



Review

Complementary Approaches with Free-living and Parasitic Nematodes to Understanding Anthelmintic Resistance

Janneke Wit ^{1,3} Clayton M. Dilks ^{1,2,3} and Erik C. Andersen ^{1,*}

Anthelmintic drugs are the major line of defense against parasitic nematode infections, but the arsenal is limited and resistance threatens sustained efficacy of the available drugs. Discoveries of the modes of action of these drugs and mechanisms of resistance have predominantly come from studies of a related nonparasitic nematode species, *Caenorhabditis elegans*, and the parasitic nematode *Haemonchus contortus*. Here, we discuss how our understanding of anthelmintic resistance and modes of action came from the interplay of results from each of these species. We argue that this 'cycle of discovery', where results from one species inform the design of experiments in the other, can use the complementary strengths of both to understand anthelmintic modes of action and mechanisms of resistance.

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 (nAChR) agonists, and amino-acetonitrile derivatives (AADs)] make up the primary defense against these parasites [1,2]. BZs 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, the prevalence of veterinary parasitic nematodes increased at an alarming rate [1,4–6]. Resistance to MLs [7–9], nAChR agonists [10,11], and AADs [12] was also quickly documented after initial use. The economic impacts of resistance in agricultural sectors around the world are massive with global losses projected in the billions of dollars per year [1,13]. In contrast to veterinary health, resistance in human parasites is not widely reported with only a few confirmed cases [14–16]. However, the selective pressures placed on human parasites by mass drug administration programs will give rise to increasing levels of resistance [17]. With the emergence of resistant human parasites and the increasing prevalence 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 mechanisms of resistance and modes of action for available anthelmintics to ensure long-term maintenance of efficacy.

***C. elegans* and *H. contortus* Create a Cycle of Discovery**

Most studies of anthelmintic modes of action and mechanisms of resistance have focused on one species at a time, including not only parasitic nematodes but also the free-living nematode *C. elegans* [18]. *C. elegans* offers many advantages, compared to parasitic nematodes, for studying the mode of action and resistance, including a short life cycle, easy laboratory maintenance and phenotyping, a high-quality reference genome, and CRISPR-Cas9 genome editing [19]. However, because *C. elegans* is not a parasite species, it lacks behaviors and processes

Highlights

The free-living nematode *Caenorhabditis elegans* has long been the model nematode to understand modes of action and anthelmintic resistance in parasitic nematodes.

The small ruminant parasite *Haemonchus contortus* is emerging as the premier parasitic nematode model to understand anthelmintic drug responses.

Results from experiments using *C. elegans* and *H. contortus* can combine to make a 'cycle of discovery' where strengths of both systems can advance anthelmintic research more than either alone.

¹Molecular Biosciences, Northwestern University, Evanston, IL 60208, USA

²Interdisciplinary Biological Sciences Program, Northwestern University, Evanston, IL 60208, USA

³These authors contributed equally to this manuscript

*Correspondence: erik.andersen@northwestern.edu (E.C. Andersen).

essential for parasitic life cycles, including host–parasite interactions. Parasitic nematodes are difficult to study empirically as a result of their requirements for hosts along with poorly developed molecular and genetic toolkits [19]. The veterinary helminth *H. contortus* is emerging as a model parasite species because of its high prevalence of resistance, a high-quality reference genome, and genetic crosses [1,20–23]. The use of both *C. elegans* and *H. contortus* provides an ideal complementary approach to study resistance against a wide range of anthelmintics. Candidate resistance alleles can be identified in either species, causally linked to resistance in *C. elegans*, and tested for relevance in parasitic anthelmintic resistance in *H. contortus*. This approach creates a cycle of discovery that has been critical in the advances in anthelmintic modes of action and mechanisms of resistance (Figure 1). Here, we describe how complementary but independent approaches have been productive and why purposefully combining results from the two species in future studies is a powerful route to discovery.

Research on BZs and AADs Shows the Power of the Cycle of Discovery

The combination of results and iterative approaches using *C. elegans* and *H. contortus* defined the mode of action and resistance mechanism for the BZ drug class. This cycle of discovery shows the power of this complementary approach. Early work on *H. contortus* tubulin extracts linked the drug to its target *in vitro*, suggesting that β -tubulin from resistant isolates might have reduced affinity for many BZs [24,25]. Following this discovery in *H. contortus*, researchers using *C. elegans* were able to identify the genetic target of BZs, *ben-1* [26], a nematode-specific β -tubulin gene. After the *ben-1* discovery in *C. elegans*, orthologous β -tubulin genes and amino acid substitutions that were correlated with BZ resistance were found in *H. contortus* [27,28]. Researchers were able to test a putative *H. contortus* resistance allele by heterologous expression in *C. elegans* [29]. In recent years, this cycle was further exploited to identify additional alleles in parasite species using population-based amplicon sequencing (e.g., nemabiome [30,31]) and confirmed that resistance is conferred by these alleles using CRISPR-Cas9 genome-editing technology in *C. elegans* [32,33].

The development of monepantel, an AAD anthelmintic, is another example of how experimental approaches using both *C. elegans* and *H. contortus* in tandem can elucidate mode of action and resistance mechanisms faster than experiments using either species alone. A selection experiment in *C. elegans* was used to identify monepantel-resistant mutants, and the resistance phenotype was mapped to *acr-23*, an alpha nAChR subunit gene [34]. Subsequent analysis of monepantel-resistant *H. contortus* populations in the same study identified mutations in the *acr-23* ortholog (see Glossary) *Hc-acr-23H* and *Hc-des-2H* as putative resistance loci [34]. A

Glossary

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.

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

Quantitative trait locus (QTL): a region in the genome containing genetic variation that is correlated with phenotypic variation.

Standing variation: population-wide differences in genomes.

Variants: changes in genomic sequences as compared to the reference genome.

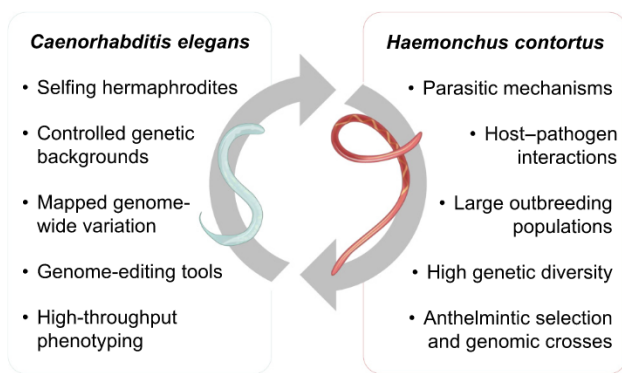


Figure 1. The *Caenorhabditis elegans* – *Haemonchus contortus* Cycle of Discovery. Advantages of *C. elegans* and *H. contortus* are listed on the left and right, respectively. The pairing of a free-living and parasitic nematode species enables experimental results from each nematode species to inform and drive new experiments in the other species.

Trends in Parasitology



subsequent *C. elegans* study found another gene, *acr-20*, that also encodes an alpha nAChR subunit, involved in AAD resistance [35,36]. The combined results between *C. elegans* and *H. contortus* established the nAChR genes as candidate genes responsible for the AAD mode of action and resistance in parasite populations. The explicit use of the cycle of discovery in the development of monepantel enabled the quick identification and testing of potential drug targets in both species.

Studies of ML and nAChR Agonists Require a More Integrated Cycle of Discovery

Although BZ and AAD research has shown the power of the cycle of discovery, ML and nAChR research could benefit from closer integration of results from the two species to enable a cycle of discovery. ML resistance research in *C. elegans* identified glutamate-gated chloride channel genes, such as *glc-1*, *avr-14*, and *avr-15*, as potential targets of ML drugs [37–40]. Like the previously described BZ experiments, heterologous expression of *H. contortus* GluCl genes can modify *C. elegans* responses to ivermectin (IVM) [41]. The *H. contortus* *avr-14* ortholog *Hco-avr-14b* expressed in the *C. elegans* IVM-resistant mutant was able to reintroduce sensitivity [41]. However, mutations in GluCl channel genes have not been identified in IVM-resistant parasite species and further studies in parasites have suggested that variation in GluCl receptors does not underlie resistance to MLs in parasite populations [22,42,43]. To study the genetics of ML resistance in *H. contortus*, a backcross approach using two independent resistant isolates and the same susceptible isolate identified genomic intervals involved in IVM resistance [21,22]. Investigators looked for correlations of individual variants or segregating haplotypes with resistance and found an interval on chromosome V using microsatellite markers [44]. In a genome-wide approach, the same **quantitative trait locus (QTL)** on chromosome V was found to be correlated with IVM resistance [22]. However, neither of these mapping approaches implicated orthologs of the previously studied candidate *C. elegans* genes, which could suggest parasite-specific mechanisms of resistance. To more closely integrate results from *C. elegans* and *H. contortus*, researchers can test candidate IVM resistance genes identified in *H. contortus* genetic crosses and also use conserved natural IVM resistance loci from *C. elegans* (discussed below).

Early nAChR research focused on selection experiments in *C. elegans* to identify resistance loci, such as *lev-1*, *unc-29*, and *unc-38* [45]. Subsequent resistance screens and selections identified additional loci essential for nAChR function, including *unc-63*, *lev-8*, *ric-3*, *unc-50*, and *unc-74* [45–47]. Following the discovery of resistance loci in *C. elegans*, it was shown that resistant parasite isolates had reduced binding of nAChR agonists to the receptor [48]. Recent studies of *H. contortus* found three main mechanisms of resistance, and each mechanism caused decreased responsiveness of the nAChR to levamisole: (i) reduced transcription of the nAChR subunit genes *Hco-unc-63*, *Hco-unc-29*, and *Hco-acr-8a* [49–54]; (ii) truncated forms of the *Hco-unc-63* and *Hco-acr-8* subunit genes [53,55,56]; or (iii) reduced expression of the genes *Hco-unc-74*, *Hco-unc-50*, and *Hco-ric-3* needed in the process of forming the nAChR [56]. Although all of these mechanisms reduce the number of functional levamisole receptors, none are correlated consistently with resistance [52,53], and causal follow-up studies are essential to understand the mechanisms of resistance. Thus far, the cycle of discovery in ML and nAChR agonist research has not led to proven resistance genes in parasitic nematodes. However, advances in technology and resources for both *C. elegans* and *H. contortus* will enhance the cycle of discovery.

Improvements to the *C. elegans*–*H. contortus* Cycle of Discovery

The successes of the *C. elegans* and *H. contortus* cycle of discovery have come from complementary approaches that mitigate disadvantages from studies in any single species. Despite these successes, both species have additional opportunities to improve the cycle of discovery

by alterations to experimental processes and available techniques. In the following section, we delineate how the cycle of discovery can be improved by adjustments to techniques and interpretations of results in both nematode species.

Incorporation of *C. elegans* Natural Diversity Enables Studies beyond a Single Genetic Background

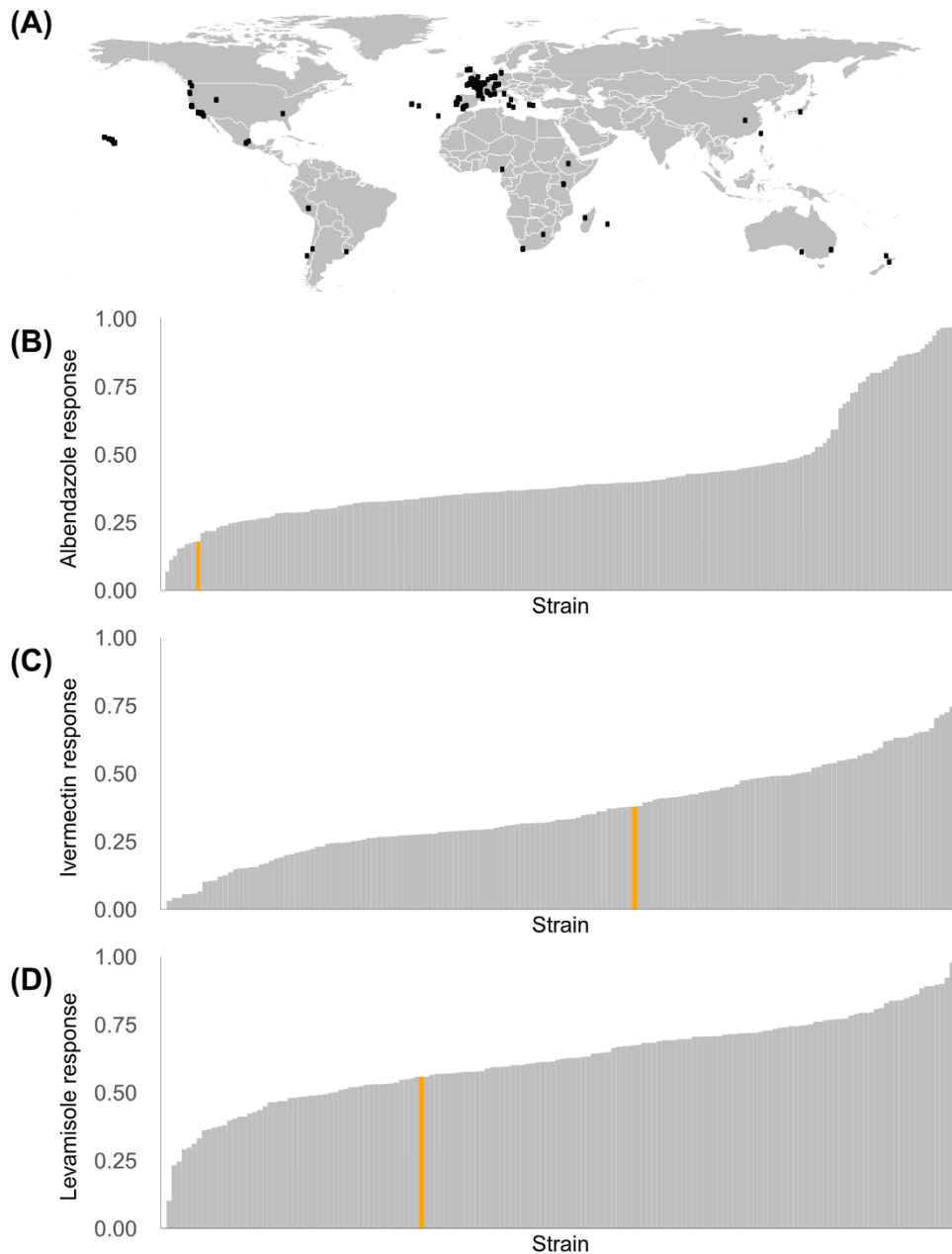
In nature, *C. elegans* is found in complex niches filled with bacteria, fungi, and other nematodes competing for resources. Most of the anthelmintic drugs used today are derivatives of natural compounds produced by bacteria and fungi [57–59]. Many parasitic nematode species spend some part of their 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* [60,61]. For this reason, parasitic nematodes and *C. elegans* have likely evolved shared or similar resistance mechanisms to survive the effects of natural anthelmintic compounds [33]. As described above, *C. elegans* genetic selection approaches have been used to identify 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 genetic diversity present across the entire species (Box 1) [18]. Wild isolates of *C. elegans* can facilitate studies of how natural diversity affects the responses to anthelmintics, which could recapitulate responses found in natural parasitic nematode populations. *C. elegans* is sampled from diverse locations worldwide (Figure 2A), and these strains are readily available from the *C. elegans* Natural Diversity Resource (CeNDR) [62]. Quantitative genetic studies correlate phenotypic differences across a set of individuals with genetic differences across the same set of individuals [63]. Genomic regions that harbor correlated genetic differences are called **QTL**. Such loci can be narrowed further to single genes and ultimately specific **variants** that underlie quantitative traits [64,65]. This approach can be applied to anthelmintic drug responses to discover modes of action and resistance mechanisms [33].

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 were correlated with resistance [33]. In addition to these single amino-acid substitutions, structural variants, including deletions, insertions, transposons, and inversions, were found in some individuals across the population. These sites represent additional alleles that could cause BZ resistance in parasite populations. Nearly 15% of *C. elegans* wild strains harbor variation in the

Box 1. One Lucky *C. elegans* Strain

Research using *C. elegans* has contributed numerous discoveries of many basic biological principles over the past 50 years [88]. Most of these studies used the laboratory-adapted N2 reference strain, and nearly all advances come from that single genetic background [18]. 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 [18]. 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 [62]. 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* β -tubulin gene that is predicted to cause resistance [33]. 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 β -tubulin genes [89]. 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.



Trends in Parasitology

Figure 2. Worldwide Collection of Approximately 249 Natural *Caenorhabditis 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 (animal length normalized from zero to one, with zero representing the most affected strain and one representing the least affected strain) is shown on the y-axis, and strains are ordered by their response on the x-axis from sensitive to resistant with the N2 strain phenotype shown in orange: (B) albendazole, (C) ivermectin, and (D) levamisole. All data in this figure were collected previously [33].

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)** to identify naturally occurring

alleles that can modify anthelmintic responses. Using this powerful strategy, the researchers identified a genomic interval on the X chromosome that underlies differences in BZ responses independent of the β -tubulin gene, *ben-1* [33]. 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.

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 ABZ [33] showed varied responses to ivermectin and levamisole (Figure 2C,D). The distributions of these ML and nAChR agonist responses across the wild population indicate strains that can be used in future anthelmintic studies. 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 [37,40]. 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 [66]. 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 [66], 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 [66]. 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 [66]. 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* and provide excellent avenues of research into novel resistance loci. Using publicly available data from CeNDR [62], 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.



Known targets of other anthelmintic classes 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 [62]. Previous work has demonstrated that several loss-of-function *acr-23* mutations cause resistance to monepantel [67], but the wild isolates could tell us how parasitic 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 [32,33]. Use of naturally occurring alleles provides insights into the diversity that is present in nature and amplifies the cycle of discovery to go beyond a single *C. elegans* genetic background.

Parasitize *C. elegans* to Understand Anthelmintic Resistance

As we discussed earlier, genetic selections using *C. elegans* have identified modes of action and resistance mechanisms for anthelmintics [26,34,37,47]. Most of these studies used mutagenized individuals or extrachromosomal arrays to express parasite genes [29,68]. In recent years, the CRISPR-Cas9 system has made genome editing not only possible but a relatively straightforward process [69]. 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* β -tubulin variation and resistance in a controlled genetic background without overexpression using extrachromosomal arrays or incomplete knockdown using RNAi [32,33,70].

Genome editing can be used for the replacement of *C. elegans* genes with the orthologous parasite genes [19]. This replacement strategy could help to 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 number of β -tubulin genes of *C. elegans* as compared to *H. contortus*. *C. elegans* has six β -tubulin genes, but *H. contortus* has only four [71]. This difference in β -tubulin number could be important for understanding resistance to BZs because fewer β -tubulin genes limits redundancy and increases potential detrimental fitness effects. With genome editing, it is possible to make *C. elegans* more similar to *H. contortus* in its β -tubulin content 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 p-glycoprotein and GluCl subunit genes with MLs, nAChRs with nAChR agonist drugs, and the *acr* family of receptor genes with AADs. One potential scenario where this strategy would fall short is when no direct ortholog is present between the species. However, this may be overcome by technologies allowing for the insertion of whole genes, such as MosCI and large-scale CRISPR integrations/insertions.

Genome editing can be performed in any strain in the CeNDR, which opens a large number of genetic contexts to test hypotheses. Recent work has shown that vast regions of genetic diversity exist in natural *C. elegans* populations, including punctuated regions of extreme genetic diversity [72]. This diversity includes gene content and number variation along with large structural variants. Strains with such extreme genetic diversity can be mined to identify the best or most translatable context relative to parasitic nematode genomes to test hypotheses related to diverse gene families.

Exploiting Genetic Background Effects on *H. contortus* Studies to Improve the Cycle of Discovery

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 can reveal these resistance variants. However, helminth populations harbor extremely large numbers of variants because of their large outbreeding populations and predicted high mutation rates [73–75]. 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 [21,22,31], because genetic diversity should be focused in genomic regions that differentiate the two genotyped samples.

Take Advantage of the Newly Improved *H. contortus* Genome

Incomplete parasitic nematode reference genomes hamper the identification of important regions underlying responses to anthelmintics. However, in recent years the *H. contortus* genome has been vastly improved [76,77]. Now that the genome has reached a high level of completion, *H. contortus* genome-wide experiments have become much more accurate and informative. Recent studies have identified new regions of the genome that underlie the ivermectin response [22,44]. Although *C. elegans* candidate genes were not present in the identified regions, these findings fit into the cycle of discovery. These regions associated with resistance contain many new genes that can be investigated using genome editing of *C. elegans* and in comparison to natural diversity in *C. elegans*. However, orthologs between the two species need to be defined, and *H. contortus* **gene models** need to be improved to make these searches more precise. For the cycle of discovery to improve, *H. contortus* mapping experiments must continue to identify novel regions and candidate genes to investigate in *C. elegans*.

Genome Editing in *H. contortus*

Currently, genome editing in nematodes is mostly limited to *Caenorhabditis* species. Some work in genome editing in parasite species has been performed successfully in *Strongyloides* spp [78–84]. When these technologies are further optimized, and can be performed in *H. contortus*, the cycle of discovery can be greatly improved and expanded. First, resistance genes discovered in *C. elegans* can be tested in *H. contortus*. These loci could be important contributors to field resistance in parasites, especially loci identified using *C. elegans* natural variation. Second, candidate genes identified from *H. contortus* mappings can be tested for resistance in both *C. elegans* and *H. contortus*, enabling comparisons between the two species.

Concluding Remarks

In the earlier sections, we first highlighted how our understanding of modes of action and resistance for anthelmintics has been advanced significantly by integrating findings from both *C. elegans* and *H. contortus*. Then, we outlined some recommended improvements to this one cycle of discovery because both *C. elegans* and *H. contortus* offer distinct advantages that synergize when combined and circumvent disadvantages from each species taken individually. The interplay of approaches and results from both systems enables a cycle of discovery for

Outstanding Questions

Does convergent evolution of anthelmintic resistance occur across nematode clades or the phylum?

Which helminth species, other than *H. contortus*, can be developed into a suitable model system?

How valuable will it be to incorporate anthelmintic resistance data from other free-living nematode species?

How much do the shared environments of free-living and parasitic nematode species influence shared anthelmintic drug modes of action and resistance?

How much of the putative clade-specific anthelmintic resistance can be explained by evolution of gene families?



anthelmintic modes of action and resistance. In this section we describe how the cycles of discovery can be expanded (see Outstanding Questions).

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, the cycle of discovery can be expanded to include additional species. Nematodes are phylogenetically classified into five clades [85]. *C. elegans* and *H. contortus* are clade V nematodes, alongside several helminths of veterinary and human importance, so these two species are ideally suited for comparative anthelmintic research in this clade. Mechanisms discovered in *C. elegans* and *H. contortus* are likely to be translatable to human helminths, especially to the clade V hookworms *Ancylostoma duodenale* and *Necator americanus*, 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.

Within clade V, incorporation of anthelmintic resistance studies from other species could expand the existing cycle of discovery and broaden the applicability to other parasite species, especially hookworms. For example, the canine hookworm, *Ancylostoma caninum*, offers the opportunity to expand discovery of anthelmintic resistance mechanisms to hookworms. Canine hosts are often less expensive than sheep, and BZ- and ML-resistant isolates have already been identified [86]. Genetic crossing approaches similar to *H. contortus* can provide a hookworm model to map anthelmintic resistance. To make this species an accessible model, priority should be placed on the generation of a high-quality reference genome and establishment of genetic crosses. Additional species within the *Caenorhabditis* clade might also enable mapping of conserved resistance loci that can be traced deeper into clade V nematodes than *C. elegans*, as has been shown for BZ responses between *C. elegans* and *Caenorhabditis briggsae* [87]. These expansions to the existing cycle of discovery will facilitate future resistance studies in clade V nematodes.

To enable discoveries beyond the clade V nematodes, we propose the development of multiple parallel cycles within each nematode clade and then comparative approaches across all of these diverse cycles. For example, the structure of the existing cycle with one free-living nematode and one parasitic nematode 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.

Acknowledgments

The authors thank Samantha Peters for the nematode drawings. We also thank Katie Evans, Dan Lu, and Sam Widmeyer for comments on the manuscript. C.M.D. was supported by the Biotechnology Training Program (NIH T32 GM008449). This work was supported by the NIH R01AI53088 grant to E.C.A. E.C.A. is a Pew Scholar in the Biomedical Sciences, supported by the Pew Charitable Trusts (00027327).

References

- Kaplan, R.M. and Vidyashankar, A.N. (2012) An inconvenient truth: global worming and anthelmintic resistance. *Vet. Parasitol.* 186, 70–78
- Kaplan, R.M. (2004) Drug resistance in nematodes of veterinary importance: a status report. *Trends Parasitol.* 20, 477–481
- Theodorides, V.J. *et al.* (1970) Strains of *Haemonchus contortus* resistant against benzimidazole anthelmintics. *Am. J. Vet. Res.* 31, 859–863
- Howell, S.B. *et al.* (2008) Prevalence of anthelmintic resistance on sheep and goat farms in the southeastern United States. *J. Am. Vet. Med. Assoc.* 233, 1913–1919
- Lamb, J. *et al.* (2017) Broad spectrum anthelmintic resistance of *Haemonchus contortus* in Northern NSW of Australia. *Vet. Parasitol.* 241, 48–51
- Wilson, D. and Sargison, N. (2007) Anthelmintic resistance in *Teladorsagia circumcincta* in sheep in the UK. *Vet. Rec.* 161, 535–536
- Wolstenholme, A.J. *et al.* (2015) The emergence of macrocyclic lactone resistance in the canine heartworm, *Dirofilaria immitis*. *Parasitology* 142, 1249–1259
- Ballesteros, C. *et al.* (2018) Clinical validation of molecular markers of macrocyclic lactone resistance in *Dirofilaria immitis*. *Int. J. Parasitol. Drugs Drug Resist.* 8, 596–606
- Lawrence, K.E. *et al.* (2006) Farm management practices associated with macrocyclic lactone resistance on sheep farms in New Zealand. *N. Z. Vet. J.* 54, 283–288
- Liu, Z. *et al.* (2005) A nicotinic acetylcholine receptor mutation conferring target-site resistance to imidacloprid in *Nilaparvata lugens* (brown planthopper). *Proc. Natl. Acad. Sci. U. S. A.* 102, 8420–8425
- Kopp, S.R. *et al.* (2009) Acetylcholine receptor subunit genes from *Ancylostoma caninum*: altered transcription patterns associated with pyrantel resistance. *Int. J. Parasitol.* 39, 435–441
- Van den Brom, R. *et al.* (2015) *Haemonchus contortus* resistance to monepantel in sheep. *Vet. Parasitol.* 209, 278–280
- Charlier, J. *et al.* (2009) Gastrointestinal nematode infections in adult dairy cattle: Impact on production, diagnosis and control. *Vet. Parasitol.* 164, 70–79
- Furtado, L.F.V. *et al.* (2019) First identification of the benzimidazole resistance-associated F200Y SNP in the beta-tubulin gene in *Ascaris lumbricoides*. *PLoS One* 14, e0224108
- Orr, A.R. *et al.* (2019) Genetic markers of benzimidazole resistance among human hookworms (*Necator americanus*) in Kintampo North Municipality, Ghana. *Am. J. Trop. Med. Hyg.* 100, 351–356
- Krücken, J. *et al.* (2017) Reduced efficacy of albendazole against *Ascaris lumbricoides* in Rwandan schoolchildren. *Int. J. Parasitol. Drugs Drug Resist.* 7, 262–271
- Ásbjörnsdóttir, K.H. *et al.* (2017) Prospects for elimination of soil-transmitted helminths. *Curr. Opin. Infect. Dis.* 30, 482–488
- Sterken, M.G. *et al.* (2015) The laboratory domestication of *Caenorhabditis elegans*. *Trends Genet.* 31, 224–231
- Zamanian, M. and Andersen, E.C. (2016) Prospects and challenges of CRISPR/Cas genome editing for the study and control of neglected vector-borne nematode diseases. *FEBS J.* 283, 3204–3221
- Sallé, G. *et al.* (2019) The global diversity of *Haemonchus contortus* is shaped by human intervention and climate. *Nat. Commun.* 10, 4811
- Redman, E. *et al.* (2012) Introgression of ivermectin resistance genes into a susceptible *Haemonchus contortus* strain by multiple backcrossing. *PLoS Pathog.* 8, e1002534
- Doyle, S.R. *et al.* (2019) Population genomic and evolutionary modelling analyses reveal a single major QTL for ivermectin drug resistance in the pathogenic nematode, *Haemonchus contortus*. *BMC Genomics* 20, 218
- Hunt, P.W. *et al.* (2010) The use of DNA markers to map anthelmintic resistance loci in an intraspecific cross of *Haemonchus contortus*. *Parasitology* 137, 705–717
- Lacey, E. and Prichard, R.K. (1986) Interactions of benzimidazoles (BZ) with tubulin from BZ-sensitive and BZ-resistant isolates of *Haemonchus contortus*. *Mol. Biochem. Parasitol.* 19, 171–181
- Lubega, G. and Prichard, R. (1990) Specific interaction of benzimidazole anthelmintics with tubulin: high-affinity binding and benzimidazole resistance in *Haemonchus contortus*. *Mol. Biochem. Parasitol.* 38, 221–232
- Driscoll, M. *et al.* (1989) Genetic and molecular analysis of a *Caenorhabditis elegans* beta-tubulin that conveys benzimidazole sensitivity. *J. Cell Biol.* 109, 2993–3003
- Kwa, M.S.G. *et al.* (1993) Molecular characterisation of β -tubulin genes present in benzimidazole-resistant populations of *Haemonchus contortus*. *Mol. Biochem. Parasitol.* 60, 133–143
- Kwa, M.S.G. *et al.* (1994) Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in β -tubulin isotype 1. *Mol. Biochem. Parasitol.* 63, 299–303
- Kwa, M.S. *et al.* (1995) Beta-tubulin genes from the parasitic nematode *Haemonchus contortus* modulate drug resistance in *Caenorhabditis elegans*. *J. Mol. Biol.* 246, 500–510
- Avramenko, R.W. *et al.* (2015) Exploring the gastrointestinal 'nemabiome': deep amplicon sequencing to quantify the species composition of parasitic nematode communities. *PLoS One* 10, e0143559
- Avramenko, R.W. *et al.* (2017) The use of nemabiome metabarcoding to explore gastro-intestinal nematode species diversity and anthelmintic treatment effectiveness in beef calves. *Int. J. Parasitol.* 47, 893–902
- Dilks, C.M. *et al.* (2020) Quantitative benzimidazole resistance and fitness effects of parasitic nematode beta-tubulin alleles. *Int. J. Parasitol. Drugs Drug Res* 14, 28–36
- Hahnel, S.R. *et al.* (2018) Extreme allelic heterogeneity at a *Caenorhabditis elegans* beta-tubulin locus explains natural resistance to benzimidazoles. *PLoS Pathog.* 14, e1007226
- Kaminsky, R. *et al.* (2008) A new class of anthelmintics effective against drug-resistant nematodes. *Nature* 452, 176–180
- Rufener, L. *et al.* (2010) Phylogenomics of ligand-gated ion channels predicts monepantel effect. *PLoS Pathog.* 6, e1001091
- Baur, R. *et al.* (2015) Monepantel irreversibly binds to and opens *Haemonchus contortus* MPTL-1 and *Caenorhabditis elegans* ACR-20 receptors. *Mol. Pharmacol.* 87, 96–102
- Dent, J.A. *et al.* (2000) The genetics of ivermectin resistance in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U. S. A.* 97, 2674–2679
- Cully, D.F. *et al.* (1994) Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. *Nature* 371, 707–711
- Vassiliatis, D.K. *et al.* (1997) Genetic and biochemical evidence for a novel avermectin-sensitive chloride channel in *Caenorhabditis elegans*. Isolation and characterization. *J. Biol. Chem.* 272, 33167–33174
- Dent, J.A. *et al.* (1997) *avr-15* encodes a chloride channel subunit that mediates inhibitory glutamatergic neurotransmission and ivermectin sensitivity in *Caenorhabditis elegans*. *EMBO J.* 16, 5867–5879
- Glendinning, S.K. *et al.* (2011) Glutamate-gated chloride channels of *Haemonchus contortus* restore drug sensitivity to ivermectin resistant *Caenorhabditis elegans*. *PLoS One* 6, e22390
- Njue, A.I. *et al.* (2004) Mutations in the extracellular domains of glutamate-gated chloride channel $\alpha 3$ and β subunits from ivermectin-resistant *Cooperia oncophora* affect agonist sensitivity. *J. Neurochem.* 89, 1137–1147
- Laing, R. *et al.* (2016) Analysis of putative resistance gene loci in UK field populations of *Haemonchus contortus* after 6 years of macrocyclic lactone use. *Int. J. Parasitol.* 46, 621–630
- Rezansoff, A.M. *et al.* (2016) Evidence from two independent backcross experiments supports genetic linkage of microsatellite Hcms8a20, but not other candidate loci, to a major ivermectin resistance locus in *Haemonchus contortus*. *Int. J. Parasitol.* 46, 653–661
- Lewis, J.A. *et al.* (1980) The genetics of levamisole resistance in the nematode *Caenorhabditis elegans*. *Genetics* 95, 905–928
- Boulin, T. *et al.* (2008) Eight genes are required for functional reconstruction of the *Caenorhabditis elegans* levamisole-sensitive acetylcholine receptor. *Proc. Natl. Acad. Sci. U. S. A.* 105, 18590–18595
- Fleming, J.T. *et al.* (1997) *Caenorhabditis elegans* levamisole resistance *Geneslev-1*, *unc-29*, and *unc-38* encode functional

- nicotinic acetylcholine receptor subunits. *J. Neurosci.* 17, 5843–5857
48. Sangster, N.C. *et al.* (1998) Binding of [3H]m-aminolevamisole to receptors in levamisole-susceptible and -resistant *Haemonchus contortus*. *Int. J. Parasitol.* 28, 707–717
 49. Fauvin, A. *et al.* (2010) cDNA-AFLP analysis in levamisole-resistant *Haemonchus contortus* reveals alternative splicing in a nicotinic acetylcholine receptor subunit. *Mol. Biochem. Parasitol.* 170, 105–107
 50. Boulin, T. *et al.* (2011) Functional reconstitution of *Haemonchus contortus* acetylcholine receptors in *Xenopus* oocytes provides mechanistic insights into levamisole resistance. *Br. J. Pharmacol.* 164, 1421–1432
 51. Barrère, V. *et al.* (2014) Novel assay for the detection and monitoring of levamisole resistance in *Haemonchus contortus*. *Int. J. Parasitol.* 44, 235–241
 52. Neveu, C. *et al.* (2010) Genetic diversity of levamisole receptor subunits in parasitic nematode species and abbreviated transcripts associated with resistance. *Pharmacogenet. Genomics* 20, 414–425
 53. Williamson, S.M. *et al.* (2011) Candidate anthelmintic resistance-associated gene expression and sequence polymorphisms in a triple-resistant field isolate of *Haemonchus contortus*. *Mol. Biochem. Parasitol.* 180, 99–105
 54. Blanchard, A. *et al.* (2018) Deciphering the molecular determinants of cholinergic anthelmintic sensitivity in nematodes: When novel functional validation approaches highlight major differences between the model *Caenorhabditis elegans* and parasitic species. *PLoS Pathog.* 14, e1006996
 55. Sarai, R.S. *et al.* (2014) Drug-efflux and target-site gene expression patterns in *Haemonchus contortus* larvae able to survive increasing concentrations of levamisole in vitro. *Int. J. Parasitol. Drugs Drug Resist.* 4, 77–84
 56. Sarai, R.S. *et al.* (2013) Acetylcholine receptor subunit and P-glycoprotein transcription patterns in levamisole-susceptible and -resistant *Haemonchus contortus*. *Int. J. Parasitol. Drugs Drug Resist.* 3, 51–58
 57. Campbell, W.C. (2012) History of avermectin and ivermectin, with notes on the history of other macrocyclic lactone antiparasitic agents. *Curr. Pharm. Biotechnol.* 13, 853–865
 58. Alivisatos, S.G.A. *et al.* (1962) Imidazolytic processes VI. Enzymic formation of benzimidazole and 5,6-dimethylbenzimidazole containing dinucleotides. *Biochim. Biophys. Acta* 58, 209–217
 59. Campbell, W.C. (2005) Serendipity and new drugs for infectious disease. *ILAR J.* 46, 352–356
 60. Frézal, L. and Félix, M.-A. (2015) The natural history of model organisms: *C. elegans* outside the Petri dish. *eLife* 4, e05849
 61. Crombie, T.A. *et al.* (2019) Deep sampling of Hawaiian *Caenorhabditis elegans* reveals high genetic diversity and admixture with global populations. *eLife* 8, e50465
 62. Cook, D.E. *et al.* (2017) CeNDR, the *Caenorhabditis elegans* natural diversity resource. *Nucleic Acids Res.* 45, D650–D657
 63. Falconer, D.S. (1996) *Introduction to Quantitative Genetics*. Pearson Education India
 64. Brady, S.C. *et al.* (2019) A novel gene underlies bleomycin-response variation in *Caenorhabditis elegans*. *Genetics* 212, 1453–1468
 65. Zdralevec, S. *et al.* (2017) Natural variation in a single amino acid substitution underlies physiological responses to topoisomerase II poisons. *PLoS Genet.* 13, e1006891
 66. Ghosh, R. *et al.* (2012) Natural variation in a chloride channel subunit confers avermectin resistance in *C. elegans*. *Science* 335, 574–578
 67. Rufener, L. *et al.* (2013) *acr-23* encodes a monoperantel-sensitive channel in *Caenorhabditis elegans*. *PLoS Pathog.* 9, e1003524
 68. Janssen, I.J.I. *et al.* (2015) Transgenically expressed *Parascaris* P-glycoprotein-11 can modulate ivermectin susceptibility in *Caenorhabditis elegans*. *Int. J. Parasitol. Drugs Drug Resist.* 5, 44–47
 69. Frøkjær-Jensen, C. (2013) Exciting prospects for precise engineering of *Caenorhabditis elegans* genomes with CRISPR/Cas9. *Genetics* 195, 635–642
 70. Kitchen, S. *et al.* (2019) Isolation and characterization of a naturally occurring multidrug-resistant strain of the canine hookworm, *Ancylostoma caninum*. *Int. J. Parasitol.* 49, 397–406
 71. Saunders, G.I. *et al.* (2013) Characterization and comparative analysis of the complete *Haemonchus contortus* β -tubulin gene family and implications for benzimidazole resistance in strongylid nematodes. *Int. J. Parasitol.* 43, 465–475
 72. Lee, D. *et al.* (2020) Balancing selection maintains ancient genetic diversity in *C. elegans*. *bioRxiv* Published online July 25, 2020. <https://doi.org/10.1101/2020.07.23.218420>
 73. Blouin, M.S. (2000) Neutrality tests on mtDNA: unusual results from nematodes. *J. Hered.* 91, 156–158
 74. Gilleard, J.S. and Beech, R.N. (2007) Population genetics of anthelmintic resistance in parasitic nematodes. *Parasitology* 134, 1133–1147
 75. Gilleard, J.S. (2013) *Haemonchus contortus* as a paradigm and model to study anthelmintic drug resistance. *Parasitology* 140, 1506–1522
 76. Laing, R. *et al.* (2013) The genome and transcriptome of *Haemonchus contortus*, a key model parasite for drug and vaccine discovery. *Genome Biol.* 14, R88
 77. Doyle, S.R. *et al.* (2020) Genomic and transcriptomic variation defines the chromosome-scale assembly of *Haemonchus contortus*, a model gastrointestinal worm. *Commun. Biol.* 3, 656
 78. Castelletto, M.L. *et al.* (2020) Recent advances in functional genomics for parasitic nematodes of mammals. *J. Exp. Biol.* 223, jeb206482
 79. Gang, S.S. *et al.* (2020) Chemosensory mechanisms of host seeking and infectivity in skin-penetrating nematodes. *Proc. Natl. Acad. Sci. U. S. A.* 117, 17913–17923
 80. Jaleta, T.G. and Lok, J.B. (2019) Advances in the molecular and cellular biology of *Strongyloides* spp. *Curr. Trop. Med. Rep.* 6, 161–178
 81. Lok, J.B. (2019) CRISPR/Cas9 mutagenesis and expression of dominant mutant transgenes as functional genomic approaches in parasitic nematodes. *Front. Genet.* 10, 656
 82. Adams, S. *et al.* (2019) Liposome-based transfection enhances RNAi and CRISPR-mediated mutagenesis in non-model nematode systems. *Sci. Rep.* 9, 483
 83. Gang, S.S. *et al.* (2017) Targeted mutagenesis in a human-parasitic nematode. *PLoS Pathog.* 13, e1006675
 84. Ward, J.D. (2018) Spotlight on CRISPR in *Strongyloides* parasitic nematodes. *Trends Parasitol.* 34, 6–9
 85. Blaxter, M.L. *et al.* (1998) A molecular evolutionary framework for the phylum Nematoda. *Nature* 392, 71–75
 86. Jimenez Castro, P.D. *et al.* (2019) Multiple drug resistance in the canine hookworm *Ancylostoma caninum*: an emerging threat? *Parasit. Vectors* 12, 576
 87. Zamanian, M. *et al.* (2018) Discovery of genomic intervals that underlie nematode responses to benzimidazoles. *PLoS Negl. Trop. Dis.* 12, e0006368
 88. Meneely, P.M. *et al.* (2019) Working with worms: *Caenorhabditis elegans* as a model organism. *Curr. Protoc. Essent. Lab. Techn.* 19, 71
 89. Hastie, A.C. and Georgopoulos, S.G. (1971) Mutational resistance to fungitoxic benzimidazole derivatives in *Aspergillus nidulans*. *J. Gen. Microbiol.* 67, 371–373