trial Registration Clinical Trials.gov NCT03459794

Registered 9th March 2018, Retrospectively registered

https://clinicaltrials.gov/ct2/show/NCT03459794?term=NCT03459794&draw=2&rank=1

Graphical Abstract



Keywords: Ivermectin; Lymphatic filariasis; Peripheral Blood Mononuclear Cells; Polymorphonuclear Cells; Cytokine

Introduction

Ivermectin is a key part of mass drug administration programs for the control and eventual elimination of the major tropical diseases lymphatic filariasis (LF) and onchocerciasis, and as such is given to hundreds of millions of people every year (Gustavsen et al., 2018; Gyapong et al., 2018; Molyneux et al., 2003; Ottesen, 2006). Ivermectin treatment of people infected with the filarial nematodes that cause these diseases results in a rapid removal of microfilariae (Mf) from the circulation or skin of LF and onchocerciasis patients, respectively (Basanez et al., 2008; Ottesen, 2006). The replacement of these Mf by the adult female parasites is also suppressed for many months by the drug treatment due to the embryostatic action of the drug (Basanez et al., 2008), which may be due to the expression of ivermectin-sensitive glutamate-gated chloride channels in the reproductive tissues of adult females (Li et al., 2014). However, in vitro, concentrations of ivermectin equivalent to the peak plasma concentrations found following treatment have little or no measurable effect on Mf motility or viability (Devaney & Howells, 1984; Storey et al., 2014; Tompkins et al., 2010; Zahner & Schmidtchen, 1994). This has led to the hypothesis that the effect of ivermectin on Mf depends in part on the host immune system (Ali et al., 2002), and some direct evidence to support this hypothesis has been obtained from studies on related animal parasites. For example, ivermectin killing of *Litomosoides carinii* (now *L. sigmodontis*) Mf in vitro was dependent on the addition of rat spleen cells (Zahner & Schmidtchen, 1994), and this observation was later extended to neutrophils, with the ivermectin-dependent killing postulated to require nitric oxide, but not physical attachment (Zahner et al., 1997). We have previously reported that the binding of canine Polymorphonuclear Cells (PMNs)

and Peripheral Blood Mononuclear Cells (PBMCs) to *Dirofilaria immitis* Mf in vitro increases in the presence of ivermectin (Vatta et al., 2014), and this correlates with the drug-resistance status of the parasite (Berrafato et al., 2019). Human PMNs and PBMCs can also bind and kill *Brugia malayi* Mf (McCoy et al., 2017), though this depends on the batch of parasites used in the assay and may reflect their overall condition (Reaves et al., 2018). Taken together, these and other data could be interpreted suggest that ivermectin has an effect not only on the parasite, but also on the host immune system (Wolstenholme et al., 2016).

The anthelmintic action of ivermectin and the other macrocyclic lactones is accepted to be due to its specific action on glutamate-gated chloride channels (Cully et al., 1994; Wolstenholme, 2012; Wolstenholme & Rogers, 2005), at which it acts as an unconventional agonist, opening the channels slowly and effectively irreversibly (Arena et al., 1995; Atif et al., 2019; McCavera et al., 2009). These channels are unique to invertebrates, however related ivermectin-sensitive channels are expressed widely in the mammalian nervous system (Estrada-Mondragon & Lynch, 2015) and other tissues, including on some cells of the immune system (Alam et al., 2006). Indeed, the sensitivity of neuronal ligand-gated channels to ivermectin is the reason for the increased susceptibility of mdr-1 deficient animals to ivermectin intoxication (Mealey, 2004). In addition, it has more recently become apparent that ivermectin can affect multiple molecular targets in mammals, including P2X receptors and the farnesoid X receptor (FXR) (Chen & Kubo, 2018; Laing et al., 2017). Ivermectin has also been shown to have anti-inflammatory properties in T cell-mediated skin disease, and this effect was independent of any effect on ligand-gated chloride channels or FXR, suggesting an

additional, so far unidentified, target (Ventre et al., 2017). The drug has antiviral properties and has even been suggested as a potential therapy for COVID-19 (Heidary & Gharebaghi, 2020; King et al., 2020), though the drug concentrations at which the anti-viral effect becomes apparent are considerably higher than those found in the plasma of MDA recipients.

If the microfilaricidal action of ivermectin is due, at least in part, to the drug facilitating immune clearance of the parasites then this could be due to one of two general mechanisms, or a combination of them. There are several reports indicating that the drug can interfere with secretion and vesicle release from the parasites, which has been hypothesized to inhibit their ability to evade host immunity (Harischandra et al., 2018; Moreno et al., 2010), and recently these extracellular vesicles were demonstrated to downregulate the phosphorylation of mTOR in a human monocyte-derived cell line (Ricciardi et al., 2021). Alternatively, ivermectin could have a direct effect on the host immune system. This second hypothesis predicts that ivermectin treatment of uninfected people should result in measurable changes in immune function or in gene expression. In the experiments reported here, we have attempted to test this prediction. Such an effect could also be reflected in an increased ability of the treated immune cells to recognize and kill the parasites, and so we have also examined whether the ivermectin altered the ability of human leukocytes to kill *B. malayi* Mf in vitro.

Materials and Methods

Ivermectin Administration to Human Volunteers and Sample Collection

Twelve volunteers, aged between 18-65 and weighing between 50-84 kg, were recruited from the Athens, Georgia, area. Subjects attended the University of Georgia

Clinical and Translational Research Unit (CTRU) twice. They were asked to fast for at least 3-4 hours prior to the first visit. At that visit, they were weighed and allocated to groups by CTRU staff using a block randomization protocol. Eighteen mls of blood (Samples A) were drawn in a fasting state and subjects were administered 150 µg/kg Stromectol®, as 3 or 4 tablets or the equivalent number of placebo tablets immediately after blood was drawn. Participants remained at CTRU for four hours then another 15ml of blood (Samples B) was drawn. A third blood sample (18ml) – Samples C - was drawn 24 hrs after administration of the drug. All the subjects completed the study. All samples were coded immediately after being taken and all analyses completed blind. Serum was prepared from 5-10 ml of blood collected as above but allowed to clot in the absence of heparin or EDTA (incubated for ~2 hours at room temperature). The liquid fraction of the blood sample was removed to a fresh tube and centrifuged at 10,000 x g for 5 min. The resulting supernatant was filter sterilized.

Complete Blood Counts

Complete blood counts (CBC) were carried out by the Clinical Pathology laboratory at Piedmont Athens Regional Hospital.

Cytokine Measurements

Human cytokine and chemokine levels were measured on a Luminex MAGPIX® instrument using the Milliplex Human Cytokine/Chemokine magnetic bead Premixed 41 Plex Kit (EMD Millipore, Billerica MA, USA). All samples were measured using three technical replicates, with two replicates of all standards and controls.

Gene Expression Analysis

PBMCs and PMNs were purified, and RNA extracted from cells isolated from each individual subject as described previously (McCoy, Reaves et al. 2017). Neutrophils were isolated using the EasySep™ Direct Human Neutrophil Isolation Kit (STEMCELL Technologies, Vancouver, BC, Canada) according to manufacturer's instructions. PBMCs were isolated using SepMate™-50 Tubes (STEMCELL Technologies, Vancouver, BC, Canada) according to manufacturer's instructions. To remove contaminating platelets, the optional extended wash step (120 x g for 10 min) of the SepMate™ protocol was included. Isolated PMNs and PBMCs were washed in PBS (centrifuged at 300 x g for 5 min), re-suspended in a 1:1 mixture of RPMI-1640-10 mM HEPES buffer and autologous serum, stored at room temperature and used within 6 hours post-isolation. Cell counts were performed on a 1:10 dilution of each preparation of cells containing 0.4% trypan blue (Gibco, Life Technologies, Grand Island, NY, USA) using a BioRad TC10™ automated cell counter (BioRad, Hercules, CA, USA). To extract RNA, 0.75 ml Trizol were added per 0.25 ml sample (5-10 x 10^6 cells). Cells were lysed by pipetting up and down. Homogenized samples were incubated for 5 min at room temperature. Chloroform (0.2 ml/1ml Trizol) was added and the sample shaken for 15 seconds followed by a 3-minute incubation at room temperature. Samples were centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase was removed and the RNA precipitated by adding 0.5 ml isopropanol (including 15 µg GlycoBlue™ Coprecipitent, Invitrogen, Carlsbad, CA) to the aqueous phase and incubating at room temperature for 10 minutes. After centrifugation at 12,000 x g for 10 minutes, 4°C the supernatant was removed and the pellet washed in 1 ml 75% ethanol per 1 ml Trizol. After a brief vortex, the sample was centrifuged at 7500 x g for 5 minutes (4°C). Ethanol

was removed and the pellet air dried for 5-10 minutes prior to resuspending in RNAse-free water. The expression levels of 770 genes for each individual subject were measured on an nCounter® SPRINT molecular profiling system, using the nCounter® Human Innate Immunity Myeloid Panel.

Microfilarial killing

PBMCs and PMNs were isolated from the samples A & C and incubated with freshly isolated *B. malayi* Mf as previously described (McCoy, Reaves et al. 2017). Briefly, assays were set up in Corning® 96 Well TC-treated microplates (Millipore-Sigma, Burlington, MA, USA). Four components were added to each well in 50 µl volumes, giving a total volume of 200 µl: ~100 *B. malayi* Mf, autologous serum and either no cells, ~150,000 PMNs, or ~150,000 PBMCs. To create the respective controls, 50 µl of RPMI-1640 was substituted for the relevant component. The tissue culture plates were incubated at 37 °C and 5% CO_2. Viable Mf were counted on a Nikon™ TS2 microscope (Nikon Instruments Inc., Melville, NY, USA) at 1, 24 and 120 hours post-experimental set up. Mf were considered to be 'viable' if there was any detectable movement observed within approximately 10-20 seconds (8, 15).

Statistical Analysis

The mean numbers of each cell type were calculated for each arm at 24 hrs post-treatment and compared to those in the pre-treatment samples using a simple t-test, with $p = <0.05$ as the level of significance. Since none of the measurements passed this test, no further analysis was performed. Cytokine levels were obtained from the Luminex data. The mean level plus or minus standard error of each analyte were calculated for each arm at each time point (0, 4, 24 hrs post-treatment). For the control and ivermectin arms,

levels at 4 hrs and 24 hrs post-treatment were compared to the levels pre-treatment using simple t-tests to determine if any individual results required further investigation, taking $p = <0.05$ as the level of significance. The change in levels 4 hrs and 24 hrs post-treatment were also compared between the placebo- and ivermectin-treated groups. Since none of the individual analyte results met this criterion, no further analysis was carried out. The Nanostring data were analysed on the proprietary software, nSolver™ (Nanostring Technologies Inc, Seattle). The data passed quality control criteria. The software calculated the $\log_2$ geometric mean levels of each mRNA measured and used t tests to determine statistically significant changes in expression between 4 and 24 hrs post-treatment for the control and ivermectin arms. We also compared the changes in expression at 4 hrs and 24 hrs post-treatment between the placebo- and -ivermectin-treated groups. The Benjamini-Yekutieli False Discovery Rate (35) method was used to account for the expectation that significant changes in genes may be correlated with or dependent on each other, and the subsequent resulting false discovery rate adjusted p-value of <0.05 used to determine those changes that were deemed to be statistically significant. In all the analyses, each individual subject was treated as a biological replicate, giving $N = 4$ for the placebo arm and $N = 8$ for the ivermectin arm.

Results

Complete Blood Counts

After completion of all the experimental measurements, the coding was broken and we found that 4 of the participants received the placebo and 8 received ivermectin. A comparison of the CBC results from the two groups (Figure 2.1) revealed no significant differences between them 24 hours after drug administration.

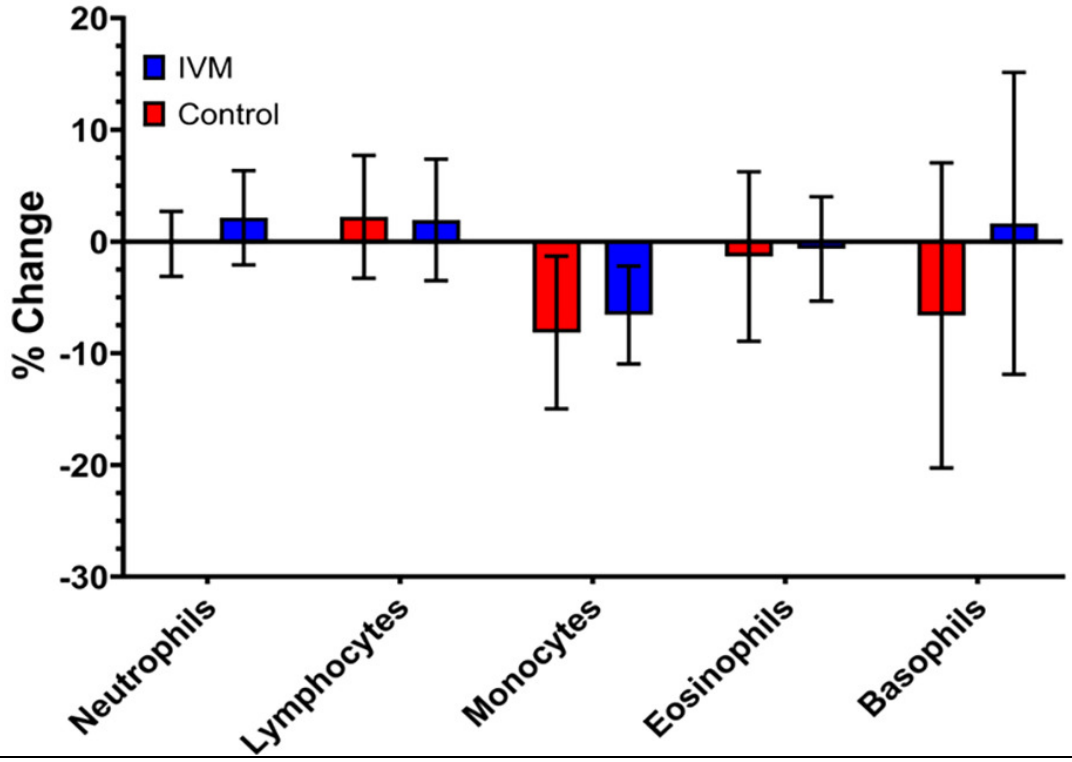
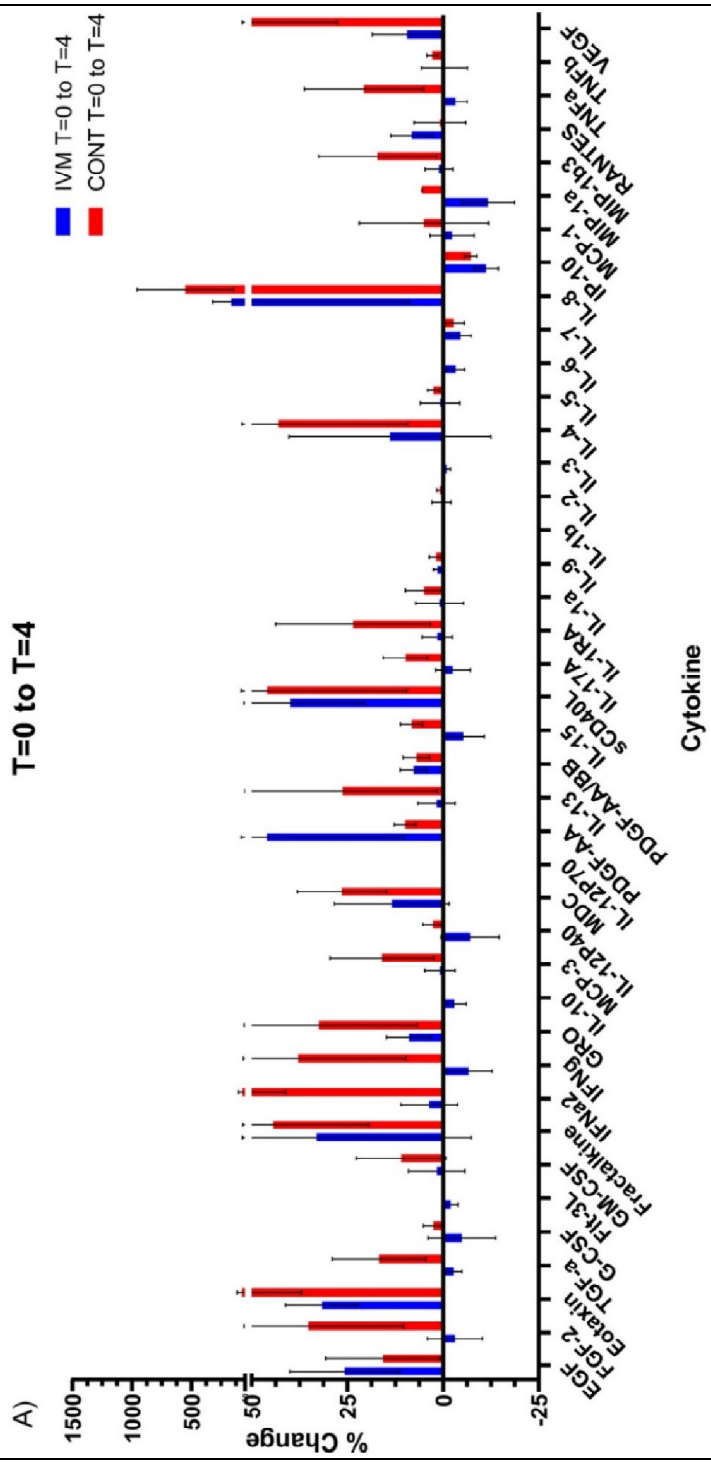


Figure 2.1: Complete blood counts show no significant changes 24 hours following ivermectin administration. The mean % change in each cell type between Samples C and A is shown; error bars indicate the SEM. Ivermectin-treated subjects are in blue, placebo-treated subjects in red.

Cytokine Levels

We measured the levels of 41 cytokines and chemokines in all the samples using the Milliplex Human Cytokine/Chemokine MAGNETIC BEAD Premixed 41 Plex Kit and compared the levels at 4 and 24 hrs post-treatment to pre-treatment levels between the two groups (Figure 2.2). This analysis was complicated by several factors. There was large individual variations in the levels of many cytokines pre-treatment (For many analytes at least one sample was below the limits of detection, which prevented a comparison of mean levels, and of the changes from the base-line measurement. Several analytes seemed to show a diurnal variation in the placebo group, as previously reported

(Petrovsky, McNair et al. 1998, Gibbs, Blaikley et al. 2012, Park, Kim et al. 2018), as evidenced by changes in levels, usually increases, between t=0 and t=4 in the control group (Figure 2.2A). Overall, there were no significant differences in the levels of any of the substances tested between the ivermectin-treated and control groups at either 4hrs or 24hrs post-treatment.


A)
T=0 to T=4
IVM T=0 to T=4
CONT T=0 to T=4
% Change
1500
1000
500
50
25
0
-25
Cytokine
EGF
FGF-2
Eotaxin
TGF-a
G-CSF
Flt-3L
GM-CSF
Fractalkine
IFNa2
IFNg
GRO
IL-10
MCP-3
IL-12P40
MDC
IL-12P70
PDGF-AA
IL-13
PDGF-AA/BB
IL-15
sCD40L
IL-17A
IL-1RA
IL-1a
IL-9
IL-1b
IL-2
IL-3
IL-4
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RANTES
TNFa
TNFb
VEGF

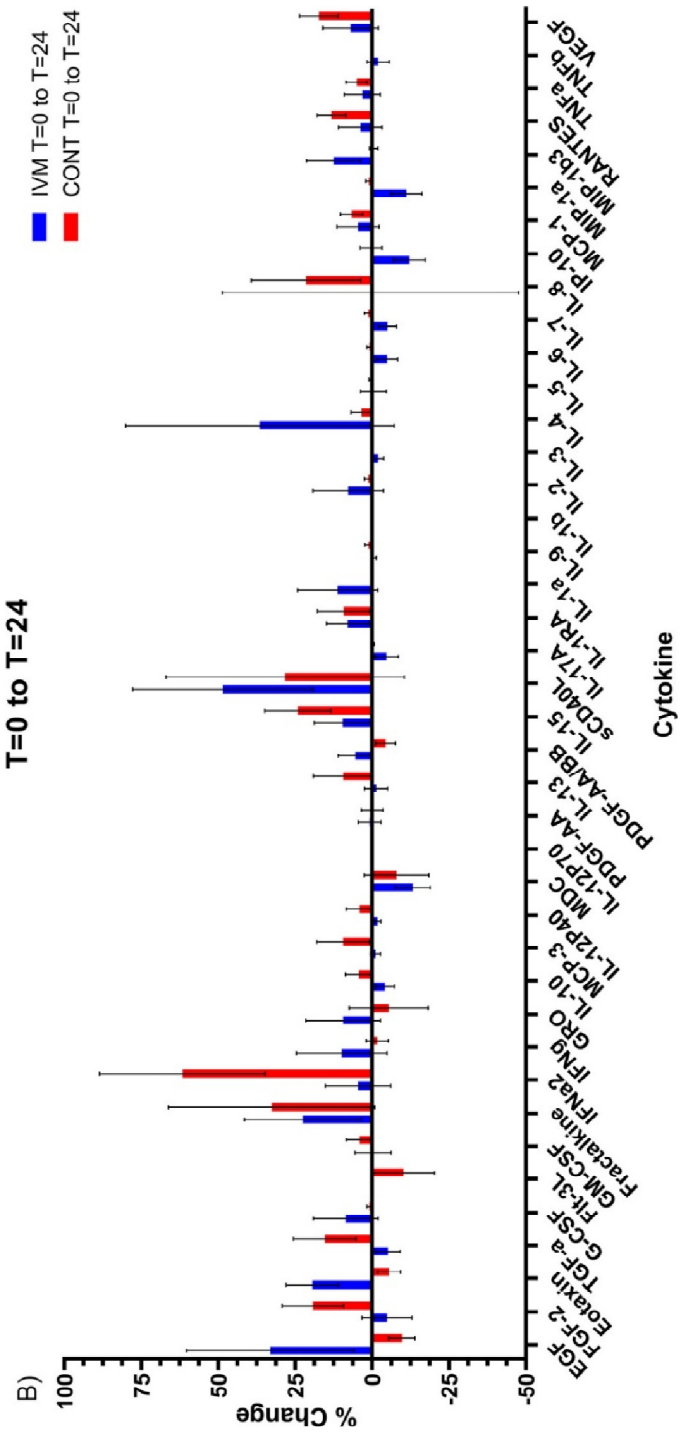

Figure 2.2: Changes in cytokine levels in ivermectin- and placebo-treated subjects at (A) 4 hrs and (B) 24 hrs post treatment. The mean of the percentage change for each individual cytokine is shown, error bars indicate SEM. Ivermectin treated subjects are blue, placebo-treated subjects in red.

Gene Expression

We extracted RNA from purified PMNs and PBMCs isolated from each of the experimental subjects and measured the expression of 770 transcripts in all these samples using Nanostring technology and the nCounter® Human Innate Immunity Myeloid Panel. The only statistically significant differences were found in the 4-hr post-treatment PBMC samples (Figure 3), where there were three transcripts that showed a reduction in expression: platelet activating factor receptor, prokineticin 2 and histone deacetylase 5 (Table 2.1). There were no changes in the expression of any of these transcripts in the PMNs at either time point, nor in the PBMCs at 24 hrs post-treatment (Figure 2.3).

Table 2.1. PBMC transcripts with a statistically significant change in expression between ivermectin- and placebo-treated samples 4 hrs after administration. The Benjamini-Yekutieli (BY) p value is that obtained after correcting for the false discovery rate (35).

Transcript	**Product**	**Log2 fold change**	**P-value**	**BY.p.value**	**Gene Sets**
PTAFR	platelet activating factor receptor	-1.04	1.01E-5	0.0349	Interferon signalling
PROK2	prokineticin 2	-2.96	2.75E-5	0.0403	Angiogenesis
HDAC5	histone deacetylase 5	-0.65	3.48E-5	0.0403	Growth factor signalling

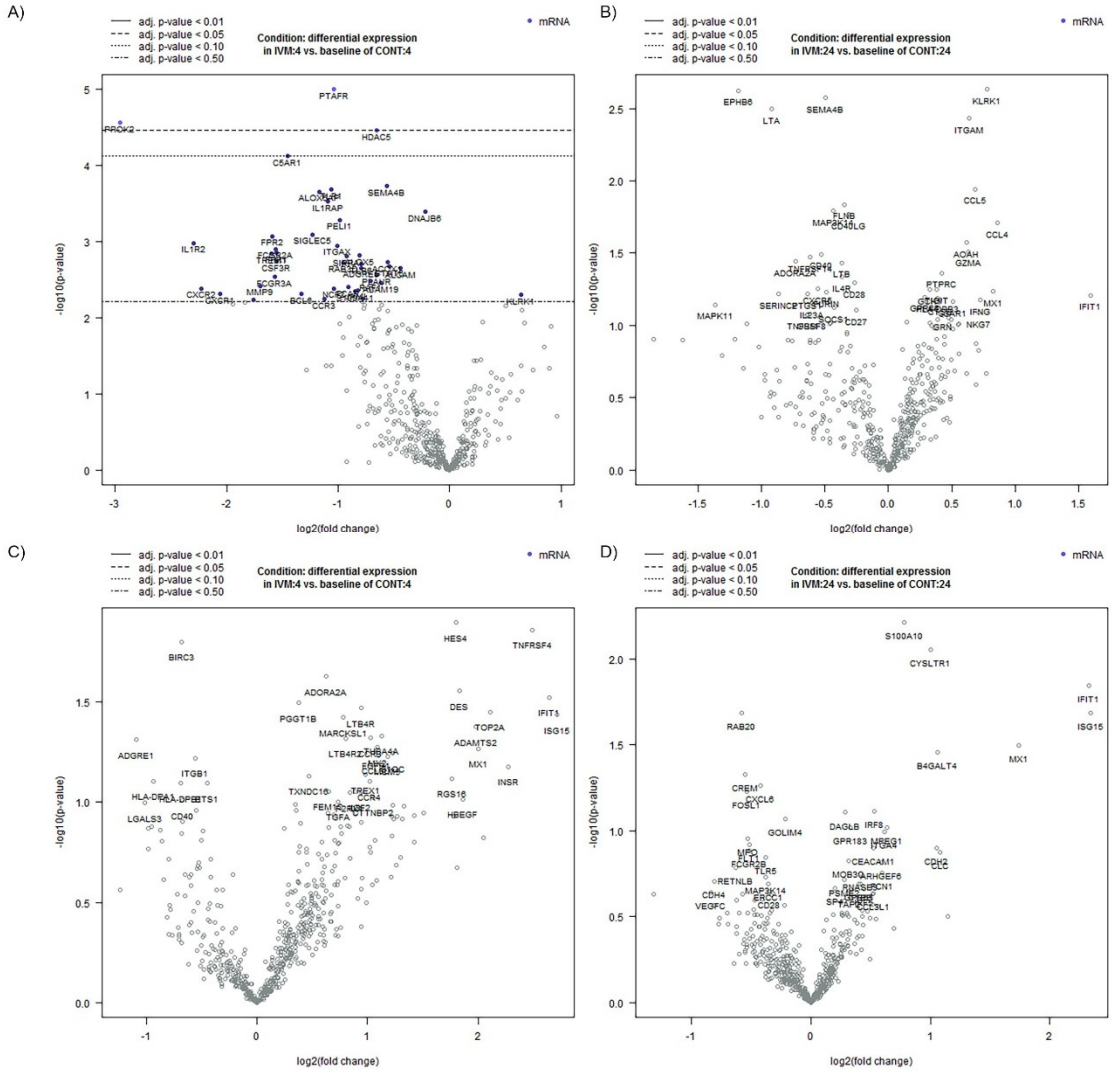

Figure 2.3. Volcano plots of changes in RNA transcript levels in PBMCs and PMNs 4 hrs and 24 hrs after administration of ivermectin or a placebo. Transcript levels were measured using the nCounter® Human Innate Immunity Myeloid Panel. A). Differential expression between ivermectin- and placebo-treated subjects in PBMCs 4 hrs after administration. B) Differential expression between ivermectin- and placebo-treated subjects in PBMCs 24 hrs after administration. C) Differential expression between ivermectin- and placebo-treated subjects in PMNs 4 hrs after administration. D) Differential expression between ivermectin- and placebo-treated subjects in PMNs 24 hrs after administration.

Leukocyte killing of Brugia malayi microfilariae

Since we have previously shown that human PMNs and PBMCs can kill *B. malayi* Mf in vitro, we tested the effect of the ivermectin treatment on the ability of

leukocytes isolated 24 hrs after treatment to do this (Figure 2.4). We observed no reduction in the survival of Mf incubated with PMNs or PMNs + PBMCs from subjects given ivermectin, compared with those from the control subjects.

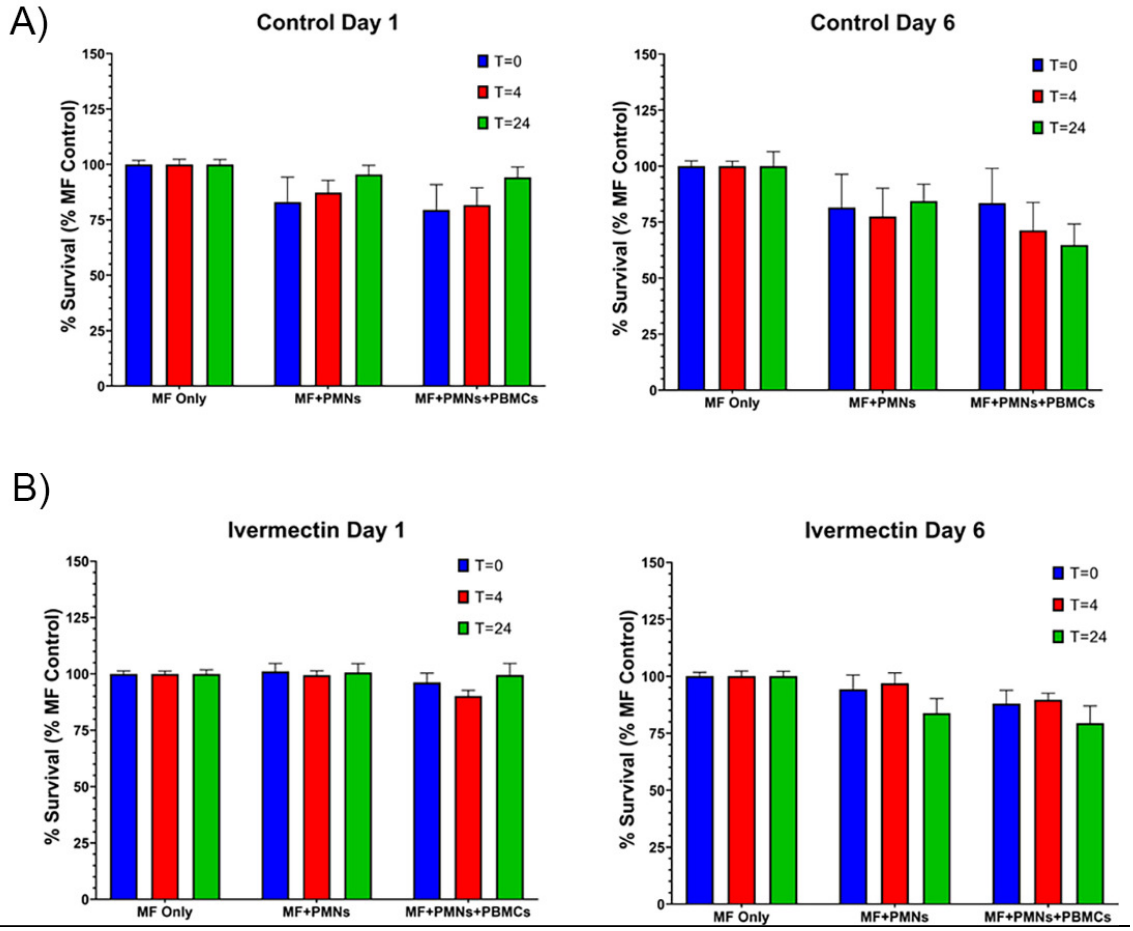

Figure 2.4. Survival of *B. malayi* Mf after incubation with PMNs or PMNs and PBMCs isolated from ivermectin- or placebo-treated subjects 4 hrs and 24 hrs after administration. A) Survival of Mf after 1- and 6-days incubation in vitro with cells from placebo-treated subjects. B) Survival of Mf after 1- and 6-days incubation in vitro with cells from ivermectin-treated subjects.

Discussion

Ivermectin is an extremely potent and effective drug, which rapidly removes microfilaria from the circulation and tissues of infected patients. It has been speculated that this rapid clearance of microfilariae involves the host immune system (Ali et al.,

2002; Wolstenholme et al., 2016) and, in vitro, it has been shown that the drug increases interactions between immune cells and filarial parasites (Vatta et al., 2014; Zahner et al., 1997). Such an involvement might result from a direct effect of ivermectin on the mammalian immune system, or from the drug interfering with the immunomodulation produced by the parasite, or a combination of the two. Previous reports have shown that ivermectin inhibits secretion, both of proteins and extracellular vesicles, by *B. malayi* Mf (Harischandra et al., 2018; Moreno et al., 2010); the experiments described here were designed to test the possibility that administration of ivermectin, at the dose given in mass drug administration programs, has, in addition, a direct effect on human cellular immunity. The study was limited in size, being originally designed to provide preliminary evidence for further and more detailed studies, but we detected no evidence for any such effect. We would not have been able to detect subtle alterations in cytokine expression or gene expression but, given the extraordinary efficacy of ivermectin treatment, if this is dependent on inducing changes in the host immune response we believe that these should have been evident in this study

These experiments focused on PBMC and PMN as we have previously found that these cell types have the ability to interact with Mf in vitro, killing the parasites under some, but not all, circumstances (McCoy et al., 2017). The complete blood counts confirmed that ivermectin did not cause any changes in the number of circulating leukocytes of any type, including eosinophils, which are frequently implicated in anti-helminth immune responses. Assessment of the effect on cytokines and chemokines was complicated by the fact that the levels of many was lower than the limits of detection of the Luminex assay, and by the variation in these levels within the control individuals. For

some analytes, there seemed to be a diurnal variation, as previously reported (Gibbs et al., 2012; Park et al., 2018; Petrovsky et al., 1998), as the levels at 0 and 24 hrs were similar, whereas those at 4 hrs varied. However, it was not possible to distinguish any specific changes due to the drug treatment. There were similar results from the gene expression measurements. In the placebo-treated PBMC samples, we found several transcripts to be significantly changed between pre-treatment and 4 hrs post-treatment, but those effects disappeared after 24 hrs. Only three genes showed any significant variation in the drug treated vs control individuals and these were all in PBMC at 4hrs post-treatment, suggesting that any drug effect on gene expression was transient. The largest effect was an almost 8-fold reduction in prokineticin 2 mRNA expression; prokineticin 2 is a pro-inflammatory peptide that is strongly upregulated in neutrophils and other inflammatory cells in response to granulocyte-colony stimulating factor or other myeloid growth factors (Monnier & Samson, 2008). This down-regulation might therefore lead to a short-lived anti-inflammatory response; in mice prokineticin 2 reduces IL-10 and IL-4 production (Franchi et al., 2008) so a reduction in expression in PBMCs might predict an increase in these cytokines. An increase in IL-4 was measured in ivermectin-treated individuals at 24 hrs but was not statistically significant. IL-10 levels did not change in either group. A prokineticin 2 antagonist also reduced interferon-γ expression in spinal lymph node cells (Abou-Hamdan et al., 2015) and reduced IFN-γ levels were seen in the drug-treated individuals 4 hrs post administration compared to controls, again these differences did not reach significance. The most statistically significant change was a smaller reduction, 2-fold, in platelet activating factor receptor mRNA (PAFR). A role has been proposed for PAFR in inflammatory pathways (Liu et al., 2019), so this down regulation is also

consistent with reduced inflammation. The third significant change was a small reduction in histone deacetylase 5 (HDAC5); this protein can also promote inflammation via activation of NF-κB (Zhao et al., 2019). Taken together, these changes are consistent with a potential anti-inflammatory effect of ivermectin (Ventre et al., 2017), but the other changes in cytokine levels or gene expression that might be expected to accompany this were not detected. Also, no change in the ability of the PBMCs and PMNs isolated from treated individuals to kill *B. malayi* Mf was found. This assay uses autologous serum so if killing were reliant on changes in compounds not tested in the Luminex assay, we should have detected it. These results suggest that any involvement of the immune system in the rapid clearance of Mf from the blood of treated individuals is due either to the inhibition of parasite-induced immunomodulation (Harischandra et al., 2018; Moreno et al., 2010) or to drug-induced damage to the worms that is difficult to detect or reproduce in vitro.

Conclusions

We found no evidence to support our hypothesis that the rapid action of ivermectin against microfilaria is mediated, at least in part, via a direct effect on the host immune system. No significant changes were found in circulating cytokine levels, or in the ability of PMNs and PBMCs to kill the Mf after the drug was administered. The expression of very few genes was significantly altered.

Appendix A: Supplementary Information

Table A1. Inclusion and exclusion criteria for participants recruited into the study.

Table A2. Nanostring results for the changes in expression of the 770 genes of the myeloid gene panel in PBMCs. In this Table all the results are compared to the Control, t= 0 sample.

Table A3. Nanostring results for the changes in expression of the 770 genes of the myeloid gene panel in PMNs. In this Table all the results are compared to the Control, t = 0 sample.

Table A4. Nanostring results for the changes in expression of the 770 genes of the myeloid gene panel in PBMCs. In this Table the changes between subjects who received ivermectin and those who received a placebo are compared at 4hrs and 24hrs post-treatment.

Table A5. Nanostring results for the changes in expression of the 770 genes of the myeloid gene panel in PMNs. In this Table the changes between subjects who received ivermectin and those who received a placebo are compared at 4hrs and 24hrs post-treatment.

Abbreviations

CBC: Complete Blood Counts; CTRU: University of Georgia Clinical and Translational Research Unit; HDAC5: histone deacetylase 5; LF: lymphatic filariasis; Mf: microfilariae; mTOR: PAFR: platelet activating factor receptor; PBMC: peripheral blood mononuclear cells; PMN: polymorphonuclear cells.

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Availability of data and materials

Data supporting the conclusions of this article are included within it. The results are available at ClinicalTrials.gov under record NCT03459794

Ethics Approval and Consent to Participate

Experiments and informed consent procedures were approved by the Institutional Review Boards of the University of Georgia (permit number 2012-10769). Human subjects recruited under the guidelines of IRB-approved protocols were provided written informed consent. Human blood material was obtained from the Clinical and Translational Research Unit, University of Georgia; experiments and informed consent procedures were approved by the Institutional Review Boards of the University of Georgia (STUDY00005069).

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CHAPTER 3

IDENTIFICATION OF NEW GENES INFLUENCING THE RESPONSE TO IVERMECTIN IN *C. ELEGANS* [2]

[2] Wilson, N.E., Reaves, B.J. & Wolstenholme, A.J. Identification of new genes influencing the response to ivermectin in *C. elegans*. To be submitted to PLOS Neglected Tropical Diseases

Abstract

In 2016, nearly 800 million tablets of ivermectin were distributed to countries for use in elimination programs for human filarial diseases. Despite its widespread use, the mode of action of ivermectin against filarial nematodes is not well understood, and its *in vivo* potency cannot be replicated *in vitro*. To better understand how ivermectin affects filarial worms, our lab previously performed a transcriptomics study to identify differently expressed genes (DEG) in *Brugia malayi* adults and microfilariae after treatment of infected gerbils. Forty-four of these DEG had *C. elegans* orthologs available as mutant strains through the *C. elegans* Genetics Center. We have assayed these mutant strains for differential sensitivity to ivermectin by measuring three phenotypes affected by ivermectin: egg production, development, and motility. We have identified several resistant and hypersensitive strains of *C. elegans* as well as differences between responses to the three assays. Overall, we identified eleven strong candidate genes as altering ivermectin sensitivity in at least one assay. These include genes related to previously implicated mechanisms of resistance such as: *che-12* (e1812), a gene required for the normal structure and function of distal ciliary structures on amphid neurons and a known dye filling defective mutant (a phenotype associated with ivermectin resistance); and *wht-4* (ok1007), a gene encoding an ABC transporter, a family of genes in which mutations have been associated with ivermectin toxicity in animals and overexpression with resistance in helminths. We also identified genes involved in pathways not previously associated with ivermectin's mechanism of action or resistance such as: *tyr-2* (ok1363), a dopachrome isomerase involved in protection of the germline from DNA-

damage induced apoptosis; and *lips-7* (ok3110), a lipase involved in lipid metabolism and longevity.

Introduction

Lymphatic Filariasis

Parasitic helminths are a major concern to both human and animal health, infecting approximately 1 billion people and causing the loss of over 14.11 million disability-adjusted life years in 2010 (Hotez et al., 2014). In the agricultural sector, helminth parasites of livestock have negative effects on production by reducing weight gain, milk production, and meat quality (Charlier et al., 2014). Filarial helminths (superfamily Filarioidea) are a widely distributed group of parasites of both humans (e.g., lymphatic filariasis, onchocerciasis, *Loa loa*, mansonellosis) and animals (canine heartworm). Heartworm (*Dirofilaria immitis*) infects over 200,000 dogs in the United States every year (1.22% prevalence in 2021) and is present with varying prevalence throughout the world (Noack et al., 2021; *Parasite Prevalence Maps*, n.d.). Both onchocerciasis and lymphatic filariasis are neglected tropical diseases targeted for elimination and elimination as a public health concern respectively by 2030 (World Health Organization, 2020). Lymphatic filariasis (caused by *Wuchereria bancrofti, Brugia malayi,* and *Brugia timori*) is the most widely distributed human filarial parasite, with approximately 859 million people at risk of infection and 51.4 million people infected (World Health Organization, 2020). Lymphatic filariasis is transmitted as infectious third-stage larvae by various species of mosquitos including members of the *Anopheles* and *Culex* genera (Taylor et al., 2010). Adult worms reside in the lymphatics and produce microfilariae (MF) which circulate in the blood stream. These MF are taken up by new mosquitos, in which they develop into third stage larvae to continue the transmission cycle. Adult worms live between six and eight years and the damage they

cause to the lymphatics prevents fluid from draining, resulting in pathology. The disease can range from asymptomatic to severe disfigurement and disability; symptoms include lymphoedema and elephantiasis, hydrocele, and secondary bacterial infections resulting from the rupture of skin lesions (Babu & Nutman, 2012; Burri et al., 1996; Taylor et al., 2010). Lymphatic filariasis resulted in a loss of approximately 1.6 million disability-adjusted life years (DALYs) annually in 2019 (World Health Organization, 2020). Additionally, infected individuals and their caretakers experience a loss of approximately 5.319 million DALYs annually due to depressive illness (2012 figures, compared to 2.78 million DALYs due to LF alone) as a result of the effects of lymphatic filariasis (Ton et al., 2015).

Lymphatic filariasis has been controlled in mass drug administration campaigns using two medication regimens: ivermectin and albendazole (IA) in areas co-endemic for onchocerciasis; and diethylcarbamazine (DEC) and albendazole (DA) in areas free of onchocerciasis (World Health Organization, 2000b, 2000a). In both regimens, MF are rapidly depleted from the circulation and the production of new MF by adult females is inhibited for several months, thereby blocking transmission (Brown et al., 2000; Gyapong et al., 2005). However, recent clinical studies have shown that a triple drug combination of ivermectin, DEC, and albendazole (IDA) is more effective at suppressing MF production and clearing adult worms and so the World Health Organization has recommended its use where appropriate (Weil et al., 2019, 2020; World Health Organization, 2017). Therefore, the use of ivermectin is expanding to hundreds of millions more people every year and the Mectizan Donation Program has accordingly increased its donation of Mectizan (ivermectin) by 100 million treatments per year to

regions where onchocerciasis is not endemic (Williams et al., 2020; World Health Organization, n.d.).

Ivermectin

Ivermectin is a member of the avermectin/milbemycin family of compounds which also includes moxidectin, milbemycin oxime, eprinomectin, and others (Campbell, 2016; Crump, 2017). Ivermectin targets glutamate-gated chloride channels (GluCls), a type of ion channel exclusive to invertebrates, resulting in its excellent safety profile (Arena et al., 1995; Cully et al., 1994; Dent et al., 2000; Guzzo et al., 2002). Ivermectin acts as an atypical agonist, binding semi-irreversibly and causing hyperpolarization of the membrane (Wolstenholme, 2012; Wolstenholme & Rogers, 2005). In *C. elegans* and other nematodes, this results in paralysis and death. However, microfilariae do not feed and ivermectin does not kill adult worms. The mechanism by which ivermectin clears MF from the bloodstream and inhibits fertility is remains unclear.

The concentration of ivermectin needed to paralyze MF *in vitro* is many times higher than the peak plasma concentrations reached in mass drug administration patients (Evans et al., 2017b; Maclean et al., 2017b; Storey et al., 2014b; Wolstenholme et al., 2016); ivermectin at a concentration of 6.1±1.1µM results in paralysis of 50% a while a peak plasma concentration of approximately 50nM (an approximately 120 fold difference) is sufficient to cause a near complete elimination of MF from the bloodstream (Storey et al., 2014b; Wolstenholme et al., 2016). This drastic difference between *in vivo* and *in vitro* efficacy seem to imply that a host component may be involved mediating the clearance of MF. Notably, the anthelmintic effects of the avermectins were discovered using an *in vivo* assay (Campbell, 2016).

Ivermectin's Mode of Action

In *Brugia malayi* MF, glutamate-gated chloride channels are located around the excretory/secretory pore (Moreno et al., 2010). This has led to the hypothesis that ivermectin clears MF by suppressing the secretion of immunomodulators. Additionally, the secretion of extracellular vesicles by *B. malayi* and *D. immitis* is also inhibited by ivermectin while resistant strains of *D. immitis* were not inhibited (Harischandra et al., 2018). EVs from *B. malayi* contained cargo including immunomodulators, were taken up by murine macrophages and induce classical macrophage activation, supporting the hypothesis that they exert an immunomodulatory effect (Zamanian et al., 2015). We have previously shown that ivermectin can stimulate the attachment of canine immune cells to the MF of *D. immitis* in a concentration-dependent manner, but this attachment does not result in killing (Berrafato et al., 2019; Vatta et al., 2014). In contrast, human immune cells are capable of killing *B. malayi* MF, however this is not affected by incubation with ivermectin (McCoy et al., 2017; Reaves et al., 2018). Killing of *B. malayi* MF was dependent on both the recognition of a heat-labile factor by immune cells as well as a secreted or surface-associated factor that inhibits recognition; this supports the hypothesis that inhibition of secretion allows recognition and clearance of MF by immune cells. However, ivermectin's lack of effect in the co-culture assay clearly indicates that the mechanism by which ivermectin clears MF is more complicated. GluCls are also expressed in the gonad tissue of adult female *B. malayi,* but it is still unknown how exactly this affects fertility (Li et al., 2014).

To better understand how ivermectin affects *B. malayi*, we previously performed a study investigating how treating gerbils infected with *B. malayi* with a single dose of

ivermectin (0.2mg/kg) affected gene expression in adult males, adult females, and MF, compared to parasites from untreated hosts (Maclean et al., 2019). This study identified 117 differently expressed genes unique to the ivermectin treated group. Differently expressed genes included those involved in endothelin signaling, cell division, motor activity, and developmental processes. Ivermectin's effects on MF gene expression occurred rapidly and reversed by 7 days post treatment, which may reflect rapid clearance of MF; effects on females did not appear until 7 days post treatment, which may reflect ivermectin's long-term suppression of MF production. These differently expressed genes may reflect the damage caused to the parasite by ivermectin treatment or the parasite's response to the drug. Therefore, characterizing these genes' effects on ivermectin sensitivity may provide insight into other pathways affected by ivermectin.

Present Study

To this end we have performed a screen of these genes utilizing mutant strains of the model nematode *Caenorhabditis elegans. C. elegans* is a widely used and well-established model for parasitic nematodes that has been instrumental in identifying and characterizing the targets of a number of anthelmintics, including ivermectin (Arena et al., 1995; Cully et al., 1994; Dent et al., 2000). It shares great genetic and physiological similarities with other members of Nematoda despite its simple and rapid life cycle (Hahnel et al., 2020) and has been frequently used to express parasite genes (Britton et al., 1999; Couthier et al., 2004; Glendinning et al., 2011). A large array of resources are available for *C. elegans* including knockout mutants (C. elegans Deletion Mutant Consortium, 2012), databases with integrated bioinformatics and data-mining tools (Angeles-Albores et al., 2018; Howe et al., 2016, 2017b), RNAi libraries (Fraser et al.,

2000; Kamath et al., 2003; Rual et al., 2004), and a collection of open-access peer-reviewed chapters covering topics in *C. elegans* and nematode biology and methodology (*Wormbook*, n.d.).

Of the 117 genes identified in our transcriptomics study (Maclean et al., 2019), we selected 28 genes to investigate using knockout mutants from the *C. elegans* Genetics Center and 11 using RNAi by feeding using the Ahringer library. The decision chart for selecting specific strains of *C. elegans* can be seen in figure 3.1. We assayed these genes for their effect on ivermectin sensitivity using three standard phenotypic assays: egg laying (fertility), development, and motility. We additionally utilized a microfluidics platform to measure pharyngeal pumping. We identified 11 different genes that affect ivermectin sensitivity including *lips-7*, involved in lipid catabolic processes, *tyr-2,* a dopachrome isomerase, and *wht-4,* an ABC-type transporter (Howe et al., 2016). We also found that ivermectin affects fertility and development independently of each other, with some genes showing opposite results in the two assays, notably *unc-22,* encoding the giant muscle protein twitchin; results in the development and pharyngeal pumping assays correlated as expected. Finally, we accumulated additional evidence that motility is not an appropriate phenotype for measuring sensitivity to ivermectin.

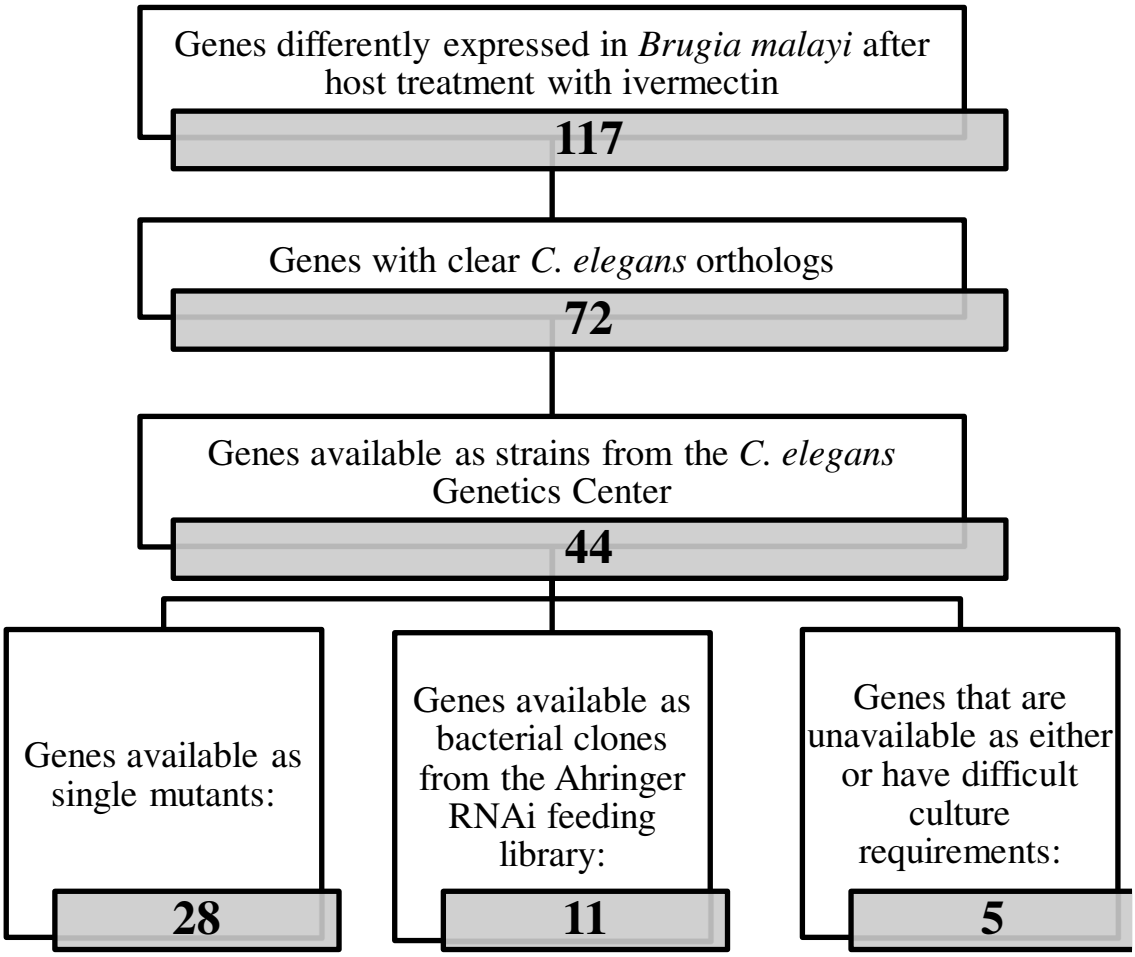


Figure 3.1: Decision chart for selecting a final list of candidate genes, strains, and clones. A full list of strains and clones used is available in tables 3.1 and 3.3.

Materials and Methods

C. elegans strains and culture conditions

All *C. elegans* strains (table 3.1), OP50, and HT115 (DE3) were received from the Caenorhabditis Genetics Center (CGC, University of Minnesota, Minneapolis, MN, USA). A list of standard solutions is provided in table 3.2. All strains were maintained at 20°C or room temperature on Nematode Growth Medium (NGM) agar plates seeded with OP50 bacteria using standard methods (Brenner, 1974). Eggs and synchronized cultures were prepared by bleaching as described (Sulston & Hodgin, 1988). Briefly, plates containing mostly eggs and gravid adults were washed with M9 into a 15mL conical tube

for 1 plate or a 50mL tube for 2 or 3 plates and centrifuged at 1000g for 1 minute or 2000g for 3 minutes, respectively. The worm pellets were treated with 15mL of an alkaline hypochlorite solution (55% diH_2O, 25% 1M NaOH (company), 20% household bleach) per starting plate. After 5 minutes of gentle inversion, the alkaline hypochlorite solution was removed, and the pellet was washed 3 times with M9 then resuspended in M9. Eggs were either used immediately or hatched in M9 in a petri dish overnight at room temperature with gentle shaking to obtain synchronized first-stage larvae (L1).

Table 3.1: Summary of all *C. elegans* strains used.
RB strains provided by *C. elegans* Gene Knockout Project at the Oklahoma Medical Research Foundation, which was part of the International C. elegans Gene Knockout Consortium, which should be acknowledged in any publications resulting from its use. Paper_evidence WBPaper00041807
VC strains provided by the *C. elegans* Reverse Genetics Core Facility at the University of British Columbia, which is part of the international C. elegans Gene Knockout Consortium, which should be acknowledged in any publications resulting from its use. Paper_evidence WBPaper00041807

	Gene Name	**Strain Name**	**Genotype**
1.	-	Bristol N2	*C. elegans* wild -type
2.	*avr-14, avr-15, glc-1*	JD608	*avr-14(ad1302); avr-15(ad1051); glc-1(pk54)*
3.	*nhr-8*	AE501	*nhr-8(ok186)*
4.	*cey-2*	RB988	*cey-2(ok902)*
5.	*wht-4*	RB1058	*wht-4(ok1007)*
6.	*tyr-2*	RB1272	*tyr-2(ok1363)*
7.	*cbn-1*	RB2619	*cbn-1(ok3646)*
8.	*him-3*	CB1256	*him-3(e1256)*
9.	*lips-7*	RB2287	*lips-7(ok3110)*
10.	*kri-1*	CF2052	*kri-1(ok1251)*
11.	*aex-5*	JT23	*aex-5(sa23)*
12.	*tap-1*	VC333	*tap-1(gk202)*
13.	*inx-14*	AU98	*inx-14(ag17)*
14.	*lpd-3*	VC1878	*lpd-3(ok2138)*
15.	*nhl-2*	RB935	*nhl-2(ok818)*
16.	*ccch-1*	RB1626	*ccch-1(ok2002)*
17.	*anc-1*	CB3440	*anc-1(e1873)*
18.	*csp-2*	RB1485	*csp-2(ok1742)*
19.	F35A5.1	RB1860	F35A5.1*(ok2406)*
20.	*unc-22*	BC177	*unc-22(s17)*

21.	*unc-54*	CB190	*unc-54(e190)*
22.	*dpy-5*	CB61	*dpy-5(e61)*
23.	*trx-5*	RB1637	*trx-5(ok2014)*
24.	*che-12*	CB3332	*che-12(e1812)*
25.	*hil-1*	VC426	*hil-1(gk229)*
26.	*pis-1*	VC3179	*pis-1(ok3720)*
27.	*aff-1*	BP600	*aff-1(tm2214)*
28.	*ogt-1*	RB653	*ogt-1(ok1474)*
29.	*ogt-1*	RB1342	*ogt-1(ok430)*
30.	*mig-6*	NW1314	*mig-6(ev701)*
31.	*fut-1*	VC378	*fut-1(gk183)*
32.	*spv-1*	RB1353	*spv-1(ok1498)*
33.	*npp-11*	RB1406	*npp-11(ok1599)*
34.	*sacy-1*	DG3430	*sacy-1(tn1385)*
35.	*mig-10*	RB1920	*mig-10(ok2499)*
36.	*gei-8*	VC1519	*gei-8(gk693)*
37.	*fkh-9*	VC1925	*fkh-9(ok1709)*
38.	*larp-1*	JK4545	*larp-1(q783)*

Name	**Composition**
Nematode Growth Medium (NGM)	1.7% (w/v) Bacto Agar, 0.25% (w/v) Bacto Peptone, 50 mM NaCl, 5 mg/L Cholesterol, 1 mM $CaCl_2$, 1mM $MgSO_4$, and 25 mM KPO_4
M9 Buffer	3 g KH_2PO4, 11.35g $Na_2HPO_4 \cdot 7H_2O$, 5 g NaCl, 1mL 1M $MgSO_4$ in 1L H_2O
LB Medium	10g/L tryptone, 5g/L yeast extract, 10g/L NaCl
LB Agar	10g/L tryptone, 5g/L yeast extract, 10g/L NaCl, 15g/L Bacto Agar
Table 3.2: List and composition of standard solutions.	

Drug Preparations

Chemicals were sourced from Sigma-Aldrich, Inc. (St. Louis, MO) except where noted. Ivermectin (IVM) was dissolved in dimethyl sulfoxide (DMSO) in the egg laying, egg development, and pharyngeal pumping assays, prepared as 100x working stocks, and stored at -20°C. When used in an assay, the IVM drug stocks were diluted to 1% (v/v) DMSO and the desired ivermectin concentration. Ivermectin was dissolved in propylene

glycol (PG; Butler Schein Animal Health, Dublin, OH) in the motility assay due to the optical requirements of the assay. It was also prepared as 100x working stocks, stored at -20°C, and diluted to 1% (v/v) PG and the desired concentration of ivermectin when used in an assay. Carbenicillin, ampicillin, tetracycline, and isopropyl β-d-1-thiogalactopyranoside (IPTG) were prepared as 1000x stocks in diH_2O and stored at -20°C. Working stocks of serotonin were prepared at 100mM for serotonin-HCl or 50mM for serotonin-creatinine in M9 Buffer. All serotonin solutions were used on the day of preparation.

Egg Lay Assay Set-Up

This assay was used to determine the effects of various mutations on *C. elegans'* ability to lay eggs in the presence of varying concentrations of ivermectin. Egg lay assays were set up in 96-well edge plates (Nunc, Thermo Fisher Scientific, Waltham, MA). Each well contained 200µL of M9 buffer with 2.5% (v/v) OP50 overnight LB medium liquid culture (not more than 5 days old), 1% DMSO, and increasing concentrations of IVM from 10pM to 10µM in one log intervals, or DMSO alone. One gravid adult hermaphrodite was added to each well and the number of eggs and larvae in each well was counted immediately after setting up (T=0) to account for any accidental transfer. The surrounding wells and moat were filled with diH_2O to reduce the edge effect. Each concentration was set up as five replicates and each experiment was repeated three times. On each day of assay preparation, an additional well containing a control N2 worm was added for each concentration to confirm assay conditions. The plates were incubated on the bench at room temperature. After 24 hours (T=24), the number of eggs and larvae in each well was counted. The T=0 count was subtracted from the T=24 count to calculate

the number of offspring produced in 24 hours, and this was normalized to the DMSO control for each experiment.

Egg development assay set up

This assay was used to determine the effects of various mutations on *C. elegans'* ability to develop from eggs to adulthood in the presence of varying concentrations of ivermectin. Approximately 50 *C. elegans* eggs were added to the wells of a 24 well plate poured with 1.5mL NGM seeded with 30µL of OP50 overnight LB medium liquid culture diluted 1:1 with LB medium. The NGM additionally contained 1% DMSO and either no drug, 1nM ivermectin, or 10nM ivermectin. The plates were left inverted at room temperature on the bench for 2-3 days based on the development rates of the strains being measured, at which time the number of eggs which had developed to at least the third larval stage were counted and normalized to the percent that developed in the DMSO only control. Each concentration was set up in triplicate and each initial experiment was repeated twice. Strains that showed inhibited development at 1nM or any detectable development at 10nM were retested using a full dilution series from 10pM to 10µM, in one log increments, with an additional 3nM concentration and DMSO only control. Each concentration was set up in triplicate, and each experiment was repeated three times.

Motility assay set up

This assay was used to determine the effects of various mutations on *C. elegans* motility in the presence of varying concentrations of ivermectin. *C. elegans* eggs were isolated and left to hatch at room temperature overnight in a 60mm petri dish with gentle shaking in 7mL of M9 with 100µL of OP50 LB medium liquid culture. First stage larvae

were collected and diluted to 3 larvae/µL. An intermediate drug dilution of 2% propylene glycol and 2x ivermectin, or 2% PG alone, in M9 buffer was prepared from the 100x ivermectin in PG stocks. 200µL of the intermediate drug dilutions and 200µL of the L1 dilution were combined and mixed thoroughly. This was divided into four wells of 100µL each in a 384 well optical plate (Nunc, Thermo Fisher Scientific). Each well therefore contained 1% PG, 1x ivermectin, and approximately 150 L1. The outermost wells and the wells bounding each unique strain on the same plate were filled with diH_2O to prevent evaporation and reduce the edge effect. The plates were left to incubate on the bench at room temperature for 24 hours. The motility of the L1s was measured using the Worminator system (Storey et al., 2014a), for 30 seconds per well. The mean motility units measured by the Worminator program were normalized to the average of the PG control wells of that strain for that day. Each experiment was repeated 3 times.

Pharyngeal Pumping Assay set-up

Approximately 500 to 1000 synchronized L1 per plate were plated on 90mm NGM plates seeded with OP50 bacteria and maintained under standard culture conditions. Approximately four days later (based on the growth rate of the strain being measured), the synchronized young adult population was washed off the plates with M9 buffer into 15mL conical tubes and left for several minutes to settle. The M9 was pulled off and the adults were washed several times with M9, each time leaving the adults to settle to the bottom of the tube. This was repeated until the adults settled quickly and the remaining M9 stayed clear. The M9 was then removed and the adults were poured on an unseeded 90mm NGM plate and left to dry. The adults were then washed off the plate into a 2mL microcentrifuge tube with M9, and the process of washing with M9 and

letting the adults settle was repeated until the M9 wash was clear. The repeated washings helped remove any remaining OP50, eggs, or larvae that may have been washed off the plate. The adults were resuspended in 800µL of M9. Adult worms, 10mM serotonin from a 100mM or 50mM stock, 1% DMSO, and either 10nM, 50nM, or 100nM ivermectin or DMSO alone were combined with M9 to a final volume of 1mL in a 1.5mL microcentrifuge tube, mixed thoroughly, and incubated for 1 hour at room temperature. Electropharyngeograms (EPGs) were recorded with the NemaMetrix (now InVivo Biosystems, Eugene, OR) ScreenChip™ system (Lockery et al., 2012; Weeks et al., 2018). Recordings were made for 30 seconds per worm in a SC40 chip, acquired with NemAcquire and analyzed with NemAnalysis (available for download from invivobiosystems.com). Each program was used on the default settings. A pharyngeal pump was defined as an event with a distinct positive excitation event (E-spike) followed by a distinct negative relaxation event (R-spike), based on the software threshold settings. Each chip was used for two days of recordings and washed with M9 when switching concentrations or strains. The time to record 20 adults from start to finish for each concentration was about 40 minutes, and the experiment was repeated on 3 separate days. The pump frequency for each concentration was normalized to the DMSO control from that day.

RNAi by feeding

A selection of genes not available through the CGC as single knockout mutants (for example, those carrying a balancing mutation) were investigated using RNA interference as described (Kamath et al., 2000) with modifications. HT115 bacteria transformed with an empty L4440 vector were used as a control and all HT115 clones

targeting genes of interest were from the Ahringer RNAi library and are listed in table 3.3. Clones were maintained on 12.5µg/mL tetracycline LB agar plates. LB media with 50µg/mL ampicillin was used to grow bacteria overnight (less than 24 hours) with shaking at 37°C. 500uL of the LB culture was spread on NGM plates containing 1mM IPTG and 25µg/ml carbenicillin, left to dry, and kept inverted overnight. The same day, eggs were isolated and synchronized overnight as described. Approximately 750 L1 were plated per plate and incubated at room temperature for 4 days, or until the worms had reached adulthood. Eggs from these worms were isolated as described and used in an egg development assay with the following modifications. Approximately 50 *C. elegans* eggs from an RNAi feeding plate were added to the wells of a 24 well plate poured with 1.5mL NGM seeded with 40µL of the corresponding dsRNA expressing bacteria from a new overnight culture of LB media with 50µg/mL ampicillin. The NGM additionally contained 1% DMSO, 1mM IPTG, 25µg/mL Carbenicillin, and either no drug, 1nM ivermectin, or 10nM ivermectin. The development plates were maintained and measured as previously described.

Gene Symbol	**Sequence**	**SJJ ID**	**Code**
htp-1	F41H10.10	IV-2H08	CUUkp3303H082Q
ptr-4	C45B2.7	X-3E18	CUUkp3305E183Q
cpl-1	T03F6.7	V-11I07	CUUkp3304I0711Q
-	ZK829.7	IV-6I22	CUUkp3303I226Q
let-19	K08F8.6	II-6I24	CUUkp3301I246Q
msh-5	F09E8.3	IV-7C05	CUUkp3303C057Q
dnj-13	F54D5.8	II-7L08	CUUkp3301L087Q
myo-3	K12F2.1	V-8O03	CUUkp3304O038Q
hmr-1	W02B9.1	I-5F19	CUUkp3300F195Q
sox-2	K08A8.2	X-3L06	CUUkp3305L063Q
lat-1	B0457.1	II-6O04	CUUkp3301O046Q
aff-1	C44B7.3	II-5C08	CUUkp3301C085Q

Table 3.3: List of all bacterial clones used for RNAi by feeding. The *C. elegans* RNAi Feeding Clones were created by Dr Julie Ahringer and provided by Source BioScience UK Limited (Nottingham, UK; www.sourcebioscience.com).

Gene Enrichment and Interaction Analysis

Gene set enrichment analysis was performed using the tool available through WormBase (Angeles-Albores et al., 2016, 2018). Gene interactions were analyzed using GeneMania (Mostafavi et al., 2008; genemania.org). The max resultant genes was set to 20, the max resultant attributes was set to 100, the network weighting was set to the default "automatically selected weighting method" or "equal by network type," and all databases available were used.

Statistical Analysis

All statistical analyses were performed in Prism 9.3. Dose response curves and IC50s were calculated using Prism's Absolute IC50 equation, with the baseline constrained to zero and the bottom constrained to greater than 0. Intervals given are the 95% symmetrical (asymptotic) approximate confidence interval. 95% confidence bands plot the likely location of the true dose-response curve. Prism identified unstable parameters for several strains which are indicated where necessary. Unstable or ambiguous parameters, including 95% confidence bands, were excluded from graphs for clarity. Additionally, 95% confidence bands cannot be plotted for data in which the analysis has calculated a best-fit value for a parameter that hits a constraint. The specifics of additional statistical tests are described in the accompanying figure.

Results

Egg Laying assay

We measured ivermectin's effect on egg laying by counting the offspring produced by single hermaphrodites after 24 hours. A hit in this assay was defined as a 10-fold or higher change in absolute IC_{50} compared to the N2 control and a clear difference

from N2 as measured by the separation of the 95% confidence intervals of the points and the fitted curves. In this assay, the N2 control had an absolute IC_{50} of 81nM (95% CI 21, 308) while the resistant control (JD608; *avr-14(ad1302), avr-15(ad1051), glc-1(pk54)*) was 5059nM (95% CI 1280, 19986), approximately 62-fold higher (figure 3.2). We tested 29 strains covering 28 genes in this assay (table 3.2). Eight gene mutations resulted in resistance (figures 3.4 and 3.5) while none resulted in hypersensitivity. Three gene mutations (*tyr-2(ok1363)*, *unc-22(s17)*, *wht-4(ok1007)*) resulted in absolute IC50's more than 100 times that of N2 (figure 3.4).

Notably, some genes had a marked increase in apparent offspring production at 1µM compared to 100nM, including N2, JT23 (*aex-5(sa23))*, and RB935 (*nhl-2(ok818))*, among others (figure 3.3B). This seemed to be due to ivermectin's inhibition of egg-laying and the resulting bag-of-worms phenotype (figure 3.6). At 100nM IVM, it was common to see that egg laying had been inhibited and the eggs had hatched inside the hermaphrodite. At 1µM, the hermaphrodites often broke open, releasing the worms and increasing the offspring count as it was impossible to distinguish between offspring from eggs that had been laid and offspring that had come from the ruptured adult. At 10µM the bag-of-worms phenotype and ruptured adults was less common.

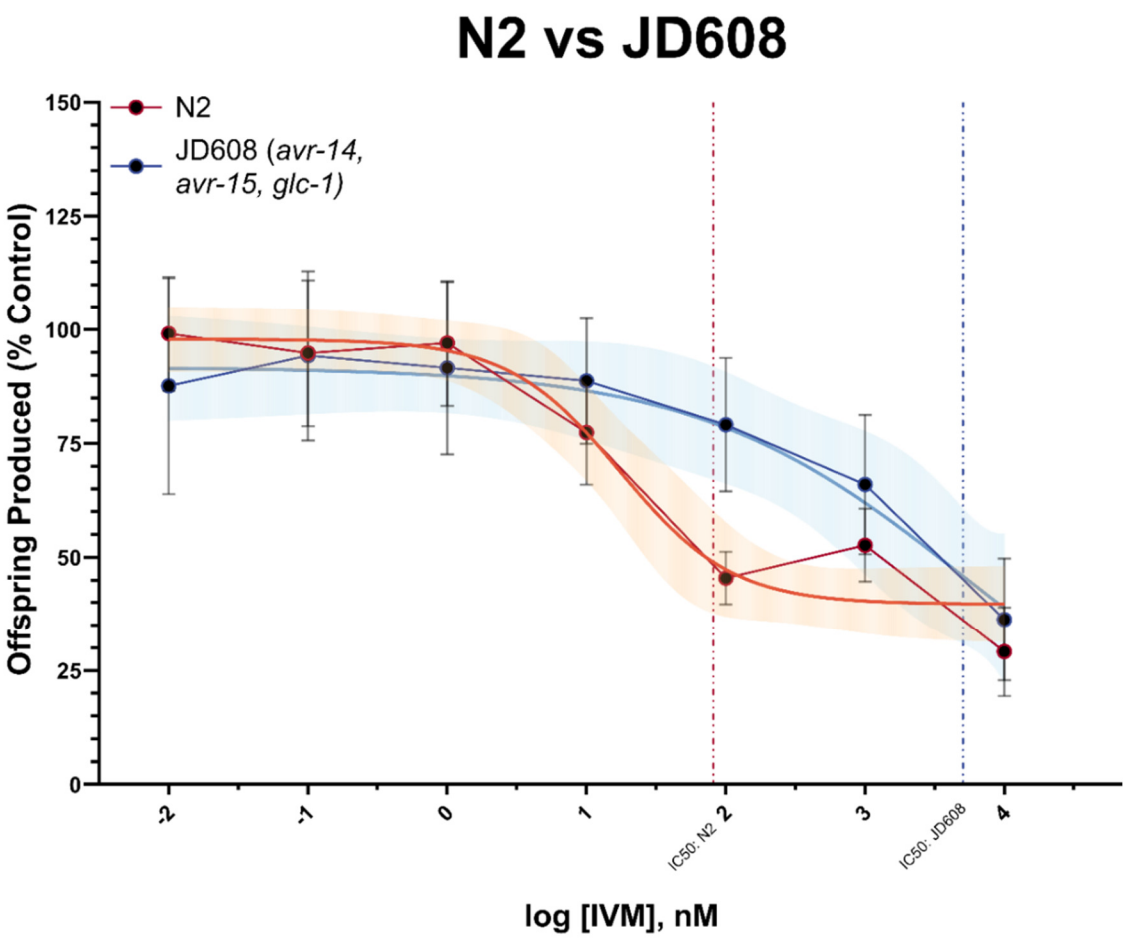


Figure 3.2: Inhibition curve of N2 (wild type) compared to JD608 (resistant control) in the egg laying assay. Individual hermaphrodites were placed in wells with OP50 and increasing concentrations of ivermectin and the offspring produced by each worm was counted 24 hours later and normalized to the DMSO control. Error bars are the 95% CI of each point, shaded regions are the 95% confidence band of the dose-response curve. Dashed lines mark the absolute IC50, n=15.

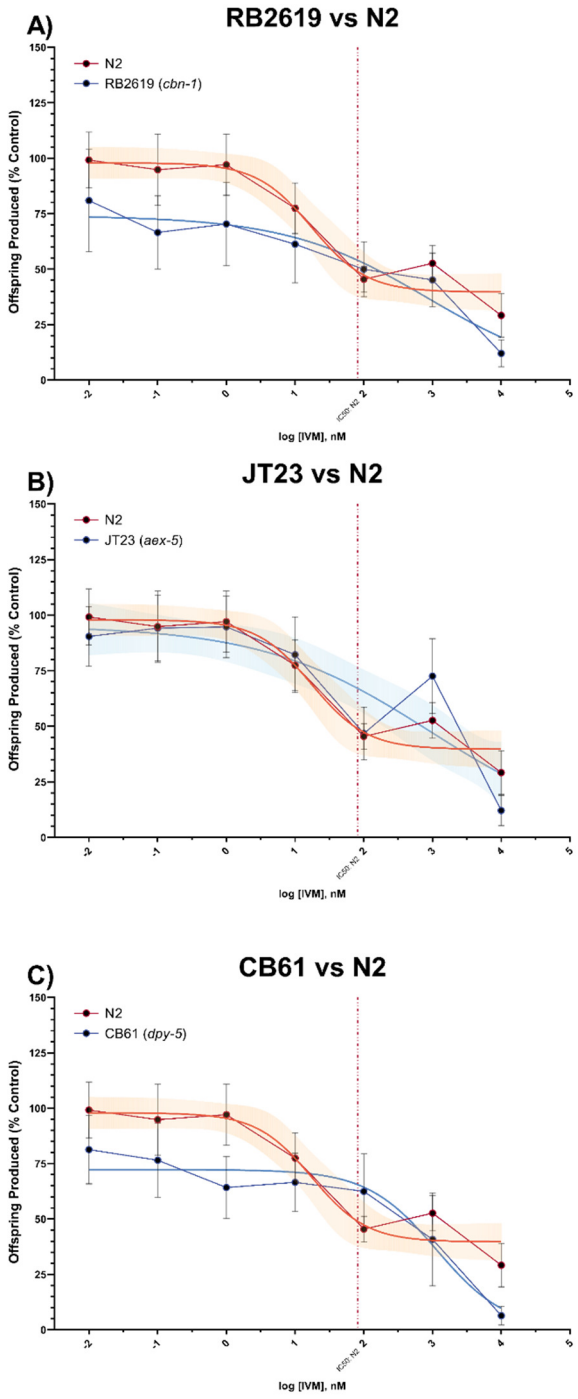
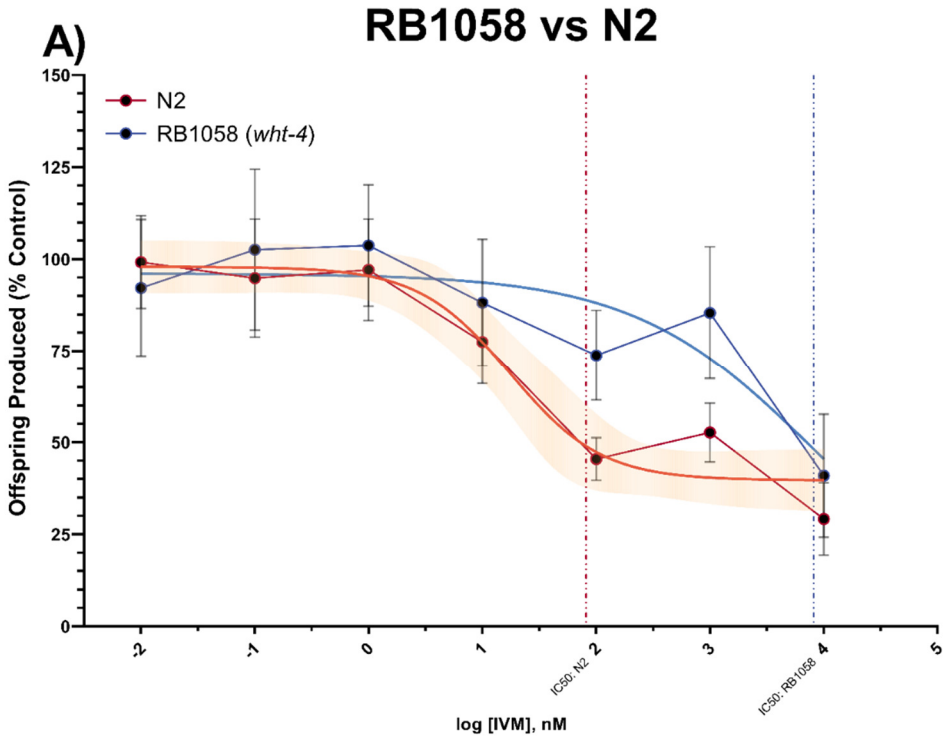
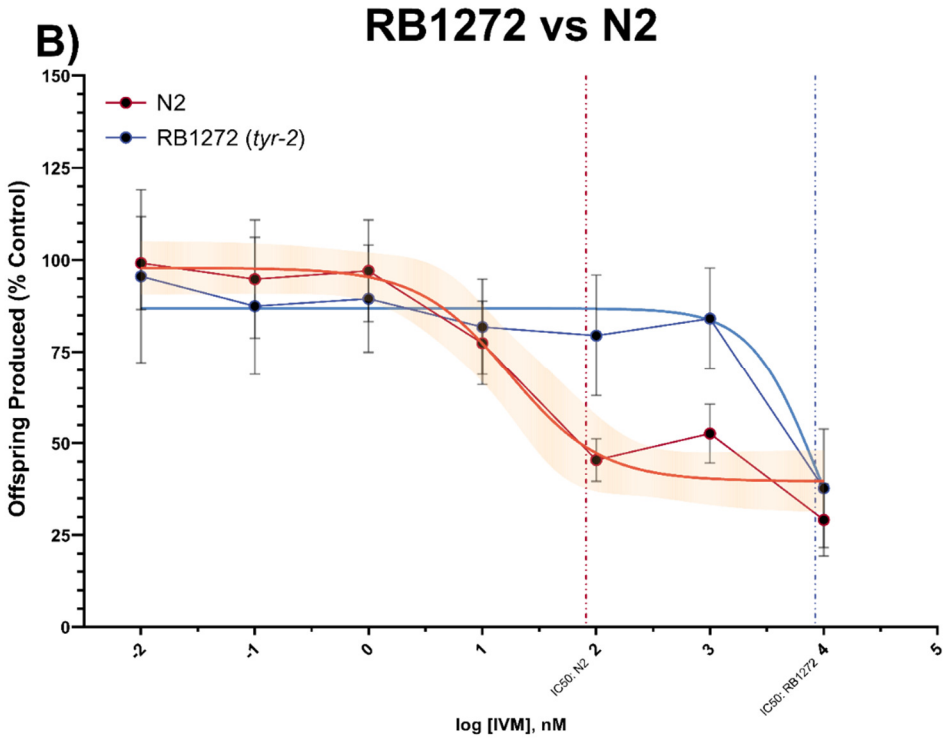


Figure 3.3: Inhibition curves of strains with high absolute IC50s that were not considered resistant in the egg laying assay due to a lack of clear difference between them and N2. A) RB2619 (*cbn*-1) vs N2. B) JT23 (*aex-4*) vs N2. C) CB61 (*dpy-5*) vs N2. Error bars are the 95% CI of each point, shaded regions are the 95% confidence band of the dose-response curve. Dashed lines mark the absolute IC50, n =15.


A)
RB1058 vs N2
N2
RB1058 (wht-4)
Offspring Produced (% Control)
150
125
100
75
50
25
0
-2
-1
0
1
IC50: N2
2
3
IC50: RB1058
4
5
log [IVM], nM


B)
RB1272 vs N2
N2
RB1272 (tyr-2)
Offspring Produced (% Control)
150
125
100
75
50
25
0
-2
-1
0
1
IC50: N2
2
3
IC50: RB1272
4
5
log [IVM], nM

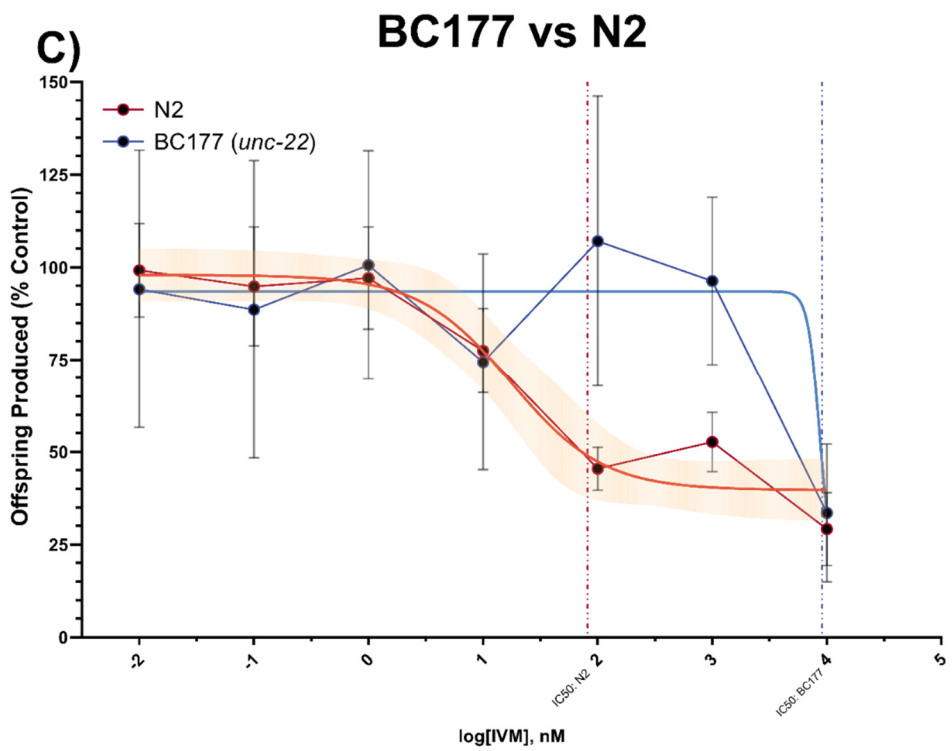


Figure 3.4: Inhibition curves of strains with absolute IC50s more than 100 times that of the N2 control in the egg laying assay. A) RB1058 (*wht-4*) vs N2. B) RB1272 (*tyr-2*) vs N2. C) BC177 (*unc-22*) vs N2. Error bars are the 95% CI of each point, shaded regions are the 95% Confidence band of the dose-response curve. Dashed lines mark the absolute IC50, n=15.

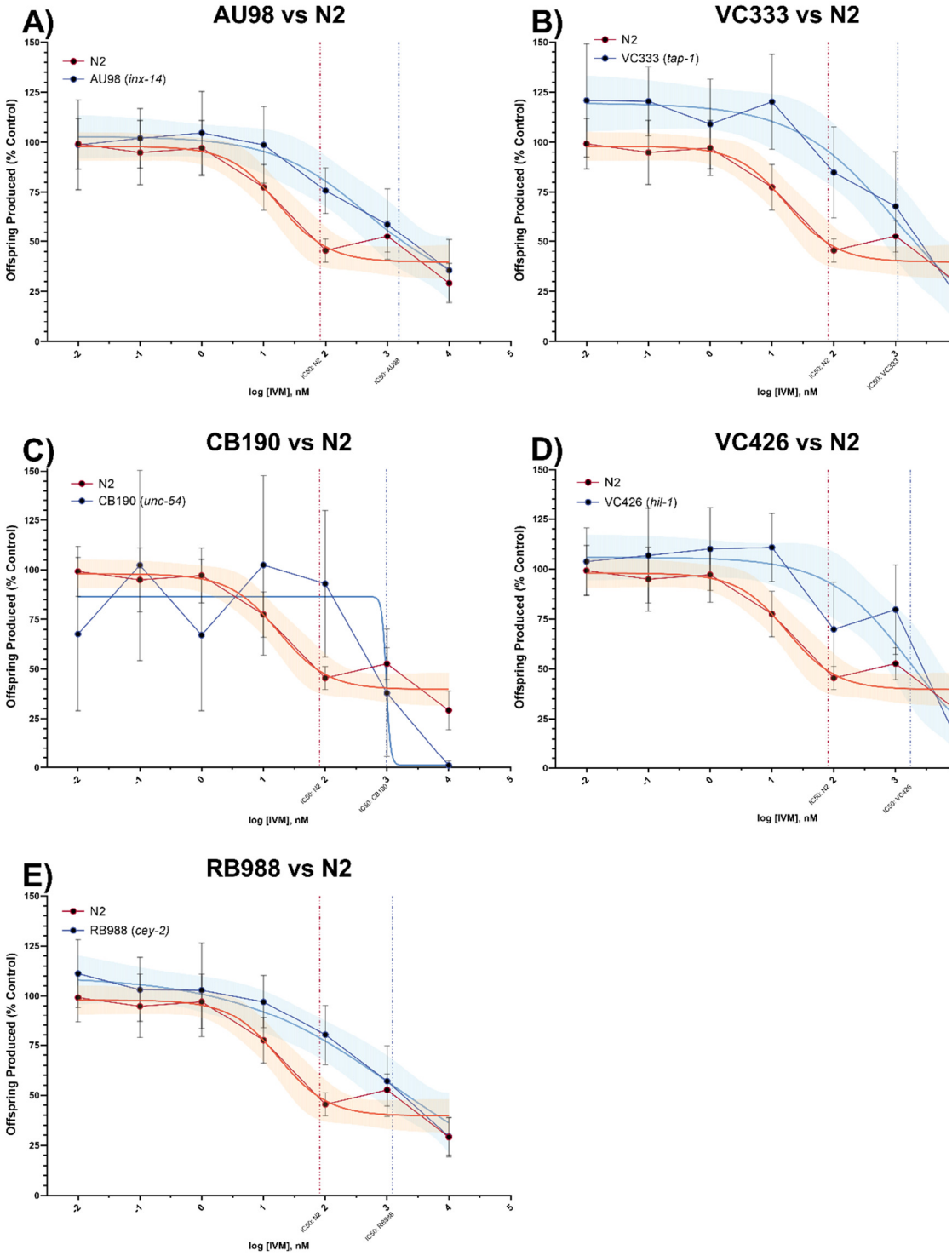


Figure 3.5: Inhibition curves of additional resistant strains in the egg laying assay. A) AU98 (*inx*-14) vs N2. B) VC333 (*tap-1*) vs N2. C) CB190 (*unc-54*) vs N2. D) VC426 (*hil-1*) vs N2. E) RB988 (*cey-2*) vs N2. Error bars are the 95% CI of each point, shaded regions are the 95% confidence band of the dose-response curve. Dashed lines mark the absolute IC50, n=15.

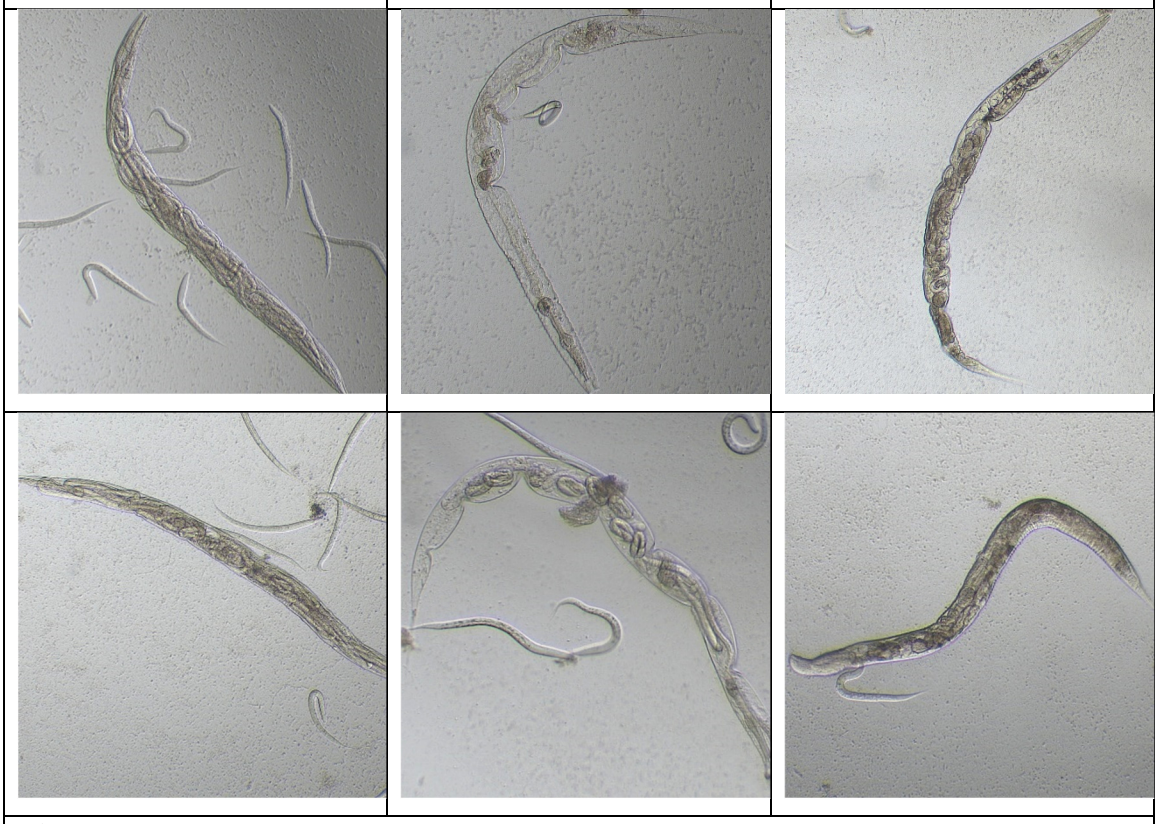


Figure 3.6: Representative images of adult hermaphrodites in the egg laying assay from strains with an increase in apparent egg laying at 1µM. At 100nM, many adults show the bag of worms phenotype. At 1µM, many adults are ruptured and the larvae are released. At 10µM, egg laying and hatching are both inhibited and many adults remain filled with unhatched eggs.

Development assay

We measured ivermectin's effect on development by allowing eggs to hatch and develop on NGM agar containing varying concentrations of ivermectin. Initial testing found that many strains developed well at 1nM IVM but did not develop at all at 10nM IVM, so we first screened all candidate strains on 1% DMSO, 1nM IVM, and 10nM IVM in triplicate on two separate weeks (n=6). Any strains that failed to develop well at 1nM (putative hypersensitive) or developed at all at 10nM (putative resistant) were then assayed on a range of concentrations from 10pM to 10µM IVM in triplicate on three

separate weeks (n=9). The absolute IC_{50} of the N2 WT strain was 2.45nM (95% CI 0.077, 78.33) while the JD608 resistant control was approximately 816.8nM (ambiguous fit, no 95% CI) (figure 3.7). In the first phase of assays, five strains developed particularly poorly at 1nM (ranging from 6.5 ± 6.2%-61.7 ± 21.0% development compared to the DMSO control; figure 3.8). One strain, CB3332 (*che-12(e1812)*), developed slightly (27.2 ± 8.2% of control) at 10nM. In the second phase of the assay, all strains developed well at lower concentrations and failed to develop at 100nM and above (excluding JD608); therefore, we performed our analysis based on the results at 1nM, 3nM, and 10nM ivermectin. The wild-type only developed to 15.4% at 3nM IVM and was entirely inhibited at 10nM while JD608 matured at all concentrations tested up to 10µM (figure 3.9). Four of the five hypersensitive candidates were significantly different from N2 at 1nM (figure 3.9). CB3332 developed less at 10nM in the second phase of the assay than the first but remained the only experimental strain to develop at 10nM at all. It was not inhibited at 3nM, in contrast to N2. Interestingly, N2 was able to lay eggs at 10nM ivermectin as shown in the egg laying assay (figure 3.2) despite being unable to develop. Additionally, BC177 and CB190 (*unc-54(e190))* were hypersensitive in this assay while being resistant in the egg laying assay; none of the mutant strains that were resistant in the egg laying assay were also resistant in the development assay, and none of the hits from the development assay showed the same resistance phenotype in the egg laying assay.

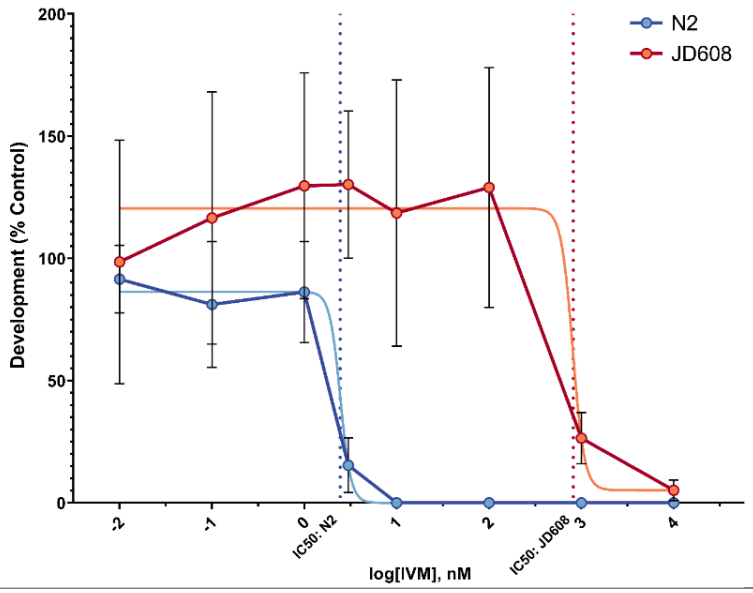


Figure 3.7: N2 (WT) compared to JD608 (*avr-14, avr-15, glc-1;* resistant control) in the egg development assay. Eggs were allowed to develop on NGM agar containing varying concentrations of ivermectin and the proportion of eggs that reached at least the third larval stage were counted and normalized to the DMSO control. Error bars are the 95% CI and dashed lines mark the absolute IC50, n=9.

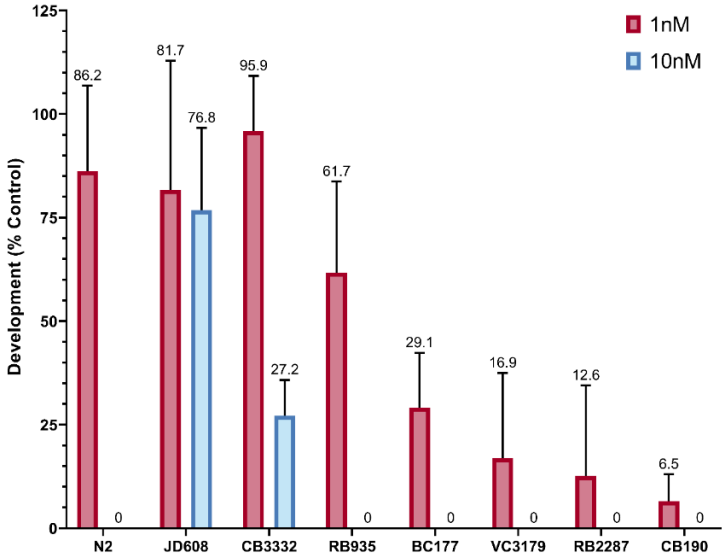


Figure 3.8: Putative resistant and hypersensitive strains from the first stage of the egg development assay. Eggs were allowed to develop on NGM agar containing DMSO, 1nM, or 10nM ivermectin and the proportion of eggs that reached at least the third larval stage were counted and normalized to the DMSO control. Strains that developed at 10nM were labeled resistant; strains that were significantly inhibited at 1nM were labeled hypersensitive. Error bars are the 95% CI. N2 (wild type) and JD608 (resistant control), n=9. All others n=6.

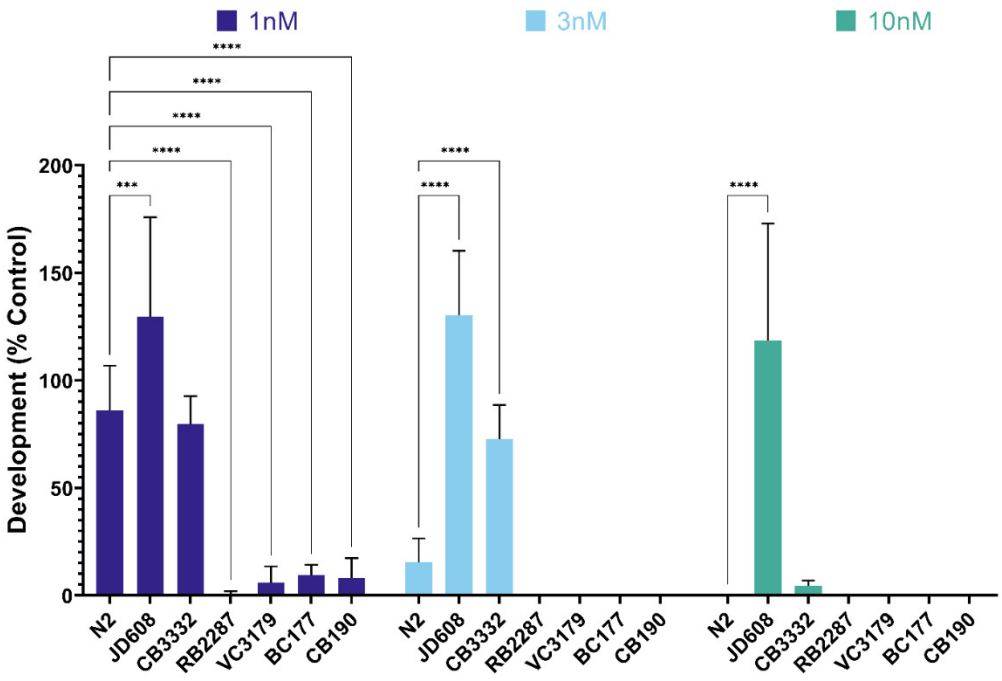


Figure 3.9: Resistant and sensitive strains from the second stage of the egg development assay. Eggs were allowed to develop on NGM agar containing varying concentrations of ivermectin and the proportion of eggs that reached at least the third larval stage were counted and normalized to the DMSO control. Error bars are the 95% CI, n=9. ***p<0.001, ****p<0.0001 compared to the N2 (WT) control, 2-way ANOVA with Dunnett's multiple comparisons test.

Motility

We measured the motility of first stage larvae (L1) using the Worminator system . Each strain was assayed on a range of concentrations from 10pM to 10µM in quadruplicate on 3 separate occasions (n=12). Motility was an extremely inconsistent phenotype, showing high variability both between replicate wells and across repeated experiments in both raw motility scores (in mean motility units) and motility as a percent of the DMSO control. The N2 strain was assayed in a total of 7 different experiments divided between two different time periods (figure 3.10). In the initial set of 3 experiments, the mean motility of the controls ranged from 3.6 to 18.2 MMU (figure 3.11) while lower concentrations of ivermectin had ranges of over 100% of control (figure 3.10). The later set of 4 experiments had control motilities ranging from 3.7 to

26.3 MMU (figure 3.11) and similarly large ranges at lower ivermectin concentrations (figure 3.10). Additionally, the later set had a much higher absolute IC50 than the first (78.53 vs 11.29). This high variability within and between experiments made it extremely difficult to draw any conclusions about any of the motility data generated from the strains screened. Figure 3.12 shows strains that were significantly different from N2 at 1µM, the lowest concentration at which both N2 experiments agreed. Several genes have already been identified as candidate resistance genes in other assays.

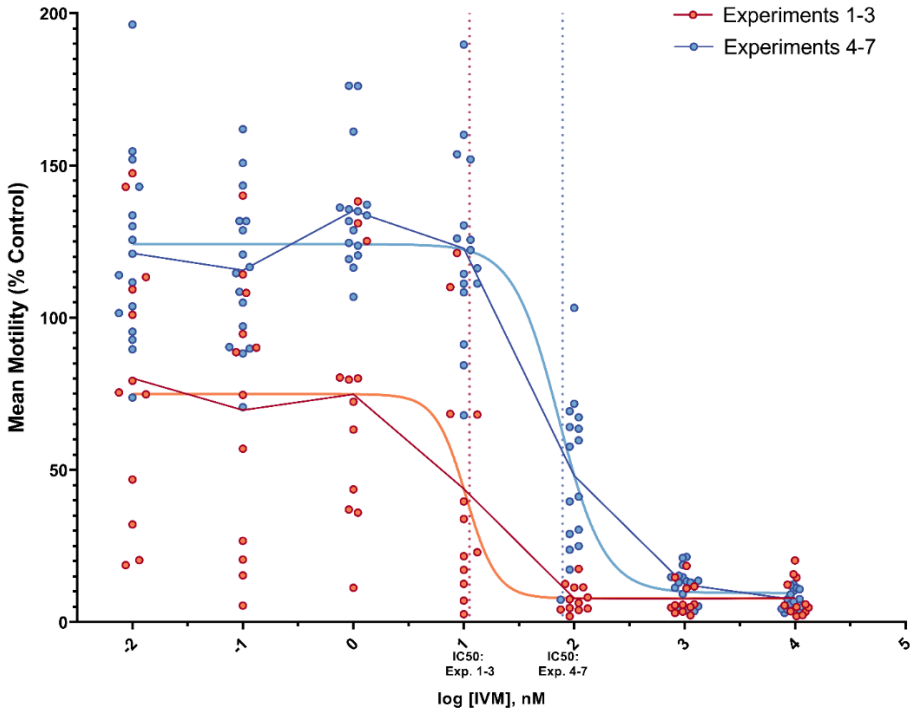


Figure 3.10: Inhibition curves of the N2 WT strain in the motility assay, performed in two different time periods. L1s were plated in quadruplicate in 384-well plates, incubated with varying concentrations of ivermectin, and assayed for motility using the Worminator system. The resulting motility scores were normalized to the DMSO controls. Each experiment is a single biological replicate (prepared from the same culture) performed in quadruplicate. Each circle represents a single well. Experiments 1-3 were performed in immediate succession of each other. Several months later, experiments 4-7 were performed in immediate succession of each other from a new stock of cryopreserved N2 to reduce the effects of genetic drift in culture. Dashed lines mark the absolute IC50.

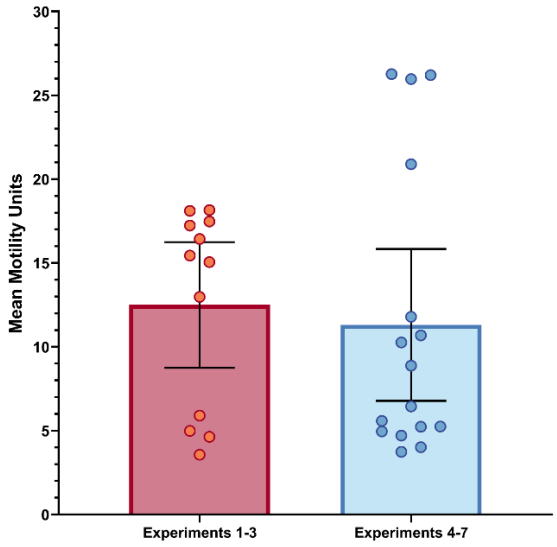


Figure 3.11: Raw motility scores of the N2 WT strain DMSO controls in mean motility units. Experiments 1-3 and 4-7 were performed as previously described. Each circle represents a single well. Error bars are 95% CI.

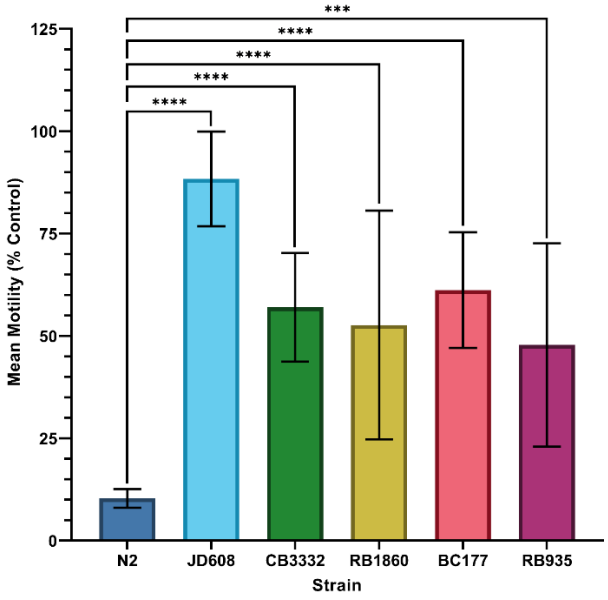


Figure 3.12: Strains significantly different from N2 at 1µM in the motility assay. L1s were plated in quadruplicate in 384-well plates, incubated with varying concentrations of ivermectin, and assayed for motility using the Worminator system. The resulting motility scores were normalized to the DMSO controls. Error bars are the 95% CI, n=12; N2, n=28. ***p<0.001, ****p<0.0001 compared to the N2 (WT) control, 2-way ANOVA with Dunnett's multiple comparisons test.

RNAi

Of the 44 genes initially identified as candidate genes for this study, 13 were available as mutant strains from the CGC but not as single gene knock out mutants, carrying for example phenotypic reporters or balancer mutations (table 3.3). We investigated these strains in the egg development assay using RNAi by bacterial feeding. RNAi of the gene *aff-1* was used as a positive control to confirm the efficacy of the bacterial feeding protocol as the *aff-1* mutant (BP600) had clear egg laying and development defects in the DMSO controls (figure 3.13). The egg laying and motility assays were not possible due to the limits of the assays and bacterial RNAi system. Of the 13 genes that were knocked down, *let-19* RNAi and *ZK829.7* RNAi worms developed poorly on 1nM IVM agar and so were progressed to the second phase (figure 3.14). As a control, N2 were fed HT115 bacteria containing an empty L4440 vector. In the second phase of the assay, neither *let-19* RNAi nor *ZK829.7* RNAi were significantly different from the HT115+L4440 control at 1nM, however neither developed at 3nM in contrast to the control (figure 3.15). Notably the HT115+L4440 fed control developed better at 3nM ivermectin than the standard N2 (79.9% vs 15.4%).

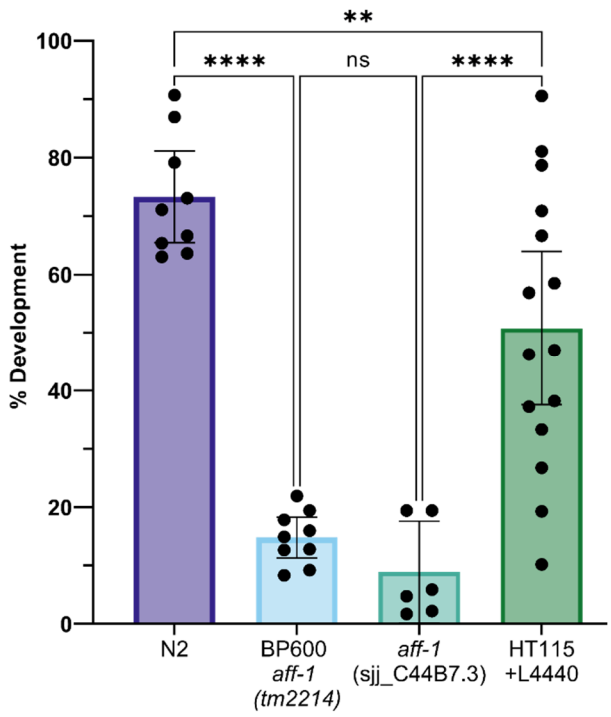


Figure 3.13: Phenocopy of BP600 (*aff-1*) by RNAi by bacterial feeding with bacterial clones expressing dsRNA targeting *aff-1*. There was no significant difference between the BP600 strain and the *aff-1* RNAi, indicating successful knockdown. Error bars are 95% CI, circles are individual replicates. N2, n=9; BP600, n=9; *aff-1* RNAi, n=6 (only phase one of the egg development assay); HT115+L4440, n=15 (phase one plus phase two of the egg laying assay). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, one-way ANOVA with Sidak's multiple comparisons test.

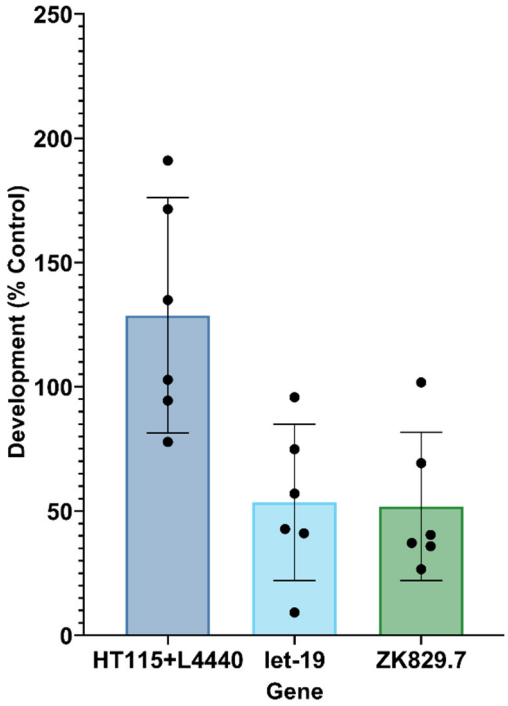


Figure 3.14: Putative genes resulting in hypersensitivity from the first stage of the egg development assay using RNAi. HT115+L4440 is the bacterial strain carrying the empty vector. Eggs were allowed to develop on NGM agar containing DMSO, 1nM, or 10nM ivermectin with IPTG and fed the specified bacterial clone. The proportion of eggs that reached at least the third larval stage were counted and normalized to the DMSO control. Error bars are the 95% CI, circles are individual replicates, n=6.

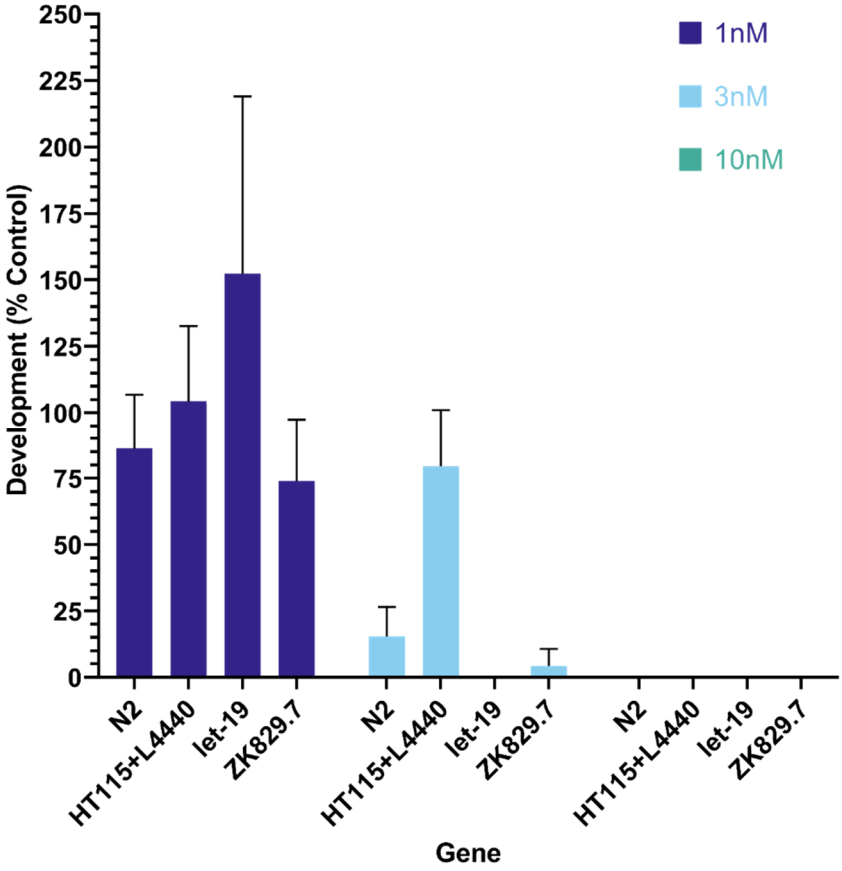


Figure 3.15: Results from the second phase of the egg development assay of genes targeted with RNAi. HT115+L4440 is the bacterial strain carrying the empty vector. Eggs were allowed to develop on NGM agar containing DMSO, 1nM, or 10nM ivermectin with IPTG and fed the specified bacterial clone. The proportion of eggs that reached at least the third larval stage were counted and normalized to the DMSO control. N2 (WT) results were presented previously and obtained as previously described; they are shown here for comparison. Error bars are the 95% CI, n=9.

Additional Strains

To further validate our methods, we included several additional strains in our study. AE501 is an *nhr-8* mutant (*ok186*) that has been shown to cause hypersensitivity in *C. elegans* and RNAi of the orthologous gene produced a similar phenotype in *Haemonchus contortus* using methods similar to those in this study. We did not find a difference between N2 and AE501 in the egg laying assay, egg development, or motility assay (figures 3.16, 3.17, and 3.18 respectively).

The high levels of N2 variability in the motility assay led to a very large number of strains being potentially significantly different from N2. To help determine if our results were due to assay variability or a true effect of the mutated genes on ivermectin sensitivity, we selected genes and strains from those that had been affected by DEC treatment in the same manner we selected the initial candidate genes. After compiling a list and removing any strains with inconvenient culture conditions (such as temperature sensitive mutants), we selected 6 at random and assayed them on each of the three assays. Our hypothesis was that our candidate genes were all affected by ivermectin treatment, so mutations in these genes may cause altered sensitivity to ivermectin. In contrast, genes affected by DEC shouldn't cause any differences in sensitivity to ivermectin. In the egg laying assay, 2 of the 6 strains were resistant, DG3430 (*sacy-1(tn1385)*) and VC1925 (*fkh-9(ok1709)*) (figure 3.19). There were no significantly different strains in the development assay. All 6 strains were far more resistant then N2 in the motility assay at 10nM and 100nM (concentrations at which there is extreme variability) but not at 1µM (the concentration at which there was little variation in the N2 assays and near complete inhibition of motility; figure 3.20); these results seem to indicate that the abundance of resistant strains in this assay is due to flaws with the assay itself.

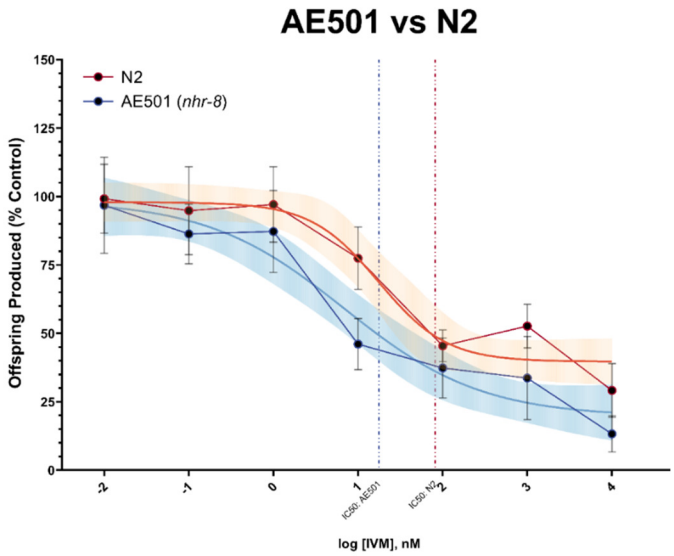


Figure 3.16: Inhibition curve of N2 (WT) compared to AE501 (*nhr-8*; reported hypersensitive) in the egg laying assay. Individual hermaphrodites were placed in wells with OP50 and increasing concentrations of ivermectin and the offspring produced by each worm was counted 24 hours later. Error bars are the 95% CI of each point, shaded regions are the 95% confidence band of the dose-response curve. Dashed lines mark the absolute IC50, n=15.

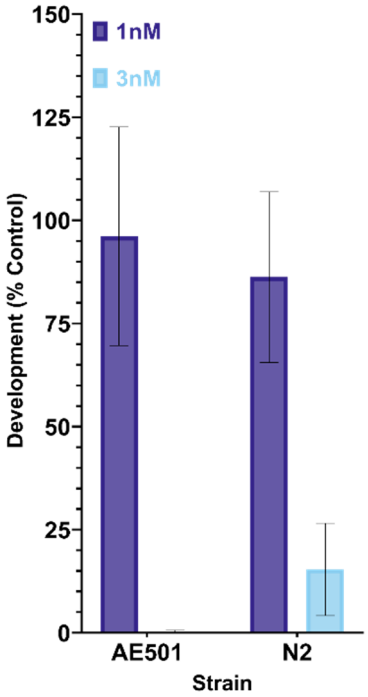


Figure 3.17: N2 (WT) compared to AE501 (*nhr-8*; reported hypersensitive) in the egg development assay. Eggs were allowed to develop on NGM agar containing varying amounts of ivermectin and the proportion of eggs that reached at least the third larval stage were counted and normalized to the DMSO control. AE501 developed normally at concentrations below 1nM and failed to develop at concentrations at and above 10µM, similar to the pattern described previously. Error bars are the 95% CI, n=9.

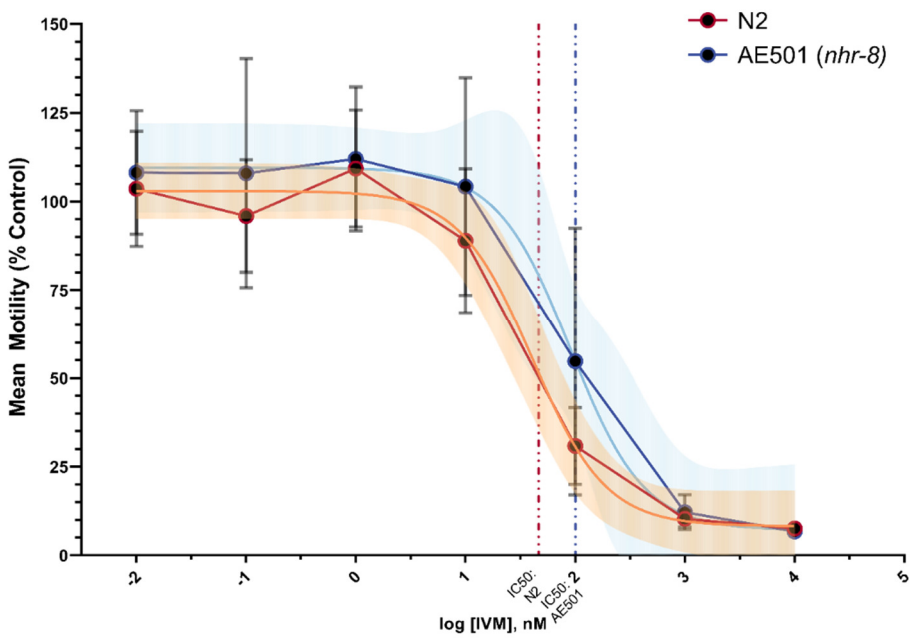


Figure 3.18: Inhibition curve of N2 (WT) compared to AE501 (*nhr-8*; reported hypersensitive) in the motility assay. L1s were plated in quadruplicate in 384-well plates, incubated with varying concentrations of ivermectin, and assayed for motility using the Worminator system. The resulting motility scores were normalized to the DMSO controls. Error bars are the 95% CI of each point, shaded regions are the 95% confidence band of the dose-response curve. Dashed lines mark the absolute IC50, n=12.

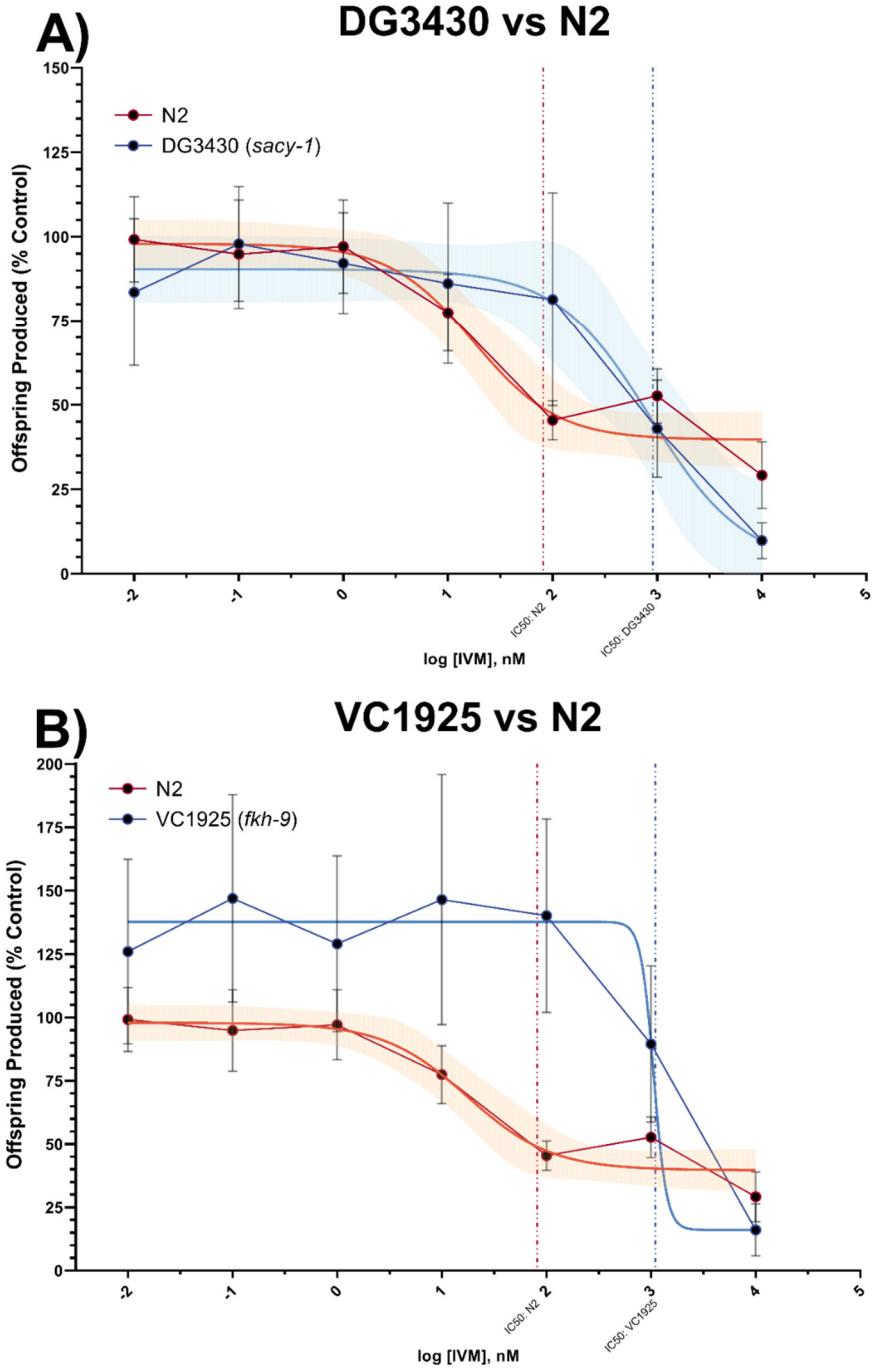


Figure 3.19: Inhibition curves of strains with mutations in genes affected by DEC treatment of hosts that differed from N2 in the egg laying assay. Individual hermaphrodites were placed in wells with OP50 and increasing concentrations of ivermectin and the offspring produced by each worm was counted 24 hours later. A) DG3430 (*sacy-1*) vs N2. B) VC1925 (*fkh-9*) vs N2. Error bars are the 95% CI of each point, shaded regions are the 95% confidence band of the dose-response curve. Dashed lines mark the absolute IC50, n=15.

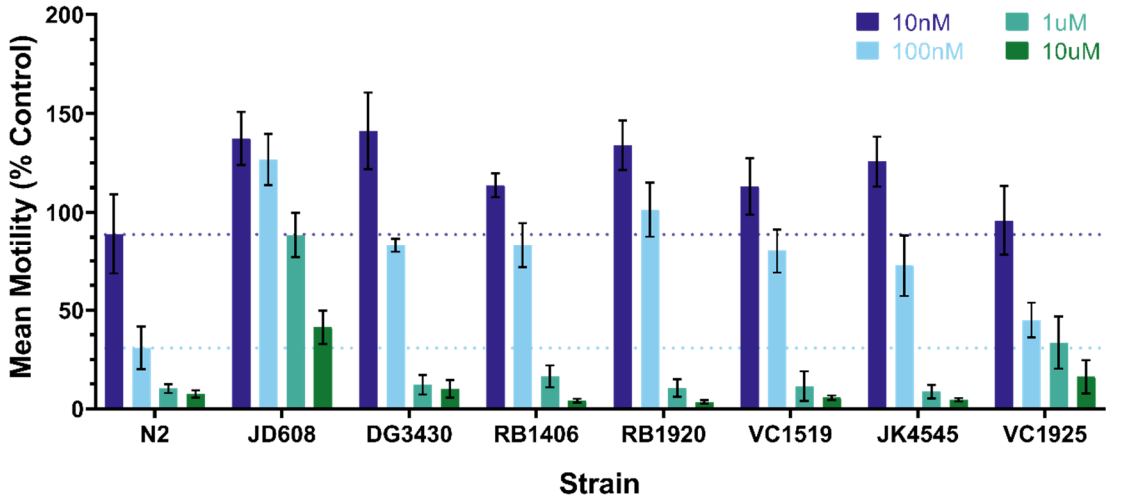


Figure 3.20: Motility assay results of strains with mutations in genes affected by DEC treatment of hosts. L1s were plated in quadruplicate in 384-well plates, incubated with varying concentrations of ivermectin, and assayed for motility using the Worminator system. The resulting motility scores were normalized to the DMSO controls. N2 (WT) and JD608 (resistant control) were assayed and presented previously and presented for comparison. Error bars are the 95% CI, n=12; N2, n=28. Dashed lines mark the mean motility of N2 at the corresponding concentration of ivermectin. Significance intentionally omitted due to lack of assay validity.

Pharyngeal Pumping

We used the results shown previously to select a subset of genes for a pharyngeal pumping assay using the InVivo Biosystems ScreenChip™ system. Ivermectin is known to inhibit pumping which would likely have a direct effect on feeding and development. Therefore, we focused on genes that were hits in the development assay, as well as hits that were expressed in the pharynx as we hypothesized that they might have a more direct effect on pumping. We also included RB653 (*ogt-1(ok1474)*) which showed some differences in pumping characteristics from N2 in preliminary testing and early proof-of-concept experiments with the ScreenChip™ system. At 10nM, 50nM, and 100nM, the pumping frequency of N2 was 109.9%, 40.3%, and 15.9% of control respectively (figure 3.21). The resistant control, JD608, was nearly identical to N2 at 10nM (110%) and only decreased slightly at higher concentrations (90.45% at 50nM and 80.27% at 100nM).

AE501, the *nhr-8* mutant, was significantly inhibited at both 50nM and 100nM ivermectin compared to N2, reproducing the hypersensitive phenotype identified by Mendez et al. Overall, each experimental strain matched its development assay phenotype: CB3332 was not inhibited at 50nM (119.3%) or 100nM (89.85%) ivermectin while both RB2287 and VC3179 were hypersensitive. The two *unc* mutants that were hypersensitive in the development assay couldn't be measured due to limitations in the dimensions of ScreenChip cartridges and the *unc* phenotype. AU98 is not different from N2 despite INX-14 being expressed in pharyngeal muscle. Our results in this assay and the development assay indicate that ivermectin resistant and sensitive strains can be easily discriminated from each other based on their response at a low number of specific concentrations when observing certain phenotypes.

In many cases, pumping when exposed to ivermectin is a binary trait. The pumping frequency of individual worms from both normal and hypersensitive strains can often exceed 100% of control at 50nM (figure 3.22); however, when pumping is counted as either present or absent during the measurement period, the results are equivalent to measuring frequency for determining whether a strain is hypersensitive, resistant, or not different from control (figure 3.23). Independent of their response to ivermectin, many strains have characteristic pumping features in the DMSO control which may give further insight into their molecular function (table 3.5). For example, many RB2287 strain worms do not pump even in the DMSO control during the recording period (figure 3.24), which may indicate that *lips-7* plays some role serotonin-induced pumping or a similar process.

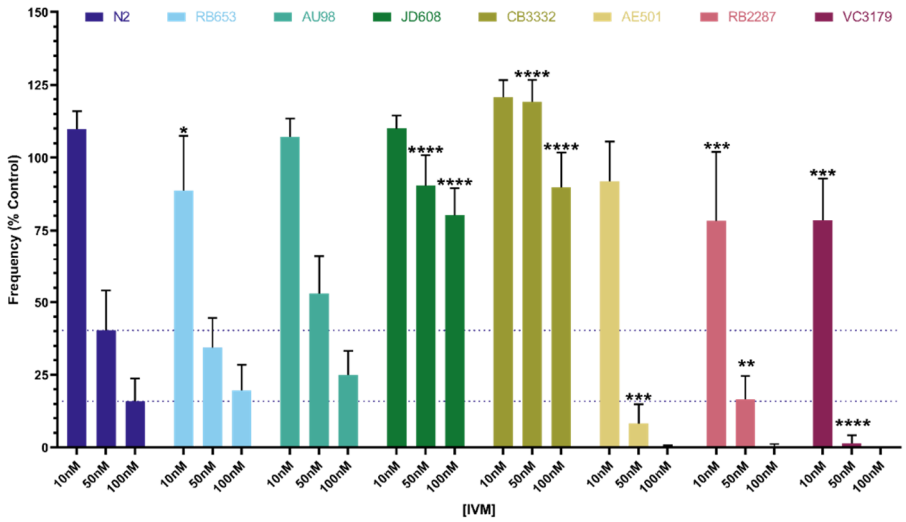


Figure 3.21: Results of the pharyngeal pumping assay. Results are presented with mostly normally sensitive strains on the left, resistant strains in the center, and hypersensitive strains on the right. Worms were incubated in 10mM serotonin and 10nM, 50nM, or 100nM ivermectin, or DMSO and the activity of the pharynx was measured with the InVivo Biosystems ScreenChip™ system. The measured frequency in each ivermectin concentration was normalized to the DMSO control. Error bars are the 95% CI, dashed lines mark the mean frequency of N2 at 50nM and 100nM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to the N2 (WT) control, 2-way ANOVA with Dunnett's multiple comparisons test, n=60.

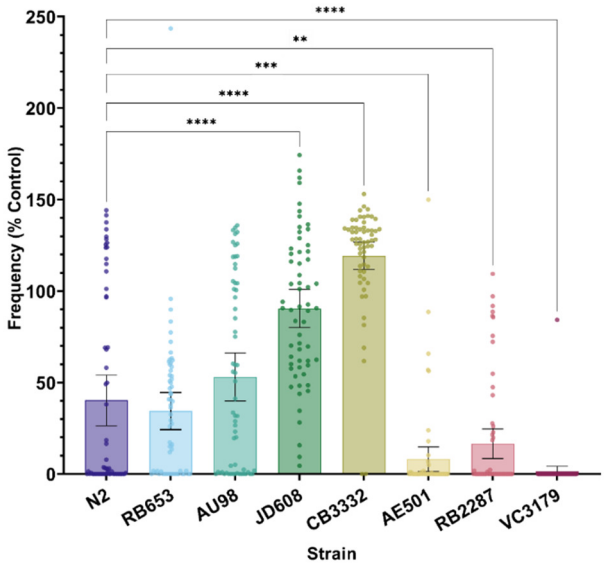


Figure 3.22: Detailed results of the pharyngeal pumping assay at 50nM ivermectin. Results are presented with mostly normally sensitive strains on the left, resistant strains in the center, and hypersensitive strains on the right. Error bars are the 95% CI, each point represents a single adult worm, n=60. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to the N2 (WT) control, 2-way ANOVA with Dunnett's multiple comparisons test.

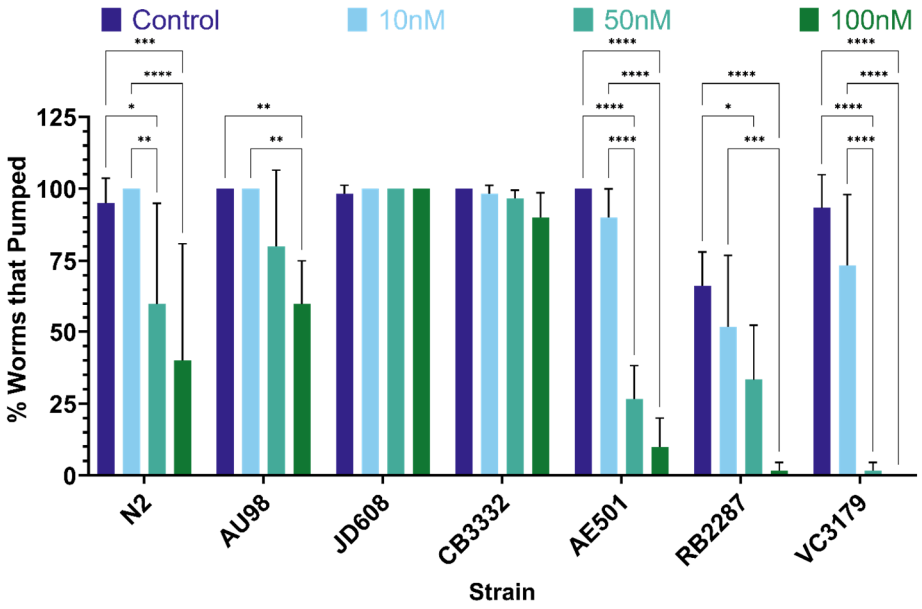


Figure 3.23: Percentage of worms that pumped in each condition in the pharyngeal pumping assay. Error bars are the 95% CI, average of 3 experiments, 20 adults per experiment. RB2287 control was the average of 4 experiments. *p<0.05, **p<0.01,***p<0.001, ****p<0.0001, 2-way ANOVA with Tukey's multiple comparisons test.

Table 3.5: Pumping characteristics of each tested strain in DMSO in the pharyngeal pumping assay. The pumping assay was performed as previously described. Frequency: the number of pharyngeal pumps per time period. Pump duration: the time between a pump's E-spike and R-spike. Inter-pump Interval: the time between one E-spike and the next E-spike and is a measure of pumping regularity. Data include 95% CI of the mean * p<.05, ** p<0.01, *** p<0.001, *** p<0.0001 compared to N2 (WT) control, one-way ANOVAs with Dunnett's multiple comparisons test.

Strain	Mean Frequency (Hz)	Mean Pump Duration (ms)	Mean Inter-Pump Interval (ms)
N2	4.9 (4.6, 5.2)	97 (93, 101)	195 (188, 202)
JD608	3.6 (3.3, 3.8) ****	129 (123, 134) ****	279 (266, 292)
AU98	5.2 (4.9, 5.4)	99 (92, 105)	193 (184, 202)
RB653	4.0 (3.5, 4.4) ***	103 (93, 113)	320 (202, 438)*
CB3332	4.7 (4.5, 4.9)	96 (92, 105)	218 (204, 232)
RB2287	1.9 (1.5, 2.2) ****	115 (108, 122) ***	544 (400, 689) ****
VC3179	4.1 (3.8, 4.4) **	123 (117, 129) ****	240 (226, 253)

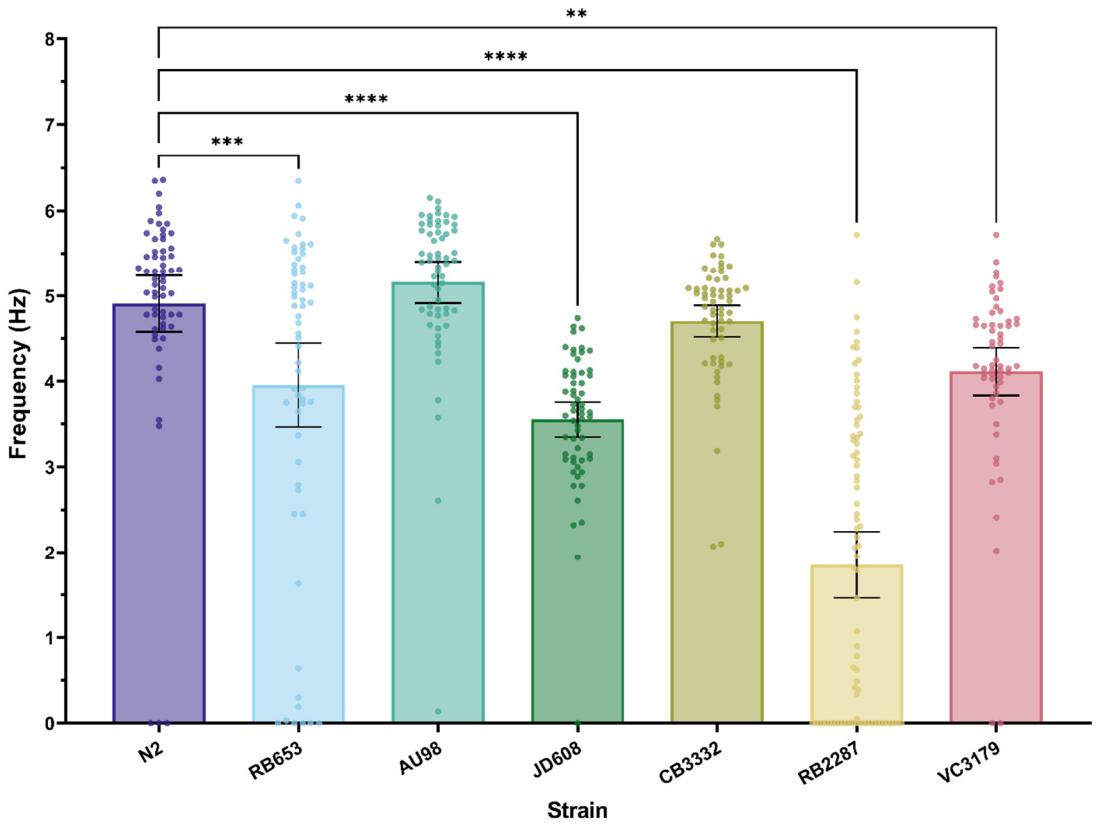


Figure 3.24: Pumping frequency of tested strains in DMSO in the pharyngeal pumping assay. Error bars are the 95% CI, each point represents a single adult worm, n=60. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to the N2 (WT) control, one-way ANOVA with Dunnett's multiple comparisons test.

Gene Enrichment and Interaction Analysis

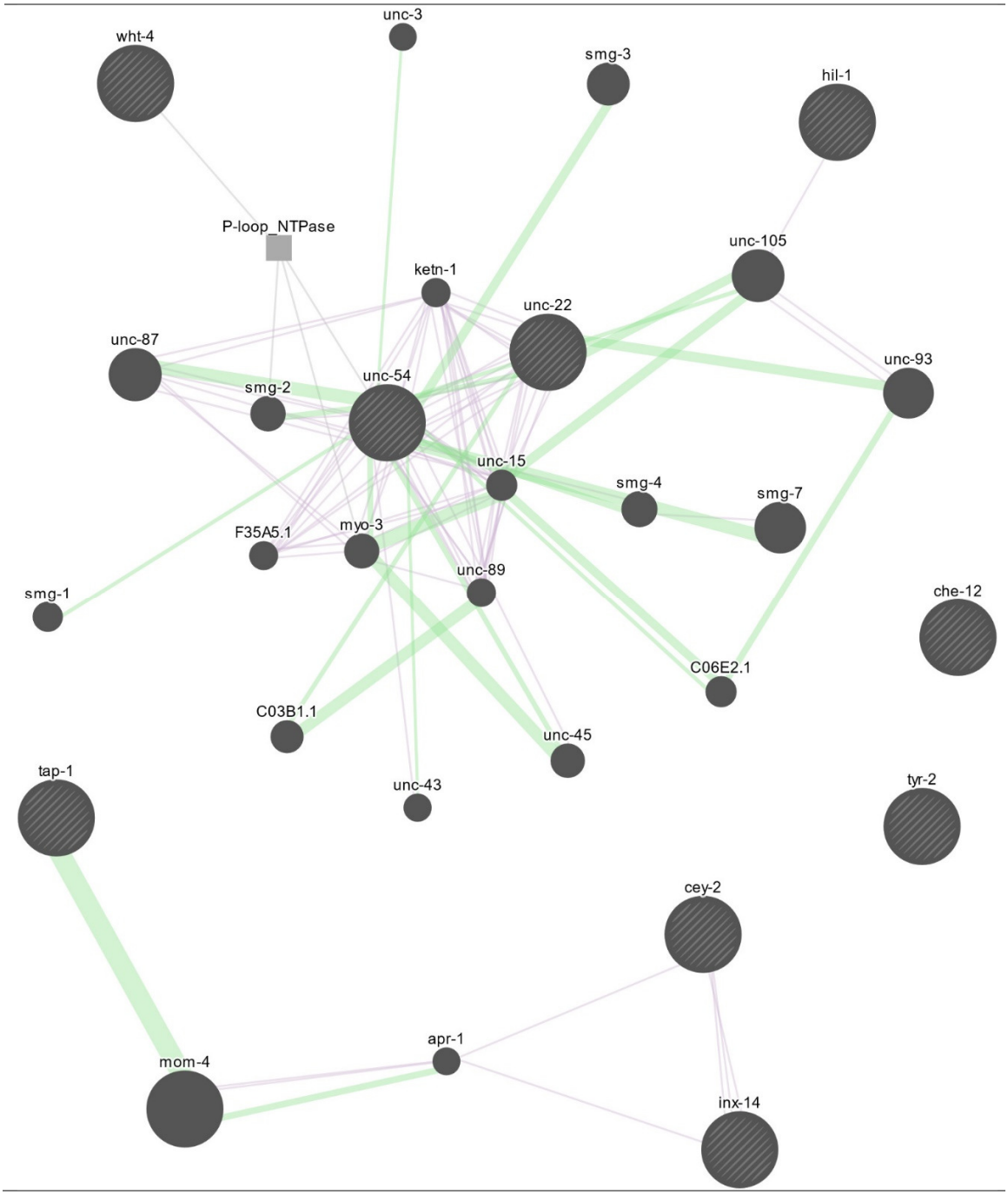
The small number of genes identified as hits in the various assays makes drawing conclusions from gene enrichment, pathway, and interaction analysis difficult; however, there are some interesting general patterns. In the set of all genes resulting in resistance in at least one assay, there were no significantly enriched tissue expression or phenotype terms. Several muscle related gene-ontology (GO) terms were enriched including the A band (q value 0.004), muscle system process (q value 0.013), myofibril (q value 0.024), kinase binding (q value 0.031), and supramolecular polymer (q value 0.034). These are likely due to *unc-22* and *unc-54*, both of which are muscle genes, and the low amount of

annotation available for many of the other genes in the set; *wht-4* had no data available for tissue expression, for example. An analysis of gene interactions connected mostly by shared protein domains and co-expression when automatic weighting was selected (figure 3.25). Interactions were connected primarily by physical interactions when weighting equal by network. *Che-12* and *tyr-2* remained separate from the network with automatic weighting while *tyr-2* and *hil-1* were separate and associated only with protein domains in the equal network weighting. GO term analysis showed a similar enrichment of muscle-related terms.

In the set of genes causing resistance in the egg laying assay there were no significantly enriched tissue expression terms, however 5 of the 9 genes were expressed in the sex organ (p value 0.0025, q value 0.091), 2 of which were specifically expressed in uterine muscle (*tyr-2* and *inx-14*). Interestingly, the two DEC affected genes that caused resistance in the egg lay assay (*sacy-1* and *fkh-9*) are both expressed in reproductive tissues, the germ line and vulva (a sub-term of sex organ) respectively. No phenotypes were significantly enriched. Like the set of all resistant gene hits, many enriched GO terms included those associated with muscle. Additional terms included cellular component assembly involved in morphogenesis (q value 0.041) and oviposition (q value 0.041), however only 1 and 2 genes were associated with those terms respectively. In the gene interaction analysis using automatic weighting, *tyr-2* was completely isolated while *cey-2, inx-14,* and *tap-1* formed a small network based on co-expression (figure 3.26). With equal weighting by network, *tyr-2* and *hil*-1 were once again isolated Overrepresented GO terms in the network included contractile fiber, supramolecular fiber, supramolecular polymer, sarcomere, anatomical structure formation

involved in morphogenesis, muscle structure development (FDR <0.0001 for all). As with the set of all resistant gene hits, these results are likely skewed by the under-annotation of many of the genes in the set and the extensive study of *unc-22* and *unc-54*. For example, Wormbase lists 360 references for *unc-22* (wormbase.org/species/c_elegans/gene/WBGene00006759) but only 4 for *wht-4* (wormbase.org/species/c_elegans/gene/WBGene00017179) and 7 for *tyr-2* (wormbase.org/species/c_elegans/gene/WBGene00010661).

Within the set of genes causing hypersensitivity in the development assay here were no overrepresented tissue expression locations. Three of the genes were annotated with the "small" phenotype, while the fourth *lips-7,* had no data available. As *unc-22* and *unc-54* are also in this set, overrepresented GO terms were all related to muscle. In the gene interaction analysis, *lips-7* and *pis-1* are only linked to the main network through co-expression with a few genes using both automatic weighting and weighting equally by network (figure 3.27). For *lips-7* both genes (*unc-105* and *myo-3)* link to both *unc-22* and *unc-54* through genetic interactions and co-expression. *Pis-1* links to *unc-40* and *sax-7*, which form a dense network of links to other genes (including *unc-22)* through shared protein domains.


unc-3
wht-4
smg-3
hil-1
P-loop_NTPase
unc-105
ketn-1
unc-22
unc-87
unc-93
smg-2
unc-54
unc-15
smg-4
smg-7
F35A5.1
myo-3
unc-89
smg-1
che-12
C06E2.1
C03B1.1
unc-45
tap-1
unc-43
tyr-2
cey-2
apr-1
mom-4
inx-14

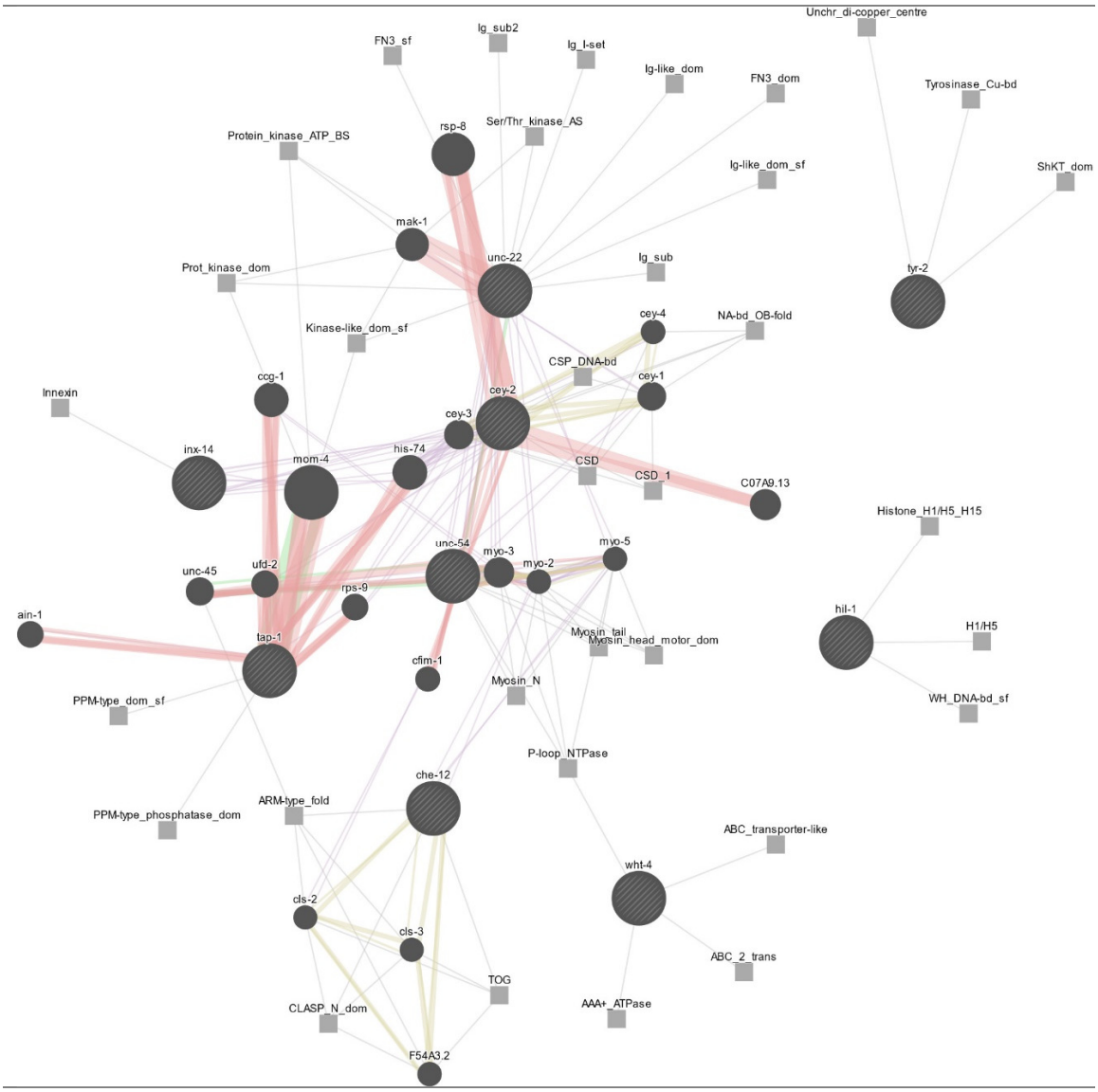
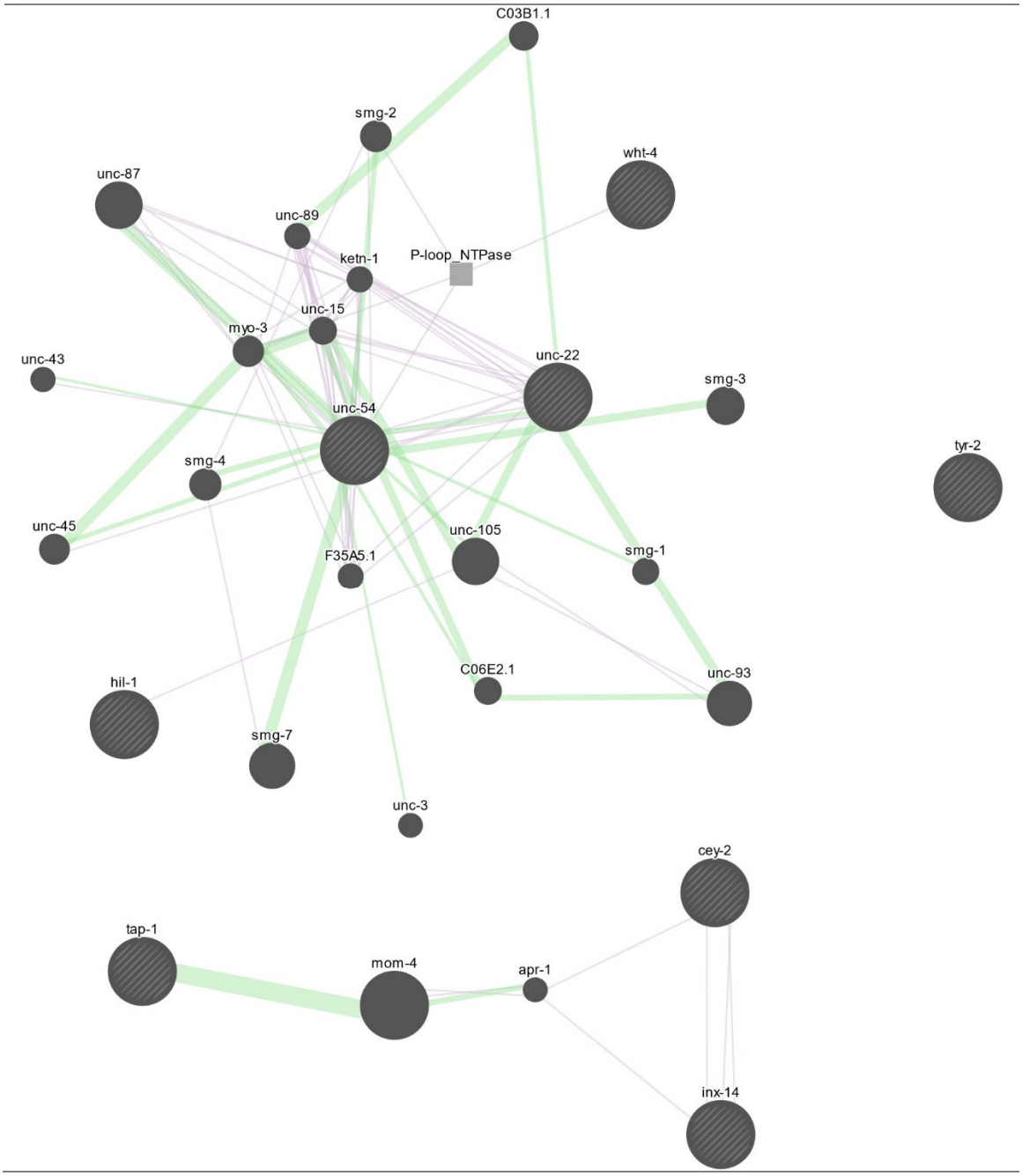


Figure 3.25: Gene interaction network for all genes resulting in a resistant phenotype in at least one assay. Top, automatically selected weighting. Bottom, equal weighting by network type. Legend: pink, physical interactions; purple, co-expression; green, genetic interactions; orange, predicted interactions; grey, interpro domains; tan, shared protein domains.


C03B1.1
smg-2
wht-4
unc-87
unc-89
ketn-1
P-loop_NTPase
unc-15
myo-3
unc-43
unc-22
smg-3
unc-54
tyr-2
smg-4
unc-45
unc-105
F35A5.1
smg-1
C06E2.1
unc-93
hil-1
smg-7
unc-3
cey-2
tap-1
mom-4
apr-1
inx-14

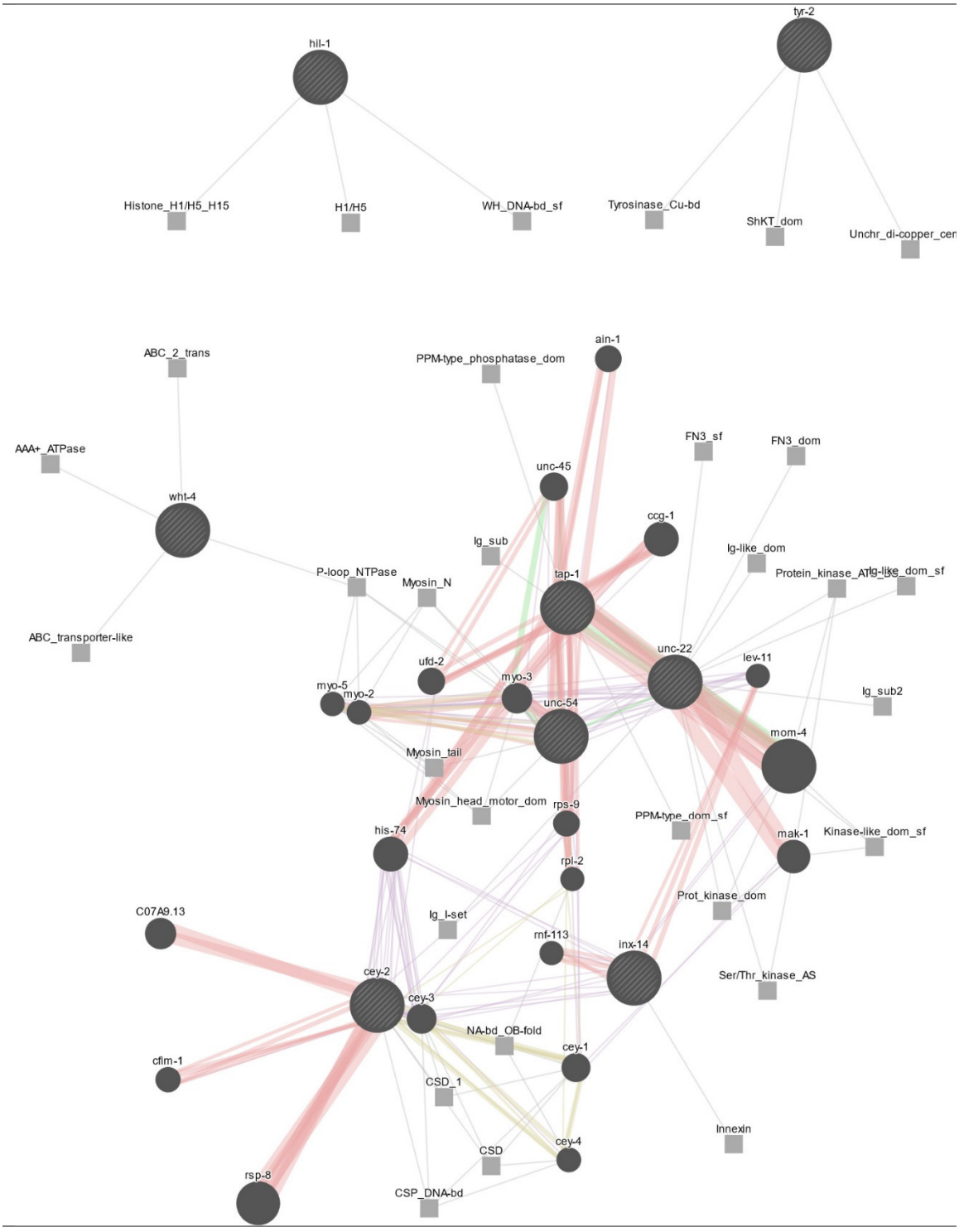
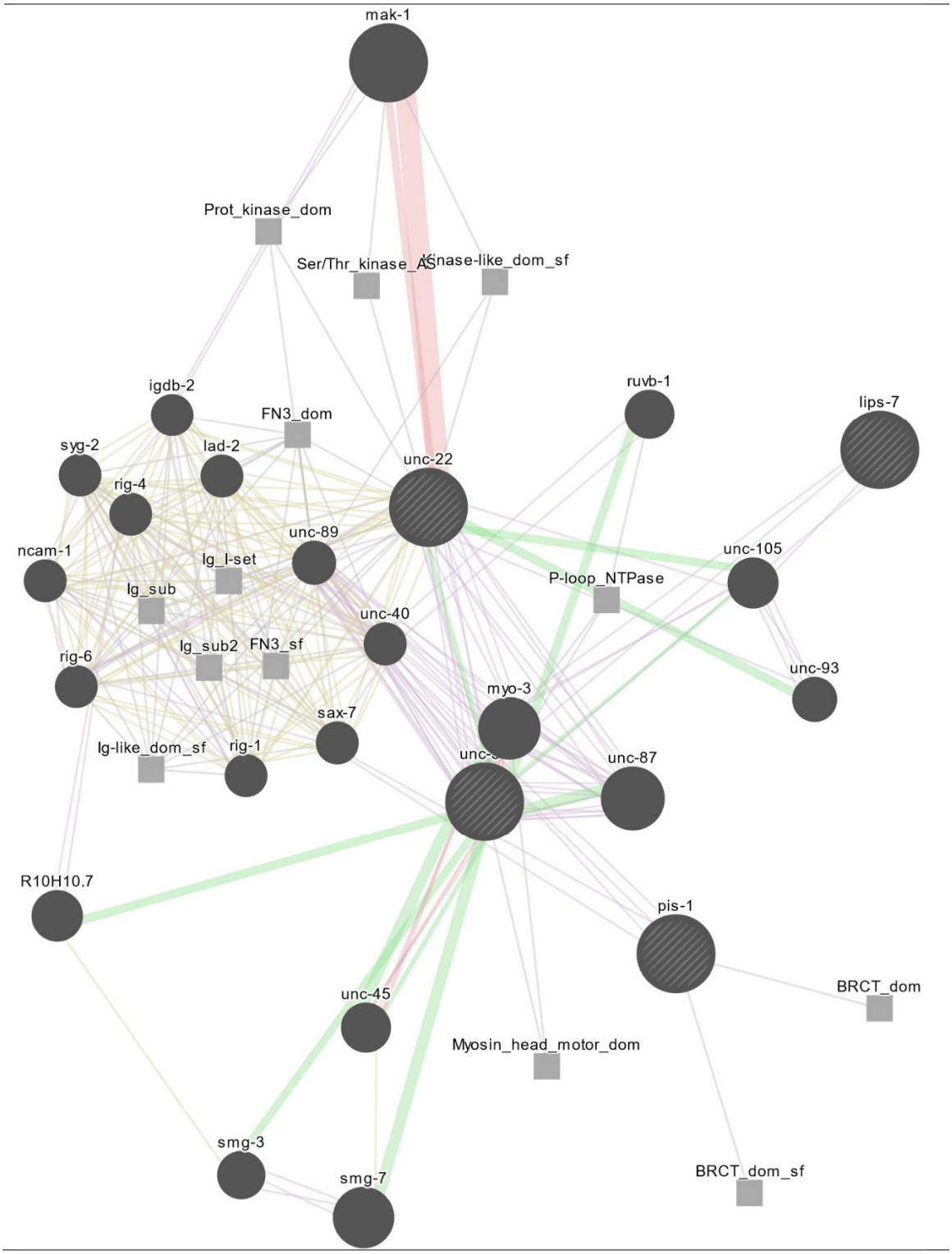


Figure 3.26: Gene interaction network for resistant gene hits in the egg laying assay. Top, automatically selected weighting. Bottom, equal weighting by network type. Legend: pink, physical interactions; purple, co-expression; green, genetic interactions; orange, predicted interactions; grey, interpro domains; tan, shared protein domains.


mak-1
Prot_kinase_dom
Ser/Thr_kinase_AS
Kinase-like_dom_sf
igdb-2
FN3_dom
syg-2
lad-2
rig-4
unc-22
ruvb-1
lips-7
ncam-1
unc-89
Ig_I-set
Ig_sub
P-loop_NTPase
unc-105
unc-40
Ig_sub2
FN3_sf
rig-6
unc-93
myo-3
sax-7
Ig-like_dom_sf
rig-1
unc-87
R10H10.7
pis-1
BRCT_dom
unc-45
Myosin_head_motor_dom
smg-3
smg-7
BRCT_dom_sf

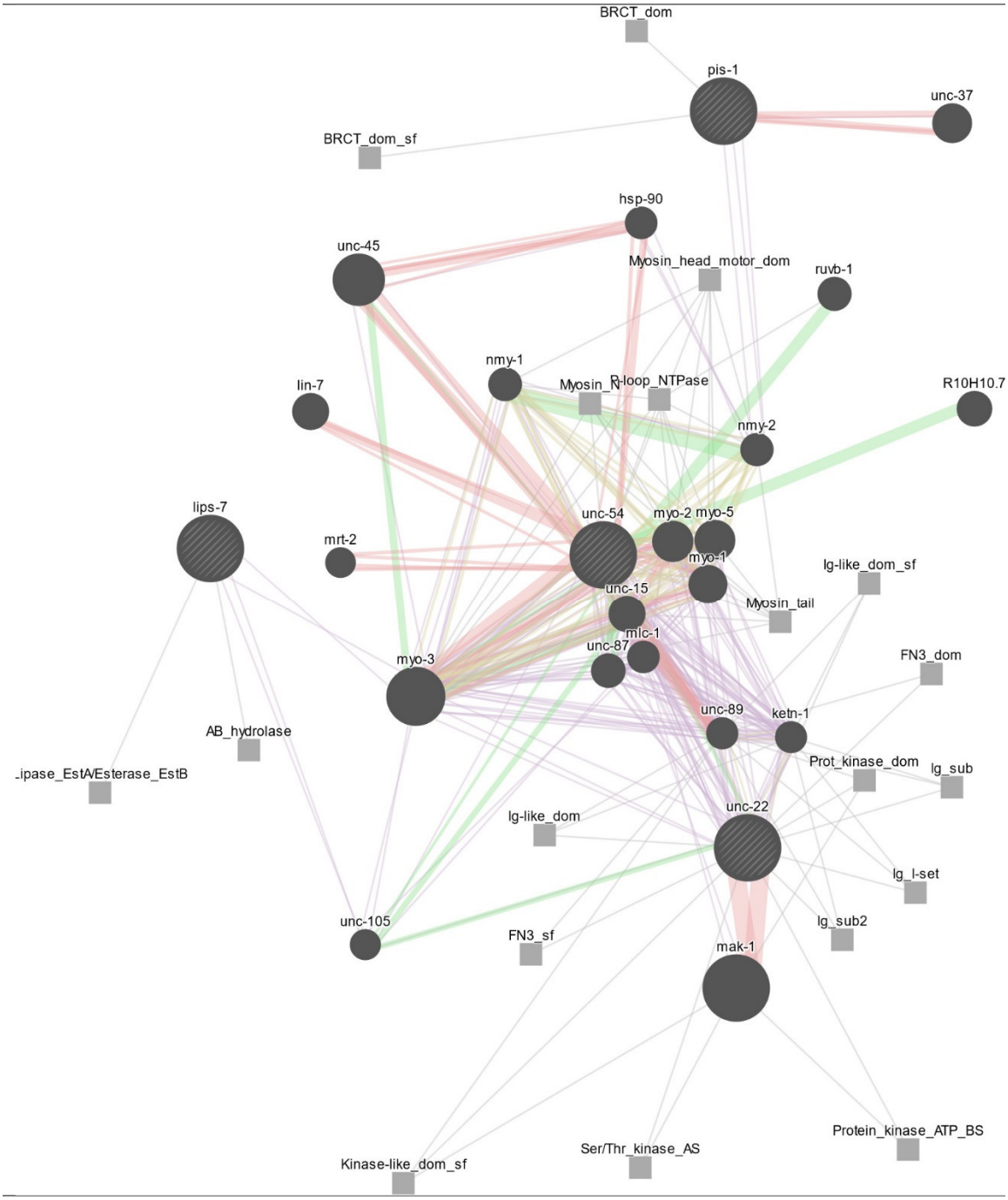


Figure 3.27: Gene interaction network for hypersensitive gene hits in the development assay. Top, automatically selected weighting. Bottom, equal weighting by network type. Legend: pink, physical interactions; purple, co-expression; green, genetic interactions; orange, predicted interactions; grey, interpro domains; tan, shared protein domains.

Discussion

With hundreds of millions of tablets distributed for use in mass drug administration programs, ivermectin is a drug of incredible public health importance for the prevention of filarial infections (de Souza et al., 2020). However, the mechanism of action of ivermectin against filarial worms is unclear. Most interestingly, ivermectin is highly effective in infected individuals but not in culture. We previously performed a transcriptomics study in which gerbils infected with *B. malayi* were treated with ivermectin and we measured which genes were differently expressed in the parasite compared to parasites from control hosts. The present study sought to further understand and characterize these genes and their contribution to the response to ivermectin through a screen of *C. elegans* mutants in several standard phenotypic assays for ivermectin resistance.

Eight gene mutations resulted in resistance in the egg laying assay while none resulted in hypersensitivity. Three gene mutations (*tyr-2(ok1363)*, *unc-22(s17)*, *wht-4(ok1007)*) resulted in absolute IC50's more than 100 times that of N2. Four strains were significantly different from N2 at 1nM: BC177 (*unc-22(s17)*), CB190 (*unc-54(e190)*), VC3179 (*pis-1(ok3720)*), and RB2287 (*lips-7(ok3110)*). The resistant candidate strain CB3332 (*che-12(e1812)*) was the only experimental strain to develop at 10nM whatsoever and was not inhibited at 3nM, in contrast to N2. Interestingly, N2 can lay eggs at 10nM ivermectin despite being unable to develop. Additionally, BC177 and CB190 (*unc-54(e190))* were hypersensitive in this assay while being resistant in the egg laying assay. These results seem to suggest that ivermectin's effect on fertility and development are independent of each other; none of the mutant strains that were resistant

in the egg laying assay were also resistant in the development assay, and none of the hits from the development assay showed the same resistance phenotype in the egg laying assay, in contrast to the resistant control with mutations in classical targets of ivermectin.

Motility was an extremely inconsistent phenotype, showing high variability both between replicate wells and across repeated experiments in both raw motility scores (in mean motility units) and motility as a percent of the control. This high variability within and between experiments makes it extremely difficult to draw any conclusions about any of the motility data generated from the strains screened. Furthermore, the additional strains we screened (selected from the list of DEC affected genes) all came up as resistant to ivermectin, while none were resistant in the development assay and only 2 were in the egg laying assay (the mutated genes in both of which have a role in reproduction, similar to other egg laying assay hits). These data add to the mounting evidence that motility is not a suitable phenotype for assessing macrocyclic lactone resistance in nematodes. In *D. immitis,* neither a larval migration inhibition assay (Evans et al., 2017a) nor a motility assay (Maclean et al., 2017a) could effectively distinguish between susceptible and resistant isolates at the L3 or MF stage respectively. Motility also could not detect resistance consistently in L3 from *Haemonchus contortus* and *Cooperia* spp. nor in L4 from *Cooperia* spp. (George et al., 2018; Paras & Kaplan, 2020).

We used the results generated in the development and egg laying assays to select a subset of genes for a pharyngeal pumping assay using the InVivo Biosystems ScreenChip™ system. Overall, each experimental strain matched its development assay phenotype. AU98 (*inx-14)* was not different from N2 despite being expressed in pharyngeal muscle. Our results in this assay and the development assay indicate that

ivermectin resistant and sensitive strains can be easily discriminated from each other based on their response at a low number of specific concentrations when observing relevant phenotypes. Additionally, adult worms are still capable of laying eggs with extended exposure (24 hours) to concentrations high enough to cause inhibition of feeding after only 1 hour, further demonstrating that egg laying is affected by ivermectin independently of development and feeding. Therefore assays only measuring one phenotype will likely miss hits that affect other processes but are still important to the question being asked.

In total there were 11 genes that affected ivermectin sensitivity, half of which were differently expressed in microfilariae, and half in adults (table 3.6). Most hits were resistant in the egg laying assay. Many genes examined in this study have limited annotation, however their functions do suggest some potential mechanisms for their effect on ivermectin susceptibility.

Table 3.6: Summary of *C. elegans* gene hits and their corresponding genes in *B. malayi*.

C. elegans Gene	IVM Phenotype in *C. elegans*	*B. malayi* Gene	Log_2 fold change in IVM	*B. malayi* Life Stage
cey-2	**Resistant**—Egg lay	Bm4155	0.587393	Mf (24 hrs)
wht-4	**Resistant**—Egg lay	Bm3156	0.508316	Mf (24 hrs)
tyr-2	**Resistant**—Egg lay	Bm4913	0.502924	Mf (24 hrs)
lips-7	**Hypersensitive**—Development; **Hypersensitive—**Pharyngeal pumping	Bm2098	0.405904	Mf (24 hrs)
tap-1	**Resistant**—Egg lay	Bm9171	0.393492	Mf (24 hrs)
inx-14	**Resistant**—Egg lay	Bm9585	0.343655	Mf (24 hrs)
pis-1	**Hypersensitive**—Development; **Hypersensitive—**Pharyngeal pumping	Bm5769	-0.35478	Adult Male (24 hrs)
unc-22	**Resistant**—Egg lay; **Hypersensitive**—Development	Bm7502	-0.50233	Adult Male (24 hrs)
unc-54	**Resistant—**Egg lay; **Hypersensitive**—Development	Bm4116	-0.53013	Adult Male (24 hrs)
che-12	**Resistant**—Development; **Resistant—**Pharyngeal pumping	Bm12054	-0.3829	Adult Male (7 days)
hil-1	**Resistant**—Egg lay	Bm5144	0.405082259	Adult Female (7 days)

The amphid neurons have been proposed as a primary route of uptake for ivermectin, as screens for ivermectin resistance repeatedly return amphid defects while strains with amphid defects are often resistant to ivermectin (Page, 2018). The gene *che-12* is involved in sensory cilium assembly and is expressed in amphid neurons. In this study, it was resistant in both the development and pharyngeal pumping assay, matching the previous report from Page that the CB3332 strain is weakly resistant. This strain was not resistant in the egg laying assay which may indicate that a different route of uptake is involved in ivermectin's action in the female gonad. Different routes of entry into

different life stages of the parasite might further complicate the mechanism of action, and of resistance.

Ivermectin may also affect lipid metabolism, possibly through an interaction with p-glycoproteins. The gene *lips-7* was hypersensitive in the development and pharyngeal pumping assays and is a triacylglecerol (TAG) lipase gene expressed in the hypodermis and head neurons (Chen et al., 2009, p. 200; Reid et al., 2014). RNAi knockdown of *lips-7* increased TAG levels (in a mutant background with reduced levels) and through its interaction with CTBP-1 might play a role linking lipid metabolism and longevity (Chen et al., 2009). CTBP-1 has some redundancy with *daf-16*, a member of the insulin/IGF-1 signaling pathway that reduces lifespan in deletion mutants, thus suggesting that CTBP-1, and *lips-7,* affect longevity through this signaling pathway. Ivermectin is a lipophilic drug and combining it with fats increases absorption (Guzzo et al., 2002); therefore alterations in the lipid levels or makeup may affect the uptake of ivermectin by *C. elegans* or the peak concentrations it can reach. Incubation of Caco-2 cells with ivermectin and oleic acid (a fatty acid found in *C. elegans)* decreased the expression of p-glycoprotein and oleic acid strongly enhanced the absorption of ivermectin by mice both 6 and 24 hours after administration (Houshaymi et al., 2019; Watts & Ristow, 2017). Additionally, it has been reported to affect lipogenesis, lipid metabolism, and ER stress pathways, alone and in combination with palmitic acid in human HepG2 hepatocytes (Yang et al., 2019).

P-glycoproteins and the wider ABC transporter family have been repeatedly implicated in both ivermectin resistance and toxicity (Ardelli & Prichard, 2007; Bourguinat et al., 2008, 2011; Kiki-Mvouaka et al., 2010; Sheps et al., 2004). In hosts, mutations in the *mdr-1* gene, a p-glycoprotein, cause sensitivity to ivermectin (and other

drugs) in several breeds of dogs (Mealey, 2004, p. 1). In nematodes, both increases in the expression of p-glycoprotein (Ardelli, 2013; James & Davey, 2009; Peachey et al., 2017; Prichard & Roulet, 2007) and mutations in the gene (Ardelli, 2013; Ardelli & Prichard, 2007; Bourguinat et al., 2008; Prichard & Roulet, 2007) increase resistance to ivermectin and often other drugs. These transporters pump a wide variety of toxic compounds out of cells and this drug-efflux action has been hypothesized to be a protection mechanism for nematodes against anthelmintics. The interaction between ivermectin and p-glycoproteins has even been explored for the treatment of multi-drug resistant cancer (Pouliot et al., 1997) and delivering ivermectin to the brain for the treatment of alcohol use disorder (Silva et al., 2020).

In contrast to what might be expected, the null mutant of the gene *wht-4* was resistant in the egg laying assay. The gene *wht-4* is an ortholog of the human ABCG subfamily and of the *Drosophila melanogaster* white gene, though little about it has been studied in *C. elegans.* The white gene is famous in *D. melanogaster* as a phenotypic marker, with a vast number of described alleles that affect eye pigmentation and behavior (Myers et al., 2021; Xiao & Robertson, 2017). However, in both humans and *D. melanogaster,* the white gene and ABCG 1 and 4 transporters are involved in lipid transport and cholesterol efflux (Engel et al., 2006; Myers et al., 2021; Woodward et al., 2011). Therefore, it is possible that *wht-4* functions similarly in nematodes and changes in its expression may also affect cholesterol levels and lipid metabolism. Ivermectin can directly affect metabolism through binding with nuclear farnesoid receptors (FXR), reducing cholesterol levels in mice (Martin et al., 2021) and altering FXR and PPARα (which regulates fatty-acid oxidation) expression in HepG2 cells (Yang et al., 2019).

However, the ABC transporter family in nematodes is more diverse than that of mammals and many have diverged greatly (Sheps et al., 2004). Therefore, it is difficult to predict what function *wht-4* may have in *C. elegans* and other nematodes and so what role it may play in ivermectin resistance. Ivermectin resistance also has been predicted to be multifactorial (Kotze & Prichard, 2016; McCAVERA et al., 2007) and so there may be other interactors playing a role that influence the response or expression of *wht-4*. *Wht-4* and *lips-7* are both upregulated in the same life stage (MF) and it is possible that one could be compensating for ivermectin's effect on the other, as the two had opposite resistance phenotypes, or that ivermectin interacts with these two genes and lipid metabolism in different ways in different tissues, as *wht-4* and *lips-7* affected sensitivity in different processes.

Many studies have compared susceptible and resistant isolates of parasitic nematodes in an attempt to identify other genes associated with resistance (Ballesteros et al., 2018; Lau et al., 2021; Redman et al., 2012; Rezansoff et al., 2016; Sanchez et al., 2020). Recently, a study involving repeated genetic crosses between resistant and susceptible *H. contortus* identified two quantitative trait loci (QTL) associated with resistance on chromosome V, one at 37-42Mbp and the second at 45-48Mbp (Doyle et al., 2019). In this study, *hil-1* was resistant in the egg laying assay and the ortholog of this gene in *H. contortus* is located at 47.8Mbp, within the smaller QTL (Howe et al., 2017a). It is linker histone-like protein involved in muscle growth and function and is expressed in the marginal cells of the pharynx, vulval muscle cells, excretory channel cell, and some head neurons (Jedrusik et al., 2002). It is associated with intermediate filaments, so mutations may affect the structure of amphid neurons. Additionally, *hil-1* was identified

in a screen for genes that protect against spontaneous mutation (Pothof et al., 2003). DNA damage may also be a mechanism for *tyr-2,* a dopachrome isomerase that is secreted from ASJ neurons to inhibit DNA-damage-induced apoptosis (regulated by the transcription factor hypoxia-inducible factor)(Sendoel et al., 2010, p. 1).

This study had several limitations. First, if ivermectin's action against filarial worms and their mechanism of resistance are multifactorial, it is likely that we were unable to detect them fully. Each gene was tested only as a single knockout strain or RNAi knockdown, nothing in combination. Notably it takes the combination of mutations in *avr-14, avr-15,* and *glc-1* to obtain such high levels of ivermectin resistance (Dent et al., 2000) and so if other resistance mechanisms are similar, we would not see their full effect unless they were tested in combination. Many of the candidate genes were differently expressed at the same life stage and time point and so might have produced different results if they were all deleted in the same strain, though this would be technically challenging. Second, all the genes we tested were in null mutant strains or RNAi knockdown, leading to no or reduced expression compared to wild type in our assays. In comparison, some genes were over expressed in ivermectin treated *B. malayi* which makes some results difficult to translate directly to an *in vivo* mechanism. Third, we don't know what downstream, potentially compensatory effects might be caused by knocking out or knocking down many of the genes in this study. Its possible that upon loss of the target gene, the expression of other genes change drastically to make up for the loss, particularly in strains without a significant growth phenotype. Additionally, genes from large gene families or involved in large pathways could have redundancies built in, so over expression could result in a phenotype, but a knockout could be

compensated for. Many of these genes require further characterization in *C. elegans* to better understand what processes they are involved in while all gene hits should be assessed with *RNAi* in *B. malayi*.

In summary, we performed a screen to identify genes affecting ivermectin susceptibility in *C. elegans* based on data from a previous *in vivo* transcriptomics study of ivermectin treated *B. malayi.* We identified 11 genes that affected susceptibility in at least one assay including genes involved in both new and previously proposed mechanisms.

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CHAPTER 4

CONCLUSIONS

Lymphatic filariasis is a disabling and disfiguring disease that causes a significant burden to the world's population (Ottesen, 2006). The primary intervention, mass drug administration, is a lengthy process that requires enough coverage and compliance to be successful. A single dose of ivermectin typically causes a rapid and long-lasting reduction in the numbers of Mf in the infected person (Basanez et al., 2008; Brown et al., 2000; Ottesen, 2006); the latter effect is caused by a long-term sterilization of the adult female parasites. As ivermectin does not kill adult worms, MDA programs using ivermectin rely instead on suppressing transmission in communities through this effect. Ivermectin has been used in human and veterinary medicine for decades, yet we still do not fully understand its mode of action. Most interestingly, ivermectin is highly effective *in vivo* but not *in vitro*. The results presented in this dissertation explore how ivermectin acts to clear filarial parasites and potential resistance mechanisms through two major focuses: the effects of ivermectin on the host and the use of a model nematode to understand ivermectin's effect on candidate parasite genes.

Ivermectin's potency *in vivo*, but not *in vitro*, suggests that a host component may be involved, likely the immune system. Human immune cells from parasite naïve patients can attach to and kill *Brugia malayi* microfilariae and ivermectin is able to bind to receptors present on human immune cells. Specific Aim 1 explored the hypothesis that ivermectin acts directly on the host immune system through a clinical study in

collaboration with UGA's CTRU. Parasite naïve patients were given the label dose of ivermectin used in mass drug administration campaigns, and we compared their immune functioning to a placebo treated group. I found that there was no difference between the groups in their complete blood counts, cytokine levels, expression of a panel of innate immunity genes, or the ability of their immune cells the kill microfilariae. This result concurs with our lab's previous *in vitro* work showing the addition of ivermectin does not improve killing by immune cells or notably change gene expression.

Though our previous results with these PMN and PBMC co-culture assays have not shown an interaction between ivermectin and human immune cells, several unanswered questions remain particularly concerning specific interactions that might be masked by the more general assays or are more reflective of real-world scenarios. The PMN (polymorphonuclear) cell population is mixed and includes primarily neutrophils but also basophils and eosinophils (Beutler, 2004). Eosinophils are widely known to be involved in the anti-parasite response and so a more pure population of eosinophils may show different results (Babu & Nutman, 2014; Beutler, 2004; Cadman & Lawrence, 2010; Cooper et al., 1999). Additionally, these assays are conducted with cells and serum from parasite naive populations while individuals who clear MF following MDA will have immune systems that have been exposed to filaria before drug administration. This results in immune system activation to form a more directed response against filarial infection that could be augmented by ivermectin treatment (Babu & Nutman, 2014; Beutler, 2004; Cadman & Lawrence, 2010). Specifically, while alternatively activated macrophages are often seen in filarial infections, these may be an immunomodulatory strategy and the nitric oxide produced by classically activated macrophages is effective

against the parasite (Harischandra et al., 2018; Zamanian et al., 2015; Zhang et al., 2009). Due to these complex interactions, it would be interesting to see the effects of using serum and cells from infected individuals and/or pre-treating cells from naïve patients with parasites or parasite antigens before adding ivermectin. An alternative hypothesis for ivermectin's differing action *in vivo* and *in vitro* is that it prevents excretion of immunomodulatory factors by MF, thereby allowing the immune system to clear the parasite (Harischandra et al., 2018; Loghry et al., 2020; Moreno et al., 2010). However, we have previously found that co-culturing MF, immune cells, and ivermectin does not increase the amount of killing compared to MF and immune cells alone despite ivermectin hypothetically eliminating secretion in MF (Reaves et al., 2018). This could relate to our finding that theres is a difference in killing between MF that had been washed and those that had been unwashed, and so a series of experiments examining pretreatment with ivermectin, washing, and/or media changes could provide further insight.

As ivermectin does not seem to have a direct effect on the host, Specific Aim 2 focused on how ivermectin affects the parasite when treated *in vivo*. Our lab previously performed a transcriptomics study in which gerbils infected with *B. malayi* were treated with ivermectin and we measured which genes were differently expressed in the parasite compared to worms from control hosts. To better understand and characterize these differently expressed genes, I used *C. elegans* as a model as it has well established molecular tools and a more convenient culture system. In Specific Aim 2, I utilized strains of C. elegans with mutations in the orthologs of the differently expressed genes we identified in *B. malayi* and measured their response to ivermectin in several standard

phenotypic assays (fertility, motility, development). I additionally utilized a microfluidics platform called the NemaMetrix ScreenChip™ which allows the collection of electrical signals from the pumping action of the nematode pharynx, a muscular feeding organ. Through this approach I have identified several genes that influence resistance and hypersensitivity to ivermectin and found that the processes of development and fertility are affected by ivermectin independently of each other. These genes identified several new pathways through which ivermectin may affect filarial nematodes including lipid metabolism and DNA damage.

There are several interesting avenues of research to consider for furthering these results. This study examined genes individually while the transcriptomics data showed many of them to be differently expressed concurrently. It would therefore be interesting to see the effect of multiple mutations on the ivermectin sensitivity of strains, particularly mutations in genes that were expressed at the same life-stage and time point and those that gave different results in different assays to see if the results were in some way additive. Some genes that I examined through null mutants were in fact over expressed and may show an opposite response to ivermectin if they were to be overexpressed in *C. elegans.* Finally, many of the knockout strains were superficially wild type, indicating that they were not essential and that their loss could be compensated for. Many of them were even part of multi-gene families, and therefore it is possible that some of these other genes could be over-expressed after the loss of one, particularly under stressful conditions like anthelmintic treatment. It would therefore be intriguing to examine the gene expression of some of these knockout mutants both with and without ivermectin

treatment, in particular genes whose loss resulted in a counterintuitive effect on ivermectin resistance such as *wht-4*.

Through the research presented in this dissertation, it was my aim to improve our understanding of ivermectin, a vital drug to human and veterinary medicine. By doing so, we can better understand mechanisms of resistance, best practices for managing resistance, and potential new drug targets that could be further developed for the benefit of humans and animals everywhere.

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APENDIX A

CHAPTER 2 SUPPLEMENTARY INFORMATION

Table A1. Inclusion and exclusion criteria for participants recruited into the study.

Inclusion Criteria	**Exclusion Criteria**
Weight over 50kg and under 84kg Aged between 18 and 65 years	-Pregnancy or nursing mothers. -Immunosuppressed individuals. -Hypersensitivity to ivermectin, cellulose, starch, magnesium stearate, butylated hydroxyanisole, or citric acid powder (inert ingredients of Stromectol). -Lactose intolerance (Lactose present in placebo) -Recent (last 3 years) travel to West or Central Africa, or any other country where onchocerciasis is present -Hepatitis/HIV -Liver or renal dysfunction -Currently taking any of the following medications (potential for drug interaction): -- Blood thinners such as Coumadin (warfarin) or aspirin -- Steroid medications (inhaled, oral or injection) -- Barbiturates -- Benzodiazepines such as Xanax or Klonopin -- Valproic acid (Lithium) -- Calcium channel blockers -- Statins (cholesterol medication)

Table A2. Nanostring results for the changes in expression of the 770 genes of the myeloid gene panel in PBMCs. In this Table all the results are compared to the Control, t = 0 sample.

Table A3. Nanostring results for the changes in expression of the 770 genes of the myeloid gene panel in PMNs. In this Table all the results are compared to the Control, t = 0 sample.

Table A4. Nanostring results for the changes in expression of the 770 genes of the myeloid gene panel in PBMCs. In this Table the changes between subjects who received ivermectin and those who received a placebo are compared at 4hrs and 24hrs post-treatment.

Table A5. Nanostring results for the changes in expression of the 770 genes of the myeloid gene panel in PMNs. In this Table the changes between subjects who received ivermectin and those who received a placebo are compared at 4hrs and 24hrs post-treatment.