Complementary approaches to understand anthelmintic resistance using free-living and parasitic nematodes

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Abstract:

Parasitic nematode infections impact human and animal health globally, especially in the developing world. Anthelmintic drugs are the major line of defense against these infections, but the arsenal is limited. Additionally, anthelmintic resistance is widespread in veterinary parasites and an emerging threat in human parasites. Discoveries of the mode of action of these drugs and mechanisms of resistance have predominantly come from studies of a related non-parasitic nematode species, *Caenorhabditis elegans*, and the parasitic nematode *Haemonchus contortus*. Here, we discuss recent progress understanding anthelmintic resistance using these two species and how that progress relates to laboratory and field-based studies of veterinary helminths. We present a powerful approach enabled by the strengths of both nematode species to understand mechanisms of resistance and modes of action of anthelmintic drugs.
Helminth drug resistance in veterinary and human health

Parasitic nematode infections of both humans and livestock constitute a major health and economic burden around the world [1]. Only four major classes of anthelmintic drugs (benzimidazoles, BZs; macrocyclic lactones, MLs; nicotinic acetylcholine receptor agonists, nAChR agonists; and amino-acetonitrile derivatives, AADs) make up the primary defense against these parasites [1,2]. Benzimidazoles were first deployed in veterinary settings in the 1960s, and resistance was identified within a few years of implementation [3]. Following these initial reports of BZ resistance, prevalence in veterinary parasitic nematodes increased at an alarming rate, including *Haemonchus contortus*, *Teladorsagia circumcincta*, and *Dirofilaria immitis* [1,4–7]. Resistance to MLs [8–10], nAChR agonists [11,12], and AADs [13] were also quickly documented. The economic impacts of resistance in agricultural sectors around the world are massive with global losses projected in the billions of dollars per year [1,14]. In contrast to veterinary health, resistance in human parasites is not widely reported with only a few confirmed cases [15–17]. However, the selective pressure placed on human parasites by mass drug administration programs will soon give rise to equivalent levels of resistance [18]. With the imminent emergence of resistant human parasites and the threat of multidrug resistant livestock parasites, broad-scale chemotherapy failure will soon become a reality for many parasitic nematode species. This looming threat necessitates continuous efforts to discover modes of action and mechanisms of resistance for available anthelmintics to ensure long-term maintenance of efficacy. Despite decades of studying anthelmintics, mechanisms of resistance for many drugs remain hypothetical.

Most studies on anthelmintic modes of action and resistance have focused on one species at a time, especially research using the laboratory-adapted strain of the free-living nematode *Caenorhabditis elegans* [19]. However, an experimental cycle of discovery between *C. elegans* and the veterinary helminth *H. contortus* provides an ideal complementary approach to study resistance against a wide range of anthelmintics (Figure 1). The genetic toolbox associated with *C. elegans* and its close
phylogenetic relationship to *H. contortus* allows researchers to use the tools available in *C. elegans* to study resistance candidates from *H. contortus* [20–22]. Here, we describe how complementary approaches have benefited studies of anthelmintic resistance mechanisms in the past and why this approach is the most productive route to discovery in the future. With a thorough understanding of the drug mode of action and resistance mechanisms, combinatorial treatments or drug rotations using anthelmintic sensitivity in refugia populations can be developed to mitigate the emergence and prevalence of resistance around the world [23].

![Diagram showing the cycle of discovery between *C. elegans* and *H. contortus*.](image)

**Figure 1.** The cycle of discovery between *C. elegans* and *H. contortus* where advances made in one organism can be tested in the other and vice versa. Our understanding of anthelmintic resistance and modes of action have been facilitated by this interplay.

**Caenorhabditis elegans - an unparalleled model to study nematode anthelmintic resistance**

Use of the model system *C. elegans* has a long history in the field of anthelmintic drug resistance because it offers many advantages compared to parasite species. *C. elegans* has a short life cycle, is easily maintained in the laboratory, and is amenable to genetic screens. It also has a well annotated genome along with many optimized molecular genetic tools, including CRISPR-Cas9 for genome editing [24,25]. Over the last 40 years, genetic screens and selections using *C. elegans* have transformed numerous fields because of the power to link genes to phenotypes of interest. Additionally,
genetic selection experiments are often used in *C. elegans* research because the laboratory strain, N2, is naturally sensitive to most anthelmintic drugs (Box 1) and resistant mutants are easy to identify. After mutagenesis, progeny are placed on a selective medium that contains an anthelmintic drug. Individuals that survive are resistant to the drug and have mutations in genes that alter drug sensitivity. Oftentimes, the identified genes encode the targets of the drugs and therefore are involved in the mode of action. In anthelmintic research, genetic selection experiments have enabled the identification of genes and mechanisms that underlie responses to several anthelmintic drug classes [26–30]. The plethora of *C. elegans* genetic tools make the mapping and identification of these mutations much easier than in parasitic nematode species. For these reasons, *C. elegans* has been the keystone model to discover modes of action and resistance mechanisms for anthelmintics.

**Benzimidazoles**

A selection experiment to identify genes that underlie BZ sensitivity yielded many resistant mutants [26]. The resistance phenotypes of these strains were mapped to a nematode-specific beta-tubulin gene, named *ben-1*. This study also found that the complete deletion of the *ben-1* locus caused resistance but did not cause any observable growth defects in control conditions, suggesting that the gene might act redundantly with other beta-tubulin genes for organismal viability. After the discovery of *ben-1*, two orthologous genes in *H. contortus*, beta-tubulin isotype-1 (*Hc-tub-8-9*) and beta-tubulin isotype-2 (*Hc-tub-12-16*), were identified [31]. An *Hc-tub-8-9* allele that caused a phenylalanine-to-tyrosine mutation at position 200 (F200Y) was found in a BZ resistant *H. contortus* population [32]. Wild-type *Hc-tub-8-9* can functionally substitute for *C. elegans* *ben-1* when overexpressed in a mutant strain lacking *ben-1* [33]. This discovery shows the translational power of *C. elegans* research, where a parasite gene can functionally substitute for the loss of an endogenous *C. elegans* gene. Additionally, a clone of *Hc-tub-8-9* with the F200Y substitution was overexpressed in the mutant *C. elegans* strain lacking *ben-1*. With this substitution, the *Hc-tub-8-9* clone was no longer able to rescue susceptibility
to BZs, which suggests that the F200Y substitution is not sufficient to confer BZ sensitivity so might be involved in resistance [33]. However, even though these studies have greatly increased our understanding of BZ resistance, a number of caveats complicate the interpretation of the results. Most importantly, these studies only correlate variation in the beta-tubulin gene with BZ resistance. To go beyond correlation, a causal relationship between beta-tubulin and BZ resistance must be defined, which is difficult to accomplish in parasitic nematode species. Causal relationships between genes and phenotypes require empirical tests of necessity and sufficiency. The overexpression experiments only show that the parasite beta-tubulin gene is sufficient for BZ sensitivity. To show that beta-tubulin is necessary for the parasite BZ response, this gene must be mutated in a defined genetic background and shown to alter the BZ response, which has been done for several parasitic nematode beta-tubulin alleles [20–22]. These approaches testing gene functions between C. elegans and H. contortus have enabled rapid progress in the understanding of BZ resistance.

**Macrocyclic lactones**

Like the discovery of BZ resistance, an ML selection experiment in C. elegans identified many resistant mutants [27]. The phenotype of one resistant mutant was mapped to a gene that encodes an alpha subunit of a glutamate-gated chloride (GluCl) channel, named avr-14. Subsequent studies identified other genes that encode alpha GluCl channel subunits (avr-15 and glc-1) that are sensitive to MLs [27,34]. C. elegans also encodes beta subunits of the GluCl channel [35]. However, these subunits are not responsive to ivermectin (IVM) in homomeric receptors although they remain sensitive to glutamate [35]. The ML anthelmintic class causes hyperactivation of GluCl channels and animal paralysis when susceptible subunits are included in the channel [36]. The three alpha GluCl subunits form heteromeric channels with beta GluCl subunits [37]. A single mutation in avr-14, avr-15, or glc-1 does not confer a high level of resistance to MLs likely because the drug can still bind to and hyperactivate the GluCl channels with other alpha GluCl subunits [38]. However, mutations in all three
genes in the same organism confer high levels of resistance because all three of the targets of the MLs (alpha GluCl channel subunits) have been lost so the drug cannot hyperactivate the channel. Interestingly, loss of all three GluCl alpha subunits causes slight defects in feeding and locomotion in laboratory conditions [38]. The identification of GluCl channel-mediated ML resistance was facilitated using *C. elegans*.

Like the previously described BZ experiments, heterologous expression of *H. contortus* GluCl genes can modify *C. elegans* response to IVM. The *H. contortus* avr-14 homolog *Hco-avr-14b* expressed in the *C. elegans* IVM resistant triple GluCl deletion mutant was able to reintroduce sensitivity to IVM [39]. However, further studies in parasites have suggested that variation in GluCl receptors does not underlie resistance to MLs in parasite populations [40]. The P-glycoproteins (Pgps) have been implicated in differential responses to the ML class of drugs [41,42]. Work in *C. elegans* found that increased expression of Pgps was associated with increased resistance to IVM [43]. In the parasite, *Parascaris equorum*, the Pgp *Peq-pgp-11* was found to be differentially expressed between resistant and sensitive isolates at certain stages of growth [44]. When *pgp-11* was deleted in the N2 background, strains became more sensitive to IVM treatment [45]. Additionally, expression of *Peq-pgp-11* in the *C. elegans* *pgp-11* deletion strain restores resistance to IVM, suggesting a conserved function in detoxification [46]. Many other studies of GluCl and Pgp genes that modulate the *C. elegans* response to MLs have advanced our understanding of ML resistance and mode of action [47–49].

**Nicotinic acetylcholine receptor agonists**

Resistance to multiple drugs in the nAChR agonist class has been studied in *C. elegans* [50]. Just like for BZ and ML anthelmintic resistance studies, levamisole resistance loci were discovered using selection experiments [28]. These experiments identified genes that underlie responses to levamisole, including an alpha subunit of the nAChR family, named *unc-38*, and two non-alpha subunits of the same family, named *lev-1* and *unc-29* [28,51]. The nAChR is either a pentomer of only alpha
subunits or a heteromer of five alpha and non-alpha subunits [52]. Like MLs activate GluCl receptors, nAChR agonists hyperactivate the target nAChR receptors [53], so loss of these receptors causes resistance. Five additional genes were discovered to be essential for the function of the levamisole-sensitive nAChR: *unc-63, lev-8, ric-3, unc-50,* and *unc-74* [54]. In addition to resistance, these mutations cause an uncoordinated phenotype where animals do not move normally on culture plates in the laboratory [51], likely indicating a significant defect in fitness.

The discovery of the *C. elegans* nAChR genes as targets of nAChR agonists led to the identification of parasite orthologs for this receptor [53,55–57]. Like the previously described studies in BZ resistance, *H. contortus* orthologs to nAChR genes were expressed in *C. elegans* to test if the genes modulated the nAChR agonist response. For example, *Hc-unc-38* expressed in a resistant *C. elegans* background was able to reintroduce susceptibility to levamisole [58]. In addition to homologs of *C. elegans* nAChR genes, some *H. contortus* specific nAChR genes have also been investigated for the ability to modify drug response. The genes, *Hco-acr-26* and *Hco-acr-27*, encode alpha and non-alpha nAChR subunits, respectively, that are not found in the *C. elegans* genome [52,56]. When overexpressed in *C. elegans*, these genes confer greater sensitivity to morantel (another nAChR agonist) than the laboratory wild-type strain [52]. When either subunit is expressed alone, the *C. elegans* response to morantel is not affected, suggesting that the *Hco-ACR-26* and *Hco-ACR-27* subunits together form a functional receptor that is sensitive to morantel. Although suggestive, this study requires additional experiments to connect these genes to nAChR agonist resistance in *H. contortus*. To prove necessity of these genes in resistance, these genes need to be deleted in a controlled *H. contortus* genetic background and shown to confer resistance, but these experiments are extremely difficult because of the lack of controlled genetic backgrounds in parasite species.

**Amino-acetonitrile derivatives**
Monepantel, the first drug in the AAD class of anthelmintics, was introduced recently, and *C. elegans* was instrumental in its development and the discovery of resistance mechanisms [59,60]. Monepantel acts as an agonist of nAChR activity, like levamisole, and animals are paralyzed after treatment [29]. For this reason, a selection experiment was used to identify monepantel-resistant mutants, and the resistance phenotype was mapped to a gene, named acr-23, that encodes an alpha nAChR subunit [30]. This result indicates that monepantel might act through the ACR-23 receptor. Unlike loss of the nAChR subunits that cause levamisole resistance, loss of acr-23 does not cause an uncoordinated phenotype or any detrimental fitness effects [30].

The involvement of genes homologous to acr-23 in monepantel resistant parasite populations was assessed in *H. contortus* [30]. Three resistant isolates were each derived from repeated rounds of monepantel treatment of three independent susceptible isolates. Mutations in acr-23 homologs were identified, including *Hc-acr-23H* and *Hc-des-2H* which are both part of the nematode-specific DEG-3 group of nAChRs. A subsequent study in *C. elegans* found another gene, named acr-20, that also encodes an alpha nAChR subunit involved in the monepantel response [61,62]. These results suggest that similar mechanisms underlie monepantel resistance in *C. elegans* and *H. contortus*.

**C. elegans** natural diversity enables discoveries of anthelmintic resistance

In nature, *C. elegans* is found in complex niches filled with bacteria, fungi, and other nematodes competing for resources. Most of the anthelmintic drugs used today are derivatives of natural compounds produced by bacteria and fungi to combat other microbes within the niche [63–65]. Many parasitic nematode species spend some part of their life cycle in soil or rotting vegetation environments (filarial nematodes are a notable exception), and this environment overlaps with known natural niches for *C. elegans* [66,67]. For this reason, parasitic nematodes and *C. elegans* have likely evolved shared or similar resistance mechanisms to survive the effects of natural anthelmintic compounds [21]. For
each of the four anthelmintic drug classes described above, genetic selection approaches have been used to identify the genes involved in modes of action and resistance. However, all of these approaches used the laboratory-adapted N2 strain, which is just one strain in the C. elegans species and does not represent the diversity of genetic backgrounds present across the entire species [19]. Wild isolates of C. elegans can facilitate studies of how natural diversity affects the response to anthelmintics, which could recapitulate responses found in natural parasitic nematode populations. C. elegans is sampled from diverse niches worldwide (Figure 2A), and these strains are readily available from the C. elegans Natural Diversity Resource (CeNDR) [68]. Quantitative genetic studies correlate phenotypic differences across a set of individuals with genetic differences across the same set of individuals [69]. Genomic regions that harbor correlated genetic differences are called Quantitative Trait Loci (QTL). Such loci can be narrowed further to single genes and ultimately specific variants that underlie quantitative traits. This approach can be applied to anthelmintic drug responses to discover modes of action and resistance mechanisms.

Using a collection of 249 C. elegans wild strains isolated from nature, a recent study measured responses to albendazole (ABZ) and found that the strains varied in their responses (Figure 2B). Many previously unidentified single amino-acid substitutions in the ben-1 gene underlied differences in BZ responses that can be correlated with resistance [21]. In addition to these single amino-acid substitutions, structural variants, including deletions, insertions, transposons, and inversions, were found in some individuals across the population. Nearly 15% of C. elegans wild strains harbor variation in the ben-1 locus, illustrative of the large amount of standing variation in the wild population and suggesting that BZ selection is prevalent in natural settings. This high level of variation increases the statistical power of genome-wide association studies (GWAS), as well as the identification of naturally occurring alleles that can modify anthelmintic responses. This approach has proven useful to identify variants that underlie the differences in responses to arsenic, topoisomerase II poisons, chemotherapeutics, toxins, and metals in C. elegans [70–75]. Using this powerful strategy, the
researchers identified a genomic interval on the X chromosome that underlies differences in BZ responses independent of the beta-tubulin gene, *ben-1* [21]. The study of natural, population-wide variation is uniquely powerful because this specific region would have been nearly impossible to identify with traditional genetic selection techniques because *ben-1* variation overshadows the small effects of other loci.
Figure 2. Worldwide collection of natural C. elegans isolates have been exposed to three anthelmintic drug classes. (A) A world map with each black dot representing a strain from the wild isolate collections maintained in the C. elegans Natural Diversity Resource. (B-D) Normalized drug response is shown on the y-axis, and strains are ordered by their response on the x-axis from sensitive to resistant with N2 shown in orange: (B) albendazole, (C) ivermectin, and (D) levamisole.
Leveraging the natural diversity in *C. elegans* has applications for mapping responses to other anthelmintics. For example, the same strains that were assayed for responses to albendazole [21] showed varied responses to ivermectin and levamisole (Figure 2C, 2D). The distributions of these ML and nAChR agonist responses across the wild population suggest strains that can be used in future anthelmintic studies. The strains with extreme sensitivity or resistance to these drugs could be excellent genetic backgrounds for selection or screening experiments. For example, a strain that is highly susceptible to an anthelmintic drug in selection experiments will enable identification of the loci underlying resistance as was shown in the identification of the *ben-1* locus using the N2 strain [26].

The laboratory-adapted *C. elegans* strain N2 enabled the identification of the GluCl channels as the loci underlying responses to the ML drugs [27,38]. However, it was not appreciated how naturally occurring variants could modulate the ML response until a natural isolate from the Hawaiian islands (CB4856) was studied [76]. By using a *linkage mapping* approach with the sensitive N2 and the resistant CB4856 strains, researchers were able to identify *glc-1* underlying the difference in abamectin responses between the two strains. The other ML responsive genes, *avr-14* and *avr-15*, were not identified because these two strains do not harbor differences in these two genes (CeNDR version 1.3.6). The CB4856 *glc-1* gene contains 77 variants as compared to the N2 strain. Three of these variants were predicted to have more deleterious effects than other variants: A20T, T346A, and a four amino-acid deletion [76], so the CB4856 strain could have a loss of *glc-1* to cause ML resistance. The N2 version of *glc-1* was used to rescue the ML response of the CB4856 strain. The authors tested the three predicted deleterious variants and found that all but the four amino-acid deletion conferred sensitivity to MLs in the CB4856 strain, indicating that the deletion is sufficient to confer resistance [76]. To confirm that the CB4856 variant is necessary to confer ML resistance, the allele must be introduced using CRISPR-Cas9 genome editing into a sensitive genetic background and shown to cause resistance. Identification of this naturally occurring *glc-1* deletion would not have been possible if research had remained limited to the N2 laboratory strain background.
Additionally, a GWA mapping using 97 wild *C. elegans* isolates was performed. This mapping also identified a region that contained *glc-1* underlying differences in the ML response. The *glc-1* gene of 53 wild strains was sequenced and 16 strains harbored the same four amino-acid deletion as the CB4856 strain [76]. The ML response phenotypes of these strains showed that this deletion was not completely predictive of the ML response because some strains with the deletion were sensitive and some strains without the deletion were resistant. Interestingly, some of the wild strains with the four amino-acid deletion had dominant resistance phenotypes, which was in contrast to the CB4856 strain that had a recessive resistance phenotype. These results suggest that the ML resistance in these strains might be independent of *glc-1*. These strains with dominant ML resistance phenotypes and no variation in *glc-1* provide excellent avenues of research into novel resistance loci. Using publicly available data from CeNDR [68], many naturally occurring variants in candidate genes that could impact responses to different anthelmintic drugs can be investigated. For example, *glc-1*, *avr-14*, and *avr-15* each contain many naturally occurring variants that range from single nucleotide changes to predicted loss-of-function variants (e.g. frame-shift mutations). However, these naturally occurring variants were not identified in the previous linkage mapping experiment because they are not present in the N2 or CB4856 strains. As mentioned above, these variants could impact organismal fitness so it is unlikely that parasitic nematodes would harbor variants in these genes. However, these wild isolates are healthy and have relatively normal fitness despite these variants. This result indicates that these variants do not affect gene function or that epistatic mechanisms allow these strains to have normal fitness. The genes underlying these epistatic mechanisms might also be relevant to resistance in parasitic nematodes.

Other anthelmintic classes with known targets also contain potentially informative natural variants. In the *lev-1*, *unc-29*, and *unc-39* genes, CeNDR shows five, two, and four naturally occurring amino-acid substitution variants, respectively, suggesting that these strains could differ in responses to nAChR agonists. Another example is the monepantel-sensitive gene, *acr-23*, which contains over 29
variants across the *C. elegans* population [68]. Previous work has demonstrated that several loss-of-function *acr-23* mutations cause resistance to monepantel [29], but the wild isolates could tell us how natural nematodes become resistant to this class of anthelmintic. These naturally occurring alleles need to be tested to determine if they confer resistance to monepantel, as has been done for BZ resistance [20,21]. Use of naturally occurring alleles provides insights into the diversity that is present in nature and not only laboratory-generated alleles from genetic selection experiments.

**Advances in understanding anthelmintic resistance in *Haemonchus contortus* and other helminths**

Since the introduction of anthelmintics in the 1960’s, resistance has been studied in a wide range of nematodes besides *C. elegans*. However, studies of specific anthelmintic modes of action and resistance in helminths have been limited because parasite life cycles make isolation and maintenance extremely challenging in a controlled laboratory setting. *H. contortus*, a gastrointestinal nematode of small ruminants, has been a long-standing model because it is easier to establish and culture in host animals than most other parasites [77,78] and its high-quality reference genome has been updated recently to a chromosome-level assembly [79]. In addition, resistance to all major classes of anthelmintics is widespread across this species [80].

**Benzimidazoles**

Resistance to BZ drugs offers the most notable success story for the understanding of anthelmintic resistance in parasitic nematodes. Mutations in nematode-specific beta-tubulin genes have been correlated with BZ resistance and loss of drug efficacy in many parasitic nematode species [77,81]. Though research using *C. elegans* was pivotal in understanding BZ mode of action and
resistance, it was early work on *H. contortus* tubulin extracts that linked the drug to its target *in vitro*, suggesting that beta-tubulin from resistant isolates might have reduced affinity for thiabendazole, albendazole, parbendazole, oxibendazole, mebendazole, oxfendazole, and fenbendazole [82,83].

The identities of the BZ resistance alleles should enable testing of parasitic nematode isolates to determine predicted levels of resistance prior to treating parasite populations with BZ drugs. Traditionally, resistance in parasite populations has been assessed using a Fecal Egg Count Reduction Test (FECRT), where egg counts before and after drug treatment are compared [84], but this method only measures resistance after the drug has been applied offering no predictive value. A molecular barcoding approach, Nemabiome, offers a screening method that can assess the presence of alleles that correlate with resistance prior to treatment [85,86]. This approach measures allele frequencies of mixed-species nematode populations from fecal samples facilitating the prediction of BZ responses by the frequency of resistant variants across the population. Nemabiome can enable farmers to determine whether BZ drugs are likely to be effective or whether an alternative treatment should be used to control parasite infections. This targeted-sequencing approach becomes more powerful as we discover and measure new genes connected to resistance, increasing the accuracy of predictions. Discoveries from *C. elegans* and *H. contortus* have impacted our understanding of BZ resistance, but the applications to parasitic nematode control have not been fully exploited.

**Macrocyclic lactones**

ML resistance studies in *H. contortus* have focused on genes suggested by *C. elegans* research. These genes encode GluCl channel subunits, P-glycoproteins, and gamma-aminobutyric acid receptors, along with genes that control the development and function of the nematode amphid sensory organs [87–90]. However, mutations in these candidate genes are not correlated with resistance. Because of the tractability of *H. contortus*, genetic crosses offer a powerful method to connect anthelmintic resistance to specific loci. These crosses are possible by surgically transplanting male L4
larvae from one parental isolate and female L4 larvae from another isolate into the abomasum, the fourth stomach compartment, of a parasite-free recipient ruminant host [40,91]. When this host is treated after infection, resistant offspring are selected. In the next generation, resistant offspring are crossed back to the susceptible parent isolate to allow crossover events to break down linkage around the resistance loci. It is important to remember that resistance might have different genetic causes in different resistant isolates, so crosses with independent isolates give additional power to discover new resistance loci. To study ML resistance, two independently backcrossed isolates were selected by crossing two independent resistant isolates with the same susceptible isolate [40,91]. Investigators looked for correlations of individual variants or segregating haplotypes with resistance and found an interval on chromosome V using microsatellite markers [92]. In a genome-wide approach, the backcrossed isolates were compared to the parental isolates, and the same QTL on chromosome V was found to be correlated with IVM resistance [40]. However, neither of these approaches implicated any of the candidate C. elegans genes studied previously.

**Nicotinic acetylcholine receptor agonists**

Levamisole resistance in *H. contortus* and *C. elegans* has been linked to genes that encode subunits of the nAChR [77]. Like BZ drugs, early research using radiolabeled ligand binding assays of *H. contortus* extracts showed that resistant isolates had reduced affinity for the nAChR [93]. Recent studies of *H. contortus* found three main mechanisms of resistance, and each mechanism caused decreased responsiveness of the nAChR to levamisole: (1) reduced transcription of the nAChR subunit genes *Hco-unc-63, Hco-unc-29,* and *Hco-acr-8a* [55,94–97], (2) truncated forms of the *Hco-unc-63* and *Hco-acr-8* subunit genes [95,98,99], or (3) reduced expression of the genes *Hco-unc-74, Hco-unc-50,* and *Hco-ric-3* needed in the process of forming the nAChR [98]. Although all of these mechanisms reduce the number of functional levamisole receptors, none are correlated consistently with resistance [94,95] and experiments are required to definitively connect variation in these genes to resistance.
Amino-acetonitrile derivatives

Before the AAD monepantel was approved for use in animals, field isolates of *H. contortus*, *T. circumcincta*, and *Teladorsagia colubriformis* that were resistant to BZ and ML anthelmintics were treated with therapeutic doses of several AADs [30]. This new anthelmintic class effectively eliminated parasitic nematode infections as shown by FECRTs. As described above, the investigators selected AAD-resistant isolates of *H. contortus* and focused their studies on the nAChR gene *Hco-acr-23* (also called *Hco-mptl-1*). These studies were facilitated again by the power of *H. contortus* genetic crosses. Animals were exposed to monepantel from the embryo to the infective larval stage (iL3) and then used to infect sheep. After eight of these in vitro selection rounds, the isolates were resistant. Because these experiments were conducted on larval stages in vitro, and resistance can depend on the selection method [100], it was unclear if *Hco-acr-23* would play a role in field resistance. To address this point, sheep hosts were infected with either monepantel-susceptible males and resistant females, resistant males and susceptible females, or the resistant parental population [101]. The offspring from these different infections across different hosts were collected both before and after monepantel treatment, pooled, and then sequenced. A QTL on chromosome II was identified that harbors both *Hco-des-2* and *Hco-acr-23* as well as *Hco-deg-3*, a candidate gene found in a different monepantel resistance study [102]. This study illustrated that approaches using a limited number of host animals and genetic crosses can discover regions that correlate with resistance in parasite populations [101]. The combined results between *C. elegans* and *H. contortus* proved that this nAChR class is responsible for the AAD resistance phenotype.

Challenges and solutions to study anthelmintic resistance in helminths

Reference genome quality
Parasitic nematode genomes range in size from 42-700 Mb [103]. All of these genomes vary in their levels of completeness, comprising many contigs rather than chromosome-level assemblies. For quantitative genetic studies, it is essential to know how markers are positioned relative to each other across a single chromosome. If the genome is fragmented and linked markers are on different contigs, then multiple QTL for resistance are inappropriately detected. The aforementioned results of multi-drug resistant T. circumcincta illustrate how a fragmented genome can hamper detection of resistance loci [104]. The reference genome for T. circumcincta is not a chromosome-level assembly but contains 81,730 contigs. This study detected Tc-lgc-54 as a candidate IVM resistance gene. However, many other contigs, besides the contig containing Tc-lgc-54, were correlated with resistance, implying that this trait could have a large number of genetic causes beyond this gene. These contigs contain no obvious candidate genes discovered in H. contortus or C. elegans, suggesting linkage to a single disjointed resistance locus might be the cause of observed correlations rather than variation on all of these contigs underlying resistance [40,104]. Chromosome-level assemblies enable the detection of linked markers and circumvent spurious QTL detection. Additionally, gene models are essential to comparative genomics between species and to narrow broader QTL to the level of genes of interest. In draft genomes, gene models are hard to predict because genes can be split across contigs and incomplete [105,106]. We require robust gene models generated from long-read RNA sequencing along with novel computational predictions to understand anthelmintic resistance in parasitic nematodes.

Novel sequencing technologies facilitate the production of chromosome-level genome assemblies. Typical high-throughput sequencing generates billions of short fragments that make genome assembly and accurate gene model prediction difficult because many small contigs are assembled [107,108]. A variety of newer technologies generate longer reads of up to hundreds of thousands of base pairs, which make chromosome-level genome assemblies possible [109]. H. contortus offers an excellent example of a parasite species where the genome has become the model
for other parasitic nematodes. Future parasitic nematode genomes should be built using multiple technologies, including short and long reads along with chromosome capture techniques [110], to generate sequences from individual nematodes.

High levels of genetic variation

All evidence suggests that anthelmintic resistance in parasite populations is heritable with resistant offspring derived from resistant parents. In the simplest case, resistant individuals have differences in the genes that control the drug response, and those genetic differences or variants are not present in sensitive individuals. A comparison between resistant and sensitive individuals should reveal these resistance variants. However, helminth populations harbor extremely large numbers of variants because of their large outbreeding populations and predicted high mutation rates [78,111,112]. Each individual nematode has a collection of variants shared by recent ancestry but also random variants. That means that resistant parasite populations have hundreds of thousands to millions of unique variants as compared to sensitive populations. To identify variants that underlie resistance, it is essential to distinguish variants linked to resistance from variants that are unique to resistant populations but do not contribute to the resistance phenotype. The discovery of resistance variants depends on the evolutionary history of the resistant and susceptible isolates used for the study. If the populations have diverged within a generation or two, then most variants unrelated to resistance will be shared by both isolates. However, if the two populations have diverged longer ago or are from different geographic locations, then both populations will have gained unique variants and disentangling the variants that cause resistance from all of the unrelated variants will be difficult if not impossible. Any unrelated variants will appear linked to resistance in a quantitative genetics approach if they are only found in resistant isolates. Sampling the same farms both before and after anthelmintic treatment offers a possible solution, as do the above described backcross approaches [40,91,113]. However, the backcross selection process is labor-intensive and requires euthanization of host livestock. It is difficult
to perform a large number of crosses for these reasons. Additionally, linkage can extend over megabases making identification of resistance genes difficult and every subsequent generation gains unique variants that likely do not contribute to resistance but confound analyses.

*Lack of widespread genome editing in parasitic nematodes*

Candidate genes are only correlated with anthelmintic resistance and need to be validated using controlled genetic backgrounds to definitely prove their role in resistance. Even if reference genome quality increases and sampling schemes are improved, genome-editing technologies are still not optimized for nearly all parasitic nematodes. The experimental goal is to replace the allele from the susceptible isolate with the allele from the resistant isolate and show that this new isolate has increased resistance. This approach is stymied by the lack of defined genetic backgrounds and obligate outcrossing. Despite these difficulties, genome editing has been performed successfully in *Strongyloides* spp. because the life cycle is amenable to laboratory manipulation [114–120]. For other parasitic nematode species, the cycle of discovery between parasitic and free-living nematodes offers promise but genome editing might still be in the distant future. *C. elegans* orthologs of parasite genes can be genetically edited or knocked out in a controlled genetic background.

*A cycle of discovery: Integrating studies from *C. elegans* and helminths*

In the above sections, we reviewed how studies of both *C. elegans* and *H. contortus* have impacted what we know about anthelmintic resistance and highlighted some ongoing issues and solutions. Importantly, our understanding of resistance has been advanced only by integrating findings from both free-living and parasitic nematode systems. We argue that this approach should be expanded because both systems offer distinct advantages that can synergize when combined and circumvent disadvantages from each species taken individually. As we described above, studies using *C. elegans* offer nearly every experimental advantage but lack the context of a parasitic nematode. Conversely, *H.*
contortus populations have been subjected to long-term treatment using all anthelmintic drug classes and resistant isolates can be sampled worldwide but experimental tools are limited. The interplay of approaches and results from both systems enables a cycle of discovery for anthelmintic modes of action and resistance.

Parasitize C. elegans to understand anthelmintic resistance

As we discussed above, genetic selections using C. elegans have identified resistance mechanisms and modes of action for anthelmintics [26,30,38,51]. Most of these studies used mutagenized individuals or extrachromosomal arrays to express parasite genes [33,46]. In recent years, the CRISPR-Cas9 system has made genome editing not only possible but a relatively straightforward process [25]. Importantly, the C. elegans CRISPR-Cas9 system has enabled the testing of long-standing hypotheses in the anthelmintic resistance field. For example, genome-editing technology enabled the functional connection between ben-1 beta-tubulin variation and resistance in a controlled genetic background without overexpression using extrachromosomal arrays or incomplete knockdown using RNAi [20–22].

It has long been observed in parasite populations that once resistance is established, it does not disappear when the drug selective pressure is removed [2,121,122]. This phenomenon has led to the hypothesis that resistance alleles do not cause a loss of fitness in natural settings. These hypotheses about specific effects on fitness can be answered using precise genome editing in C. elegans. Confirmation that the previously described beta-tubulin mutations F167Y, E198A, and F200Y confer resistance without affecting fitness required the creation of single nucleotide variant strains and then linking these mutations to resistance and fitness effects. After editing in the laboratory reference strain N2, the F167Y, E198A, and F200Y mutations all conferred resistance without causing a loss in fitness [20]. Recently, the candidate alleles E198L and E198V were identified in the parasites Teladorsagia circumcincta and Trichostrongylus axei (Avramenko et al. 2019). The E198V allele was only found with
the E198L allele but at much lower allele frequency, suggesting that the E198V allele causes a fitness detriment. Genome-edited *C. elegans* were used to show that the E198V allele conferred resistance but also a significant fitness cost (Dilks et al. 2020). This interplay between hypotheses generated from studies of *H. contortus* and experimental testing in *C. elegans* is an excellent example of the cycle of discovery that has been developed over the last 30 years.

Genome editing can be used for the replacement of *C. elegans* genes with the orthologous parasite genes. The edited strains enable testing of the effects of novel gene classes on anthelmintic responses [24]. This replacement strategy could help identify and study resistance genes in drug classes that have proven difficult to translate between *C. elegans* and parasites (e.g. MLs). Additionally, genome-edited *C. elegans* can be used to create models that more accurately reflect the parasite of interest. One potential example is the beta-tubulin complements of *C. elegans* as compared to *H. contortus*. *C. elegans* has six beta-tubulin genes, but *H. contortus* only has four [123]. This difference in beta-tubulin complements could be important in understanding resistance to BZs because fewer beta-tubulin genes reduces redundancy and increases potential detrimental fitness effects. With genome editing, it is possible to make *C. elegans* more similar to *H. contortus* in its beta-tubulin complement and measure the impacts of these changes on resistance and fitness. This same experimental setup could be applied to a number of gene families and drug classes such as the *pgp* and GluCl subunits with MLs, nAChRs with nAChR agonist drugs, and the *acr* family of receptor genes with AADs.

**The interplay of mode of action and resistance**

To prioritize candidate genes in the study of anthelmintic resistance, it is essential to understand the drug mode of action. Thus far, investigations of parasitic nematode anthelmintic resistance have only been possible using *C. elegans* results and comparisons. However, the predictive value of *C. elegans* resistance genes has been contentious because *C. elegans* and parasitic nematodes differ in
responses to some anthelmintic drugs [124]. We outline in this section that the genetics of drug resistance and fitness are balanced such that the usefulness of *C. elegans* as a model of parasitic nematodes can be determined by these two parameters.

When the mechanism of resistance is the loss of the anthelmintic drug target function, the fitness of *C. elegans* (e.g. growth rate and/or reproductive potential) must not be affected by this change in the drug target to be relevant to parasitic nematode anthelmintic resistance. Resistance to the BZ and AAD anthelmintic drug classes offer the best examples of this interplay between drug resistance and fitness. For BZ and AAD anthelmintic drugs, the loss of the drug targets *ben-1* or *acr-23*, respectively, cause resistance but do not cause any detrimental fitness effects [20,21,26,30]. The lack of measurable fitness costs is likely caused by genetic redundancy. Evolutionary selection for loss of a gene can occur easily if the selective force is an anthelmintic drug. We hypothesize that the direct translation of these *C. elegans* results to parasitic nematode anthelmintic resistance comes from the fact that loss of the drug target does not cause a fitness detriment in parasitic nematodes.

The applicability of *C. elegans* results to parasitic nematode anthelmintic resistance for ML and nAChR agonists has been brought into question. As outlined above, *C. elegans* has been critical to understand mode of action for these drug classes but resistance has not translated from *C. elegans* to parasitic nematodes. This perceived failure is likely because the targets of these anthelmintic drug classes are required for nematode fitness. For example, loss of the genes that encode subunits of the nAChR cause levamisole resistance in *C. elegans* but they also cause movement disorders (uncoordinated phenotypes) that drastically affect organismal fitness, especially in the wild. If these mutations occurred in parasitic nematode species exposed to nAChR agonists, then these nematodes might be resistant to the drug but would not be able to migrate and/or colonize hosts, mate, or feed properly. Anthelmintic resistance in the field could not act through the targets of these drugs unless the fitness effects were mitigated by *suppressor mutations*. For these drug classes, the mode of action and field resistance mechanisms in parasites are separate. *C. elegans* might still be informative for the
discovery of resistance genes beyond the drug targets. Importantly, wild *C. elegans* strains with natural loss of the anthelmintic target could provide opportunities to look beyond the genes directly involved in the drug mode of action, like xenobiotic and drug metabolism genes.

**Concluding remarks**

Discoveries of anthelmintic drug modes of action and resistance have been facilitated by studies using comparative results from *C. elegans* and *H. contortus* experiments. However, these nematode species are highly related to each other at the genetic level. To discover anthelmintic drug modes of action and resistance that are generalizable across diverse nematode species, we should consider expanding the number of species included in the cycle of discovery. Nematodes are phylogenetically classified into five clades [125]. *C. elegans* is a clade V nematode, alongside several helminths of veterinary and human importance, including *H. contortus*, so *C. elegans* is ideally suited for comparative anthelmintic research in this clade. Importantly, mechanisms discovered in *C. elegans* and *H. contortus* are likely to be translatable to human helminths, providing the only way to discover the means to mitigate both the development of resistance and its spread. However, any conclusions from this clade might not be generalizable to nematodes in the other clades that also include devastating human and veterinary parasites.

We propose the development of multiple parallel cycles of discovery within each nematode clade and then comparative approaches across all of these cycles of discovery. For example, the structure of the existing cycle with one free-living nematode and one parasitic nematode (*C. elegans* and *H. contortus*) can be replicated in clade IV, which includes the free-living genus *Panagrellus* and the parasitic genus *Strongyloides*. In clade III where parasites such as *Brugia malayi*, *Onchocerca volvulus*, *Dirofilaria immitis*, *Loa loa*, and several *Ascaris* species are found, no free-living nematodes are known. In this case, free-living nematodes basal to clade III could be studied (*e.g.* Chromadorida). In all of these new cycles of discovery within each clade, genomic resources (*i.e.* chromosome-level genome
assemblies, population-wide sequences), high-throughput assays to measure anthelmintic responses, and genome-editing tools must be developed. Comparative approaches across all of the separate clades could enable discovery of general principles of anthelmintic drug modes of action and resistance that have been conserved since the most recent common ancestor of nematodes. Progress in the field of anthelmintic resistance can be amplified by the active interplay of results gleaned from studies comparing the genes and mechanisms of resistance in free-living and parasitic nematode species.

**Highlights**

- The free-living nematode *Caenorhabditis elegans* has long been the model nematode to understand anthelmintic resistance and modes of action in parasitic nematodes.
- The small ruminant parasite *Haemonchus contortus* brings several advantages to the understanding of anthelmintic drug responses.
- Anthelmintic research should use the combined strengths of both systems (*C. elegans* and *H. contortus*) in a cycle of discovery.

**Outstanding questions**

- How valuable will it be to incorporate anthelmintic resistance data from other free-living nematodes?
- Which helminth species, other than *H. contortus*, can be developed into a suitable model system?
- How much do the shared environments of free-living and parasitic nematode species influence shared anthelmintic drug modes of action and resistance?
- Does convergent evolution of anthelmintic resistance occur across nematode clades or the phylum?
- How much of the putative clade-specific anthelmintic resistance can be explained by evolution of gene families?

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**Box 1. One lucky *C. elegans* strain**

Research using *C. elegans* has contributed numerous discoveries of many basic biological principles over the past fifty years [126]. Most of these studies used the laboratory-adapted N2 reference strain and nearly all advances come from that single genetic background [19]. These studies could have had drastically different outcomes if another strain had been selected as the laboratory strain. The N2 strain was isolated in 1951 from Bristol, England, but it was not cryopreserved until 1969.
In the 18 years between isolation from the field and cryopreservation, genetic variation accumulated as the strain adapted to different laboratory environments, which makes the N2 strain different from typical wild C. elegans strains [68]. For this reason, one strain does not represent the full diversity found in this species.

Progress in parasitic nematode BZ resistance was enabled by the lucky coincidence that a BZ sensitive strain was chosen as the laboratory reference strain. Approximately 15% of all wild C. elegans sampled to date harbor variation in the ben-1 beta-tubulin gene that is predicted to cause resistance [21]. If one of these resistant strains were chosen as the laboratory reference, it would have been more difficult to identify ben-1 and to understand the BZ mode of action. Amazingly, anthelmintic resistance researchers got lucky twice. Genetic selection experiments in Aspergillus nidulans also serendipitously chose a laboratory strain that harbors susceptible alleles in beta-tubulin genes [127]. To minimize bias in future anthelmintic resistance studies, C. elegans researchers should measure anthelmintic resistance across a genetically diverse panel of wild strains because any one strain could harbor natural resistance alleles. This approach will greatly improve the chances to discover novel anthelmintic modes of action and/or resistance mechanisms.

Glossary

**Contig**: A set of overlapping DNA sequences that together represent a consensus region of the genome. When the whole genome is fully assembled, one contig is equivalent to one chromosome.

**Gene model**: A representation of mRNA for a protein-coding gene. It contains UTRs, exons, introns, and splice sites. If a gene encodes multiple mRNAs using alternative splicing, it will have multiple gene models.

**Genome-wide association studies**: A test of correlation between genotype and phenotype for a set of wild strains

**Linkage mapping**: A test of correlation between genotype and phenotype for a set of inbred lines that are derived from a cross of two individuals

**Mutagenesis**: The process of generating mutations

**Ortholog**: A gene for which homologous sequences that originated from the same common ancestor are present in different species.

**Standing variation**: Population-wide differences in genomes

**Suppressor mutation**: A second mutation that counteracts the phenotypic effects of another mutation.

**Variants**: Changes in genomic sequences as compared to the reference genome
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