Natural variation in *Caenorhabditis elegans* responses to the anthelmintic emodepside

Janneke Wit[a], Briana C. Rodriguez[a], and Erik. C. Andersen[a,‡]

[a]Molecular Biosciences, Northwestern University, Evanston, IL 60208

[‡]**Corresponding Author:**

Erik C. Andersen, Ph.D.

Department of Molecular Biosciences

Northwestern University

4619 Silverman Hall

2205 Tech Drive

Evanston, IL 60208

847-467-4382

erik.andersen@northwestern.edu

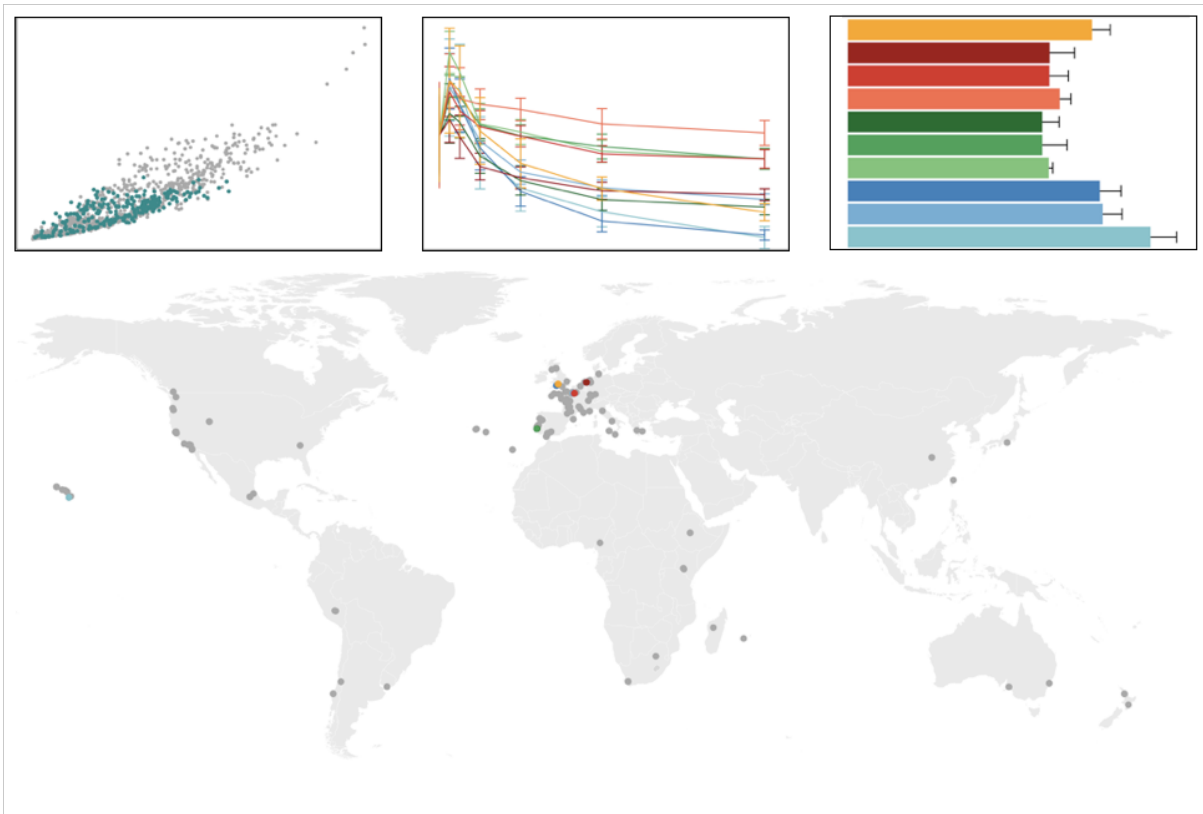
Janneke: 0000-0002-3116-744X
Briana: 0000-0002-5282-0815
Erik: 0000-0003-0229-9651

Journal: *IJP DDR*[1]

Keywords: Emodepside, natural variation, *C. elegans*, anthelmintics, hormetic effect

[1] Note: Supplementary data associated with this article.

Graphical abstract



Highlights (3-5, max 85 characters each):

- Emodepside responses vary across the *C. elegans* species.
- Wild strains of *C. elegans* model natural differences in parasite emodepside responses.
- Variation in the emodepside target *slo-1* and other loci correlate with resistance.
- Low doses of emodepside cause a hormetic effect on offspring production.

Abstract

Treatment of parasitic nematode infections depends primarily on the use of anthelmintics. However, this drug arsenal is limited, and resistance against most anthelmintics is widespread. Emodepside is a new anthelmintic drug effective against gastrointestinal and filarial nematodes. Nematodes that are resistant to other anthelmintic drug classes are susceptible to emodepside, indicating that the emodepside mode of action is distinct from previous anthelmintics. The laboratory-adapted *Caenorhabditis elegans* strain N2 is sensitive to emodepside, and genetic selection and *in vitro* experiments implicated *slo-1*, a BK potassium channel gene, in emodepside mode of action. In an effort to understand how natural populations will respond to emodepside, we measured brood sizes and developmental rates of wild *C. elegans* strains after exposure to the drug and found natural variation across the species. Some of the observed variation in *C. elegans* emodepside responses correlates with amino acid substitutions in *slo-1*, but genetic mechanisms other than *slo-1* coding variants likely underlie emodepside resistance in wild *C. elegans* strains. Additionally, the assayed strains have higher offspring production in low concentrations of emodepside (a hormetic effect), which could impact treatment strategies when parasites are underdosed. We find that natural variation affects emodepside sensitivity, supporting the suitability of *C. elegans* as a model system to study emodepside responses across natural nematode populations.

47 **1. Introduction**

48 Helminth infections are a major threat to animal and human health, and
49 control measures depend heavily on a small arsenal of anthelmintic drugs.
50 Resistance against most anthelmintic drug classes is widespread and documented
51 for several species (Kotze and Prichard, 2016; McKellar and Jackson, 2004). New
52 anthelmintics with a distinct mode of action can be used to treat populations resistant
53 to multiple anthelmintics, but the introduction of new compounds is rare (Epe and
54 Kaminsky, 2013). One of the newest anthelmintics, the cyclooctadepsipeptide
55 (COPD) emodepside, has been commercially available since 2007 (Epe and
56 Kaminsky, 2013). It is a semisynthetic derivative of a natural metabolite from the
57 fungus *Mycelia sterilia* (Harder and von Samson-Himmelstjerna, 2001). As a broad
58 spectrum anthelmintic, emodepside is efficacious against gastrointestinal nematodes
59 and filarial nematodes (Harder et al., 2003; Zahner et al., 2001) and is currently
60 approved for treatment of helminth infections of cats and dogs in combination with
61 praziquantel (Altreuther et al., 2005). Field resistance has not been reported since its
62 introduction (Prichard, 2017). Importantly, emodepside is effective against multi-drug
63 resistant parasitic nematode strains, including ivermectin- and levamisole-resistant
64 *Haemonchus contortus* (Harder et al., 2005; von Samson-Himmelstjerna et al.,
65 2005).

66 Responses to COPD have been studied in both parasitic nematodes and the
67 free-living nematode *Caenorhabditis elegans*. Initial *in vitro* studies using *Ascaris*
68 *suum* suggested that the COPD PF1022A, the parent compound in emodepside
69 synthesis (Jeschke et al., 2005), displaces GABAergic ligands from somatic muscle
70 preparations (Harder et al., 2005). However, later work comparing the effect of
71 GABA and emodepside on the rate of relaxation of contracted *A. suum* muscle

72 showed that emodepside does not act directly on the GABAergic pathway (Willson et
73 al., 2003; Willson J, Holden-Dye L, Harder A, Walker RJ, 2001). Another promising
74 lead was the identification of a putative target protein, HC110-R, from a *H. contortus*
75 cDNA library (Saeger et al., 2001). Alignment revealed HC110-R had 48% identity
76 and 76% similarity to the *C. elegans* latrophilin receptor LAT-1. Although predicted to
77 be a heptahelical transmembrane protein, the exact function of HC110-R is unknown
78 (Mühlfeld et al., 2009). Latrophilin is a G protein-coupled receptor in the secretin
79 receptor family and a Ca^{2+}-independent receptor of alpha-latrotoxin (Welz et al.,
80 2005). *C. elegans* larvae express *lat-1* in pharyngeal muscle, and adults express it in
81 both pharyngeal and non-pharyngeal neurons (Willson et al., 2004). In the laboratory
82 strain N2, emodepside inhibits pharyngeal pumping, egg-laying, as well as
83 locomotion (Bull et al., 2007). Putative null mutations in *lat-1* are less sensitive to
84 emodepside-induced inhibition of pharyngeal pumping, but locomotor activity is
85 inhibited (Guest et al., 2007; Willson et al., 2004). This inhibition of locomotion
86 suggests that emodepside affects additional pathways independent of *lat-1*.

87 A subsequent mutagenesis screen using *C. elegans* identified mutations in
88 the Ca^{2+}-activated K^+ channel (BK-channel) gene *slo-1* in nine emodepside resistant
89 mutants (Guest et al., 2007). These mutants were identified as highly resistant to
90 inhibition of both pharyngeal pumping and locomotor activity by emodepside. Gain-
91 of-function mutations in *slo-1* show decreased locomotion and pharyngeal pumping
92 similar to emodepside-treated nematodes (Davies et al., 2003), suggesting that
93 emodepside activates SLO-1 signaling. Additionally, a putative *slo-1* null allele, *slo-*
94 *1(js379)*, responded to emodepside treatment like mutants from the screen (Guest et
95 al., 2007). Tissue-specific rescue experiments in the putative *slo-1* null background
96 showed that emodepside inhibited locomotion by *slo-1* expressed in both neurons

97 and body wall muscle (Guest et al., 2007). However, feeding was inhibited by
98 emodepside effects on pharyngeal-specific neurons alone and not through muscle.
99 Subsequently, emodepside was shown to open SLO-1 channels expressed in
100 *Xenopus laevis* oocytes (Kulke et al., 2014). Taken together, these results suggest
101 that emodepside acts mainly through a *slo-1* dependent pathway, and that the drug
102 opens SLO-1 channels to inhibit locomotion and pharyngeal pumping in *C. elegans*.

103 The above studies on emodepside mode of action and resistance in *C.*
104 *elegans* focused on the N2 laboratory strain and mutants in that genetic background.
105 Although *C. elegans* is a great model organism for parasitic nematodes (Bürglin et
106 al., 1998; Dilks et al., 2020; Hahnel et al., 2018), studies that use only a single strain
107 can be biased by rare variation or genetic modifiers specific to a single genetic
108 background (Sterken et al., 2015). The observation that emodepside affects multiple
109 nematode species suggests that its mode of action is conserved throughout the
110 phylum. It is unlikely that one *C. elegans* strain represents all possible genes and
111 variants that contribute to emodepside sensitivity. The use of multiple isolates in drug
112 response studies increases the likelihood of elucidating mechanisms of resistance
113 and drug mode of action shared by multiple strains and species (Hahnel et al., 2020;
114 Wit et al., 2020). Natural variation across the *C. elegans* species is archived in the *C.*
115 *elegans* Natural Diversity Resource (CeNDR) (Cook et al., 2017) and offers a
116 powerful approach to look for genetic variation that underlies the different responses
117 to emodepside, as has been done for other drugs (Brady et al., 2019; Evans and
118 Andersen, 2020; Hahnel et al., 2018; Zamanian et al., 2018; Zdraljevic et al., 2019,
119 2017).

120 Here, we measured emodepside responses in a set of *C. elegan*s wild strains
121 to demonstrate that the effect of this anthelmintic on development and brood size

122 depends on the genetic background. Across a set of nine wild strains and the
123 laboratory strain N2, we show that natural coding variation in *slo-1* is correlated with
124 differences in response to emodepside, but that additional variation impacts
125 emodepside responses. This result illustrates the need for broader comparisons of
126 anthelmintic resistance within a species, as variation in genes other than *slo-1* might
127 affect emodepside susceptibility. Additionally, it highlights the power of using *C.*
128 *elegans* natural variation for studies of emodepside mode of action and resistance
129 because this variation might recapitulate diversity present in parasite populations.
130

131 **2. Materials and Methods**

132

133 **2.1 Strains**

134 Animals were maintained at 20ºC on modified nematode growth medium
135 (NGMA) containing 1% agar and 0.7% agarose seeded with the *E. coli* strain OP50
136 (Andersen et al., 2014). The laboratory strain N2 and a set of nine wild strains from
137 the *C. elegans* Natural Diversity Resource (CeNDR) were used to study the
138 response to multiple doses of emodepside and to determine the EC_{50}. Additionally,
139 two *slo-1* putative loss-of-function mutant strains, BZ142 and NM1968, were
140 obtained from the *Caenorhabditis* Genetics Center.
141

142 **2.2 High-throughput fitness assays**

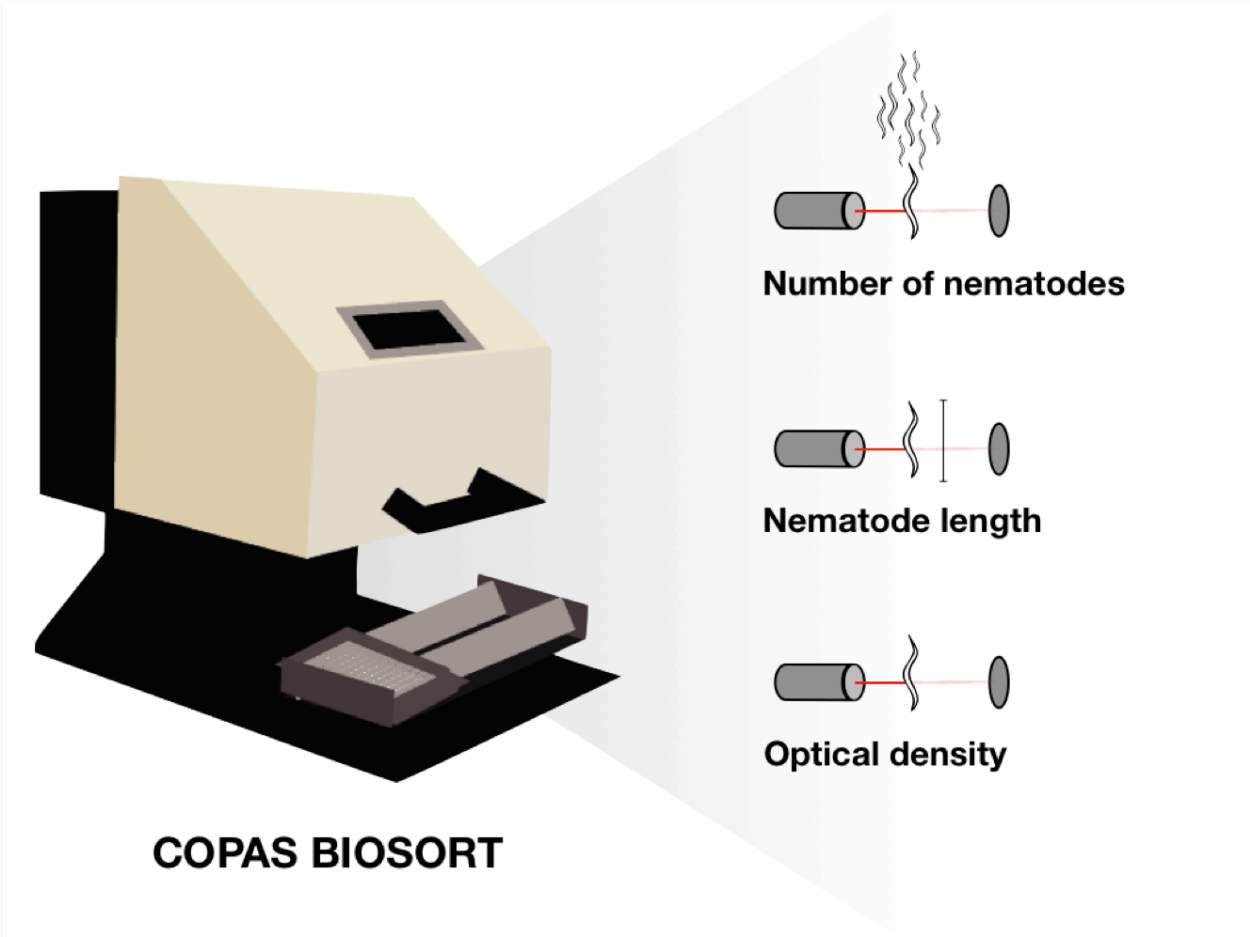
143 The high-throughput fitness assays (HTAs) were performed using the COPAS
144 BIOSORT (Union Biometrica, Holliston MA) as described previously (Hahnel et al.,
145 2018; Zdraljevic et al., 2017). In summary, the strains were grown in uncrowded
146 conditions to avoid the induction of dauer for four generations on NMGA plates at

147 20ºC prior to each assay. Gravid adults from the fifth generation were bleach-
148 synchronized, and embryos were titered at one embryo per microliter of K medium
149 (Boyd et al., 2012) into 96-well microtiter plates and incubated overnight. Hatched L1
150 larvae were fed with 5 mg/mL HB101 lysate (Pennsylvania State University Shared
151 Fermentation Facility, State College, PA (García-González et al., 2017)) and cultured
152 for 48 hours at 20ºC with constant shaking. Three L4 larvae were then sorted into
153 new microtiter plates containing K medium, 10 mg/mL HB101 lysate, 50 μM
154 kanamycin, and either 1% DMSO or emodepside dissolved in 1% DMSO.

155 After sorting, animals were cultured and allowed to reproduce for 96 hours at
156 20ºC with constant shaking. For accurate nematode length measurements, the
157 samples were treated with sodium azide (50 mM in M9) to straighten their bodies
158 before analysis using the COPAS BIOSORT. The COPAS BIOSORT is a large
159 particle flow measurement device (**Figure 1**), which measures time-of-flight (TOF),
160 extinction (EXT), and fluorescence of objects passing through the flow cell using
161 laser beams. Animal length and optical density measure nematode development
162 because animals get longer and more dense as they progress through development.
163 If animals are negatively affected by emodepside, they are expected to be smaller,
164 less optically dense, and have smaller brood sizes. Animal optical density is
165 corrected for animal length (median.norm.EXT) for each object in each well. Object
166 counts are used to calculate brood size (norm.n), which is the number of objects
167 passing the laser corrected for the number of parent animals sorted into the well.

168 To determine concentrations to measure differences in emodepside
169 responses across wild strains, a dose response assay was performed using three
170 genetically divergent *C. elegans* strains (N2, CB4856, and DL238) and four
171 increasing concentrations of emodepside (19.6, 39.1, 78.1, and 156.3 nM). A second

172 dose response with 9.8, 19.6, 39.1, 78.1, 156.3, and 312.5 nM emodepside was
173 performed using nine wild strains (JU751, WN2001, NIC258, NIC265, NIC271,
174 JU782, DL238, CB4932, and JU2586), two putative *slo-1* null mutants (BZ142 and
175 NM1968), and the N2 strain. These 12 strains were assayed in six separate assays
176 with four replicates in each assay. Raw phenotypic data were processed for outliers
177 and analyzed using the R package *easysorter* (Shimko and Andersen, 2014) as
178 described previously (Hahnel et al., 2018). For each strain, all phenotypic values
179 were normalized by deducting the average trait value in control (DMSO) conditions.



180 **Figure 1**

181 Using a COPAS BIOSORT, three independent traits were used to measure nematode responses to
182 emodepside: brood size, nematode length (μm), and optical density.

183 **2.3 Half maximal effective concentration (EC_{50}) calculations**

184 To test if emodepside had an effect on any of the three traits across the range
185 of concentrations, extreme outliers per dose were identified and removed if values
186 were greater or less than three times the interquartile range from the first or third
187 quartile, respectively, using the identify_outliers from the R package *Rstatix*
188 (Kassambara, 2020). A Kruskal-Wallis test was performed for each strain (phenotype
189 ~ dose) using the *rstatix* package (Kassambara, 2020). For strains where
190 emodepside had a phenotypic effect, the concentration with the highest response
191 was determined. To calculate the half maximal effective concentration (EC_{50}), the
192 concentration at which 50% of the drug effect is reached, we could not fit from the
193 control condition because of the hormetic effect (explained below). Instead, we fitted
194 a linear model (developmental trait or brood size ~ dose) to the data from the dose
195 with the peak phenotypic value to the highest concentration (312.5 nM) assayed and
196 calculated the concentration at the midpoint of the phenotypic effect. These EC_{50}
197 values were calculated for each strain in each of the six assays and then the mean
198 and standard deviation of each EC_{50} were calculated.

199

200 **2.4 Data availability**

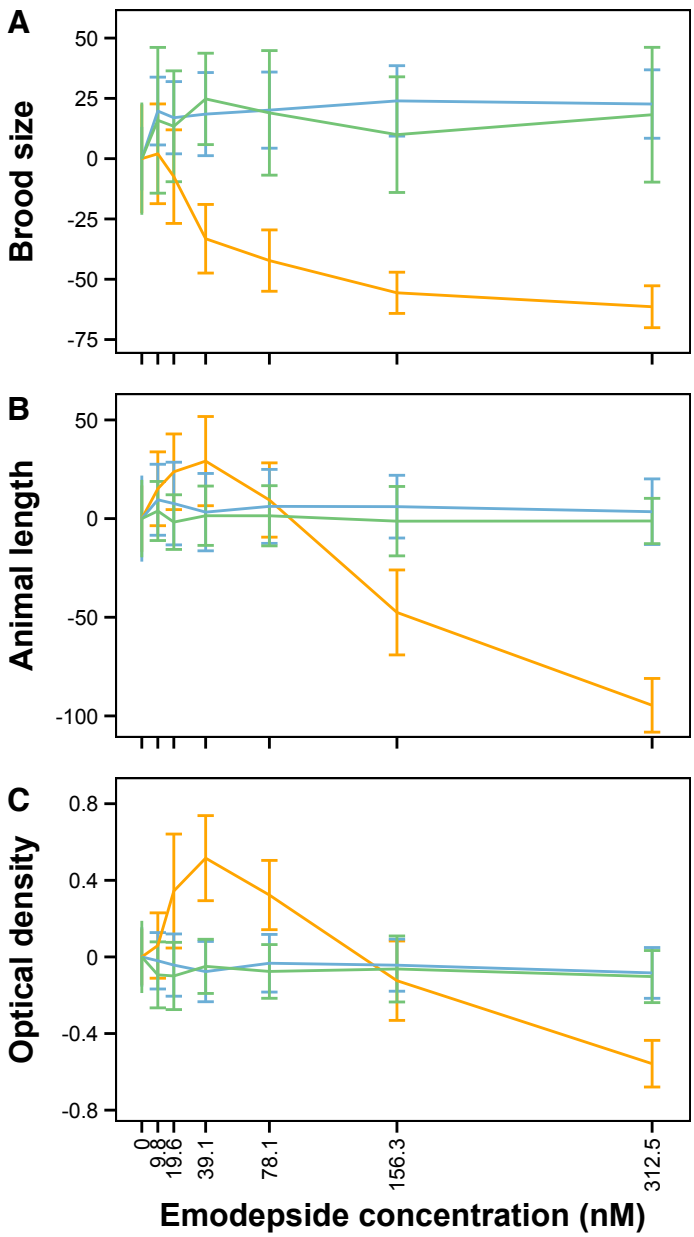
201 **Supplementary File 1** contains the phenotypic values for N2 and two putative
202 *slo-1* null mutants in response to increasing concentrations of emodepside.
203 **Supplementary File 2** contains the phenotypic values for N2, CB4856, and DL238
204 in response to increasing concentrations of emodepside. **Supplementary File 3**
205 contains the raw extinction (EXT) and time of flight (TOF) measurements for N2 and
206 two wild *C. elegans* strains (CB4856 and DL238) in control conditions and 78.1 nM
207 emodepside. **Supplementary File 4** contains the phenotypic values for the N2 strain

208 and nine wild *C. elegans* strains in response to increasing concentrations of
209 emodepside. All data and scripts to generate figures can be found at
210 https://github.com/AndersenLab/emodepside_manuscript.
211
212 **3. Results**
213
214 **3.1 Putative *slo-1* null mutants are resistant to emodepside in the high-**
215 **throughput reproduction and development assays**
216 We assayed emodepside resistance as a function of nematode reproduction
217 and development. These traits were measured for thousands of animals using a
218 previously developed high-throughput assay (HTA) (see Methods, **Figure 1**)
219 (Andersen et al., 2015; Brady et al., 2019; Evans et al., 2020, 2018; Evans and
220 Andersen, 2020; Hahnel et al., 2018; Zamanian et al., 2018; Zdraljevic et al., 2019,
221 2017). In this assay, three L4 larvae were sorted into each well of a 96-well plate and
222 allowed to grow and reproduce for 96 hours in the presence of DMSO or
223 emodepside dissolved in DMSO. Each well contained these three parents and their
224 offspring. After 96 hours, animal length and optical density, which are both proxies
225 for nematode developmental stage (Andersen et al., 2015), were measured for all
226 progeny in the well. Animals grow longer and more dense over time, and
227 anthelmintics slow this development. Therefore, shorter and less optically dense
228 animals after 96 hours show that emodepside had a detrimental effect on
229 development. In addition to development, we also measured brood size as the
230 average number of progeny produced within the 96-hour window. Although ultimate
231 brood size and demography of the population influence statistical summaries of
232 nematode development as measured by size (mean.TOF) or optical density

233 (median.norm.EXT), a smaller brood size shows emodepside sensitivity
234 (**Supplementary Figure 1**).

235 To confirm that our HTA could be used to quantitatively measure *C. elegans*
236 emodepside resistance, we measured animal development and brood size for two
237 putative *slo-1* null mutant strains, BZ142 and NM1968, and the laboratory strain, N2,
238 across a range of concentrations. The *slo-1* mutants were shown previously to be
239 resistant to emodepside based on locomotion and pharyngeal pumping assays
240 (Guest et al., 2007), and the N2 strain is known to be sensitive to emodepside.
241 Brood size and development were both inhibited in the N2 strain (Kruskal-Wallis,
242 brood size: $p = 1.49x10^{-42}$, animal length: $p = 4.32x10^{-37}$, optical density: $p = 1.25x10^{-}$
243 ^{39}), suggesting that the N2 strain is indeed sensitive to emodepside in the HTA.
244 Although development was not affected by emodepside for either mutant strain
245 (Kruskal-Wallis, BZ142 animal length: p = 0.27 and optical density: p = 0.15, and
246 NM1968 animal length: p = 0.60 and optical density: p = 0.12, **Figure 2,**
247 **Supplementary File 1**), the mutant strains both had higher brood sizes than the N2
248 strain in emodepside (Kruskal-Wallis, BZ142: $p = 4.87x10^{-11}$ and NM1968: p =
249 $6.97x10^{-3}$). Brood sizes of these putative null mutant strains were not affected even
250 at high concentrations of emodepside (Kruskal-Wallis, brood size in increasing
251 concentrations of emodepside: BZ142, p = 0.30 and NM1968, p = 0.26). This result
252 confirms that the mutant strains are indeed resistant to emodepside. By contrast,
253 both BZ142 and NM1968 had lower brood sizes than the N2 strain in control
254 conditions, indicating that *slo-1* plays a role in reproduction (**Supplementary Figure**
255 **2, Supplementary File 1**). Both deletion strains also had smaller average animal
256 lengths and lower optical densities than the N2 strain in control DMSO conditions
257 (**Supplementary Figure 2, Supplementary File 1**), which again demonstrates that

258 the putative *slo-1* null mutants are less fit than the N2 strain in control conditions.
259 These results recapitulate previous studies and illustrate the applicability of the HTA
260 to study emodepside responses in *C. elegans*.



261 **Figure 2**

262 Dose response curves for (A) brood size, (B) animal length, and (C) optical density of the N2 strain
263 (orange) and two *slo-1* putative null mutant strains (BZ142 = blue, NM1968 = green). Phenotypic
264 response values on the y-axis are corrected for the average strain response in control DMSO
265 conditions.

266 **3.2 Natural differences in emodepside response are heritable**

267 Previous studies of *C. elegans* resistance to emodepside have been
268 conducted using the N2 strain or mutant strains in the N2 genetic background.
269 Assaying natural variation in *C. elegans* was previously shown to be a powerful tool
270 to identify genetic variation that correlates with differences in benzimidazole
271 responses (Hahnel et al., 2018). To test if the response to emodepside varies by
272 genetic background, we exposed a panel of three genetically divergent *C. elegans*
273 wild strains (N2, CB4856, and DL238) to increasing concentrations of emodepside.
274 At 78.1 nM emodepside, the phenotypic variation was maximized among strains and
275 minimized within replicates of the same strain as shown by broad-sense heritabilities
276 of 88% for brood size, 61% for animal length, and 60% for optical density
277 (**Supplementary Figure 3, Supplementary File 2**). At this concentration, N2
278 animals were both shorter and less optically dense in the presence of emodepside
279 compared to animals grown in control (DMSO) conditions, showing that development
280 was delayed (**Figure 3A, Supplementary File 3**). The CB4856 and DL238 strains
281 were less affected by this emodepside concentration (**Figure 3B, Supplementary**
282 **File 3**). These differential responses across the strains and the high heritabilities
283 suggest that genetic factors underlie natural variation in emodepside responses.

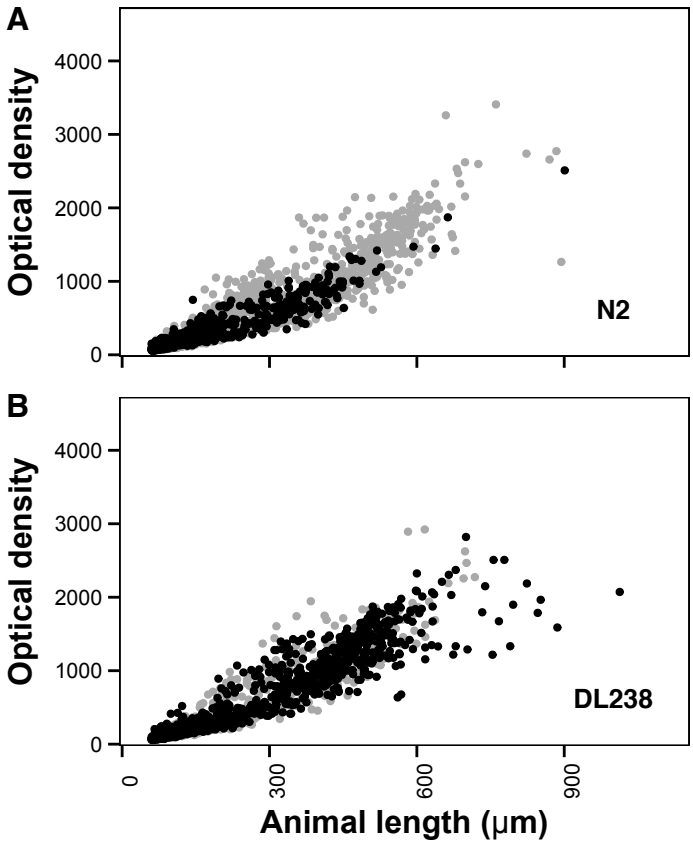


Figure 3

Plots of length and optical density values are shown for each nematode from (A) the sensitive laboratory-adapted strain N2 strain and (B) a more resistant wild strain from Hawaii, DL238. The gray points are nematodes grown in the presence of DMSO (control conditions), and the black points are nematodes grown in the presence of DMSO and 78.1 nM emodepside (emodepside conditions). *C. elegans* grows longer and more optically dense as it ages, and anthelmintic effects can be measured as changes in the demography of animals such that developmental delay is observed as smaller and less dense animals. Here, the DL238 strain was resistant to emodepside because it grew equally well in both control and emodepside conditions.

293 **3.3 Emodepside affects brood size and development in a dose-dependent**
294 **manner**

295 To describe the effects of genetic background on development and brood size
296 in response to emodepside in more detail, we selected nine genetically diverse wild
297 strains and the N2 strain for a second dose response assay to more highly replicate
298 natural differences across the species. We detected significant variation in the dose-
299 dependent responses to emodepside among strains (broad-sense heritabilities at
300 78.1 nM: 59.5% for brood size, 70.8% for animal length, and 70.8% for optical
301 density). The sensitive laboratory strain N2 falls in the middle of this range,
302 demonstrating that some wild strains are more susceptible to emodepside than the
303 N2 strain and other strains are more resistant (**Figure 4, Supplementary File 4**). At
304 the highest concentration of 312.5 nM emodepside, development and brood size
305 were reduced for all strains. Although high concentrations of emodepside were
306 shown to have detrimental effects on brood size and development, low
307 concentrations of emodepside actually produced larger brood sizes compared to
308 control conditions (**Figure 5, Supplementary Figure 4, Supplementary File 4**).
309 This positive effect on fitness at low concentrations of the drug is called a hormetic
310 effect (Bukowski and Lewis, 2000). For developmental traits the identification of a
311 potential hormetic response is confounded by increased reproduction, because
312 strains that develop further in low concentrations of emodepside start producing a
313 second generation. This second generation increases the observed brood size, but
314 also decreases the average length and optical density of the population because the
315 next generation of early larval stage animals are short and not optically dense
316 (**Supplementary Figure 1**). Regardless of the presence of a hormetic effect for

317 development, the increased brood size at low doses of emodepside suggests
318 emodepside causes a hormetic effect on reproduction.

319 We next calculated the concentration with half of the maximal drug effect
320 (EC_{50}) for each of the strains and all three traits (**Figure 4 D-F, Supplementary File**
321 **4**). For all traits, the EC_{50} was significantly affected by genetic diversity across the
322 strains that were assayed (Kruskal-Wallis, brood size: $p = 0.0422$, animal length: $p =$
323 $4.56x10^{-7}$, optical density: $p = 1.21^{-6}$). Overall, these results demonstrate that natural
324 variation in *C. elegans* affects the emodepside response, indicating that this model
325 provides an excellent system to study the genetics of emodepside mode of action
326 and resistance.

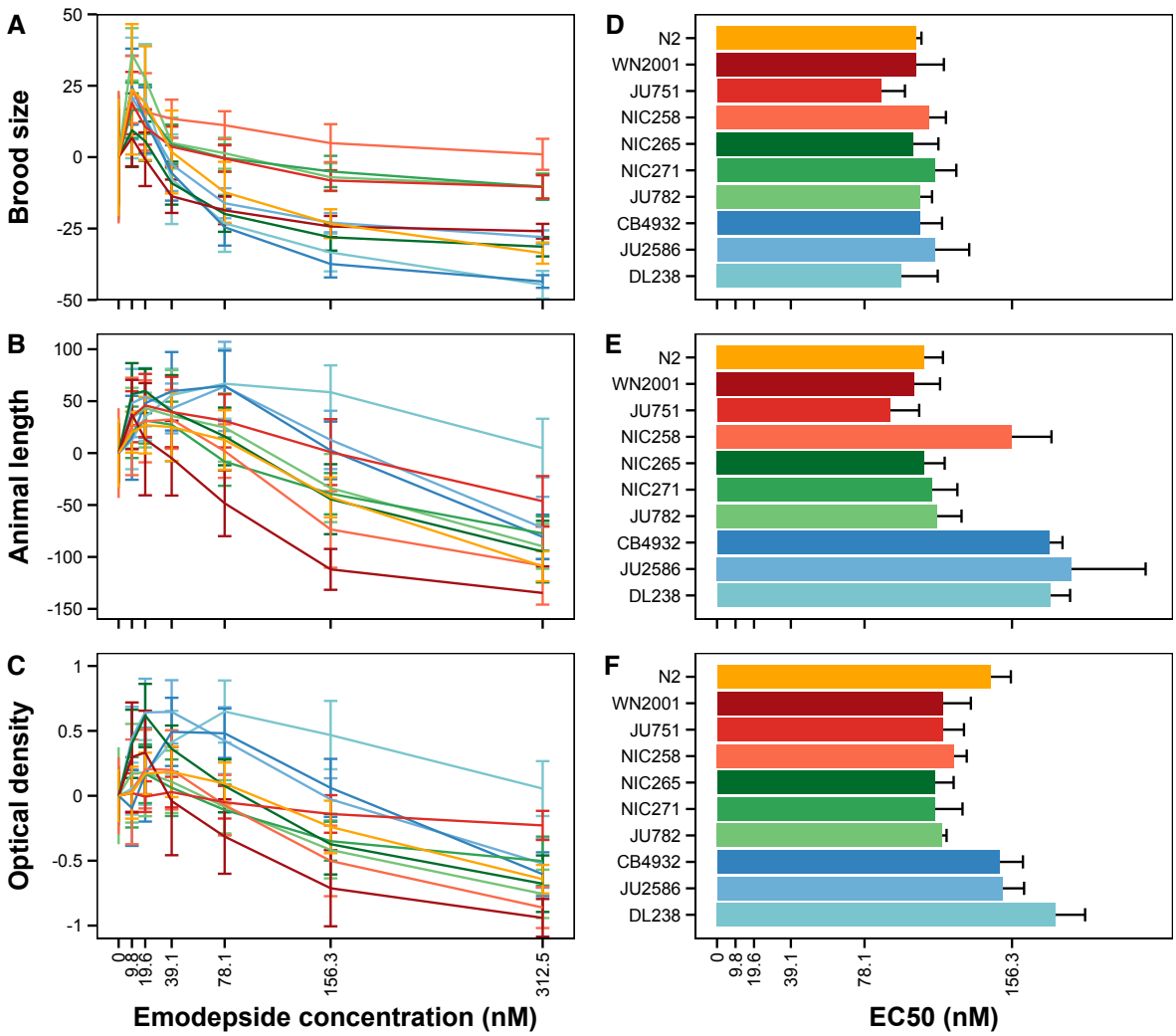


Figure 4

Dose response curves of nine wild *C. elegans* strains and N2 of (A) brood size, (B) animal length, and (C) optical density are shown on the left. Phenotypic response values on the y-axis are corrected for the average strain response in control DMSO conditions. Average EC_{50} values per strain for (D) brood size, (E) animal length, and (F) optical density are shown on the right.

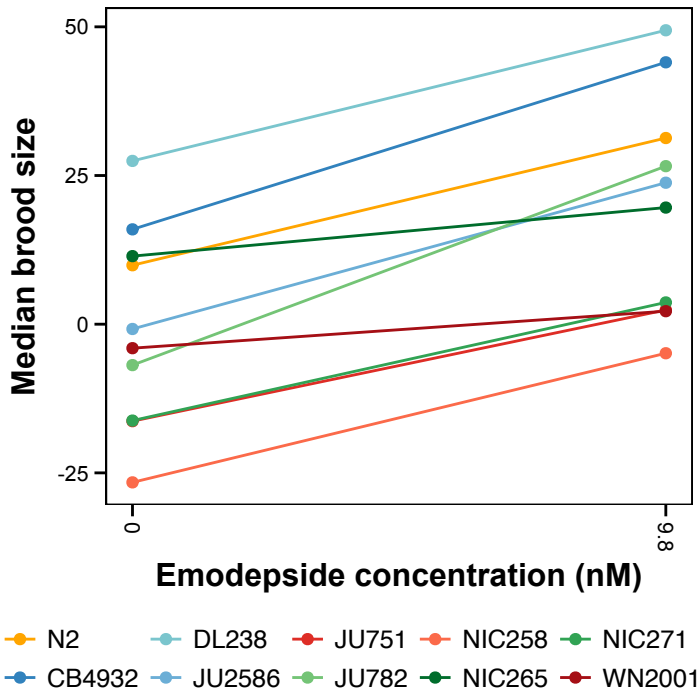


Figure 5

Plot of median brood sizes at the control condition (DMSO) and at 9.8 nM emodepside for ten *C. elegans* strains. Statistical significance of the phenotypic response in DMSO compared to 9.8 nM emodepside was calculated using a pairwise Wilcoxon test. All strains, except NIC265, showed a significant ($p < 0.05$) hormetic effect.

3.4 Natural variation in candidate gene *slo-1* correlates with resistance in reproduction but not development in wild C. elegans strains

Emodepside has been shown to directly interact with and open the *C. elegans* SLO-1 channel (Kulke et al., 2014), and putative *slo-1* null mutants are resistant to emodepside treatment (**Figure 2**, **Supplementary File 1** (Guest et al., 2007)). Because of these results, we expected that genetic variation in *slo-1* with a predicted moderate or high deleterious effect on gene function would correlate with emodepside resistance. Of nine wild strains assayed here, four (NIC258, NIC265, NIC271, and JU782) have the same four predicted variations in SLO-1 (Arg134Trp, Leu327Phe, Cys328Leu, and Arg678Leu) that causes deleterious amino acid

348 substitutions with a summed BLOSUM score of -6 (Henikoff and Henikoff, 1992).
349 This variation correlated with higher EC_{50} values for brood size but lower EC_{50} values
350 for development (Kruskal-Wallis, brood size: $p = 0.0221$, animal length: $p = 0.263$,
351 optical density: $p = 3.35x10^{-5}$).

352 We also investigated natural variation in another candidate gene for
353 emodepside resistance, *lat-1* (Saeger et al., 2001; Willson et al., 2004). All nine wild
354 strains in the dose response assay harbor natural variants in *lat-1*. To investigate if
355 that variation is predicted to affect *lat-1* function, we summed BLOSUM scores for
356 each of the wild strains. Only the DL238 strain had a negative BLOSUM score (-1),
357 and this score was not correlated with resistance across all strains (Kruskal-Wallis,
358 brood size: $p = 0.223$, animal length: $p = 0.223$, and optical density: $p = 0.117$). In
359 this set of ten strains, variation in *lat-1* does not underlie differences in emodepside
360 responses. Because strains vary in emodepside responses, our results indicate that
361 amino acid variation in *slo-1* or *lat-1* does not explain all differences in emodepside
362 responses, suggesting that additional genetic mechanisms affect the response to
363 emodepside.

364

365 **4. Discussion**

366 Emodepside is a broad range anthelmintic with a distinct mode of action
367 compared to other anthelmintics (Epe and Kaminsky, 2013). Previous studies of
368 emodepside sensitivity and phenotypic effects in *C. elegans* have focussed on the
369 laboratory strain N2 (Bull et al., 2007; Guest et al., 2007; Willson et al., 2004). In this
370 strain, emodepside inhibits egg-laying, pharyngeal pumping, development, and
371 locomotion. In the present study, sensitivity to emodepside was measured across
372 wild *C. elegans* strains, the N2 strain, and two putative *slo-1* null mutants (BZ142

373 and NM1968) using a large-particle flow cytometer high-throughput assay (HTA)
374 (**Figure 1**). Resistance to emodepside caused by the putative loss-of-function *slo-1*
375 mutants was confirmed using the HTA. Additionally, the effects of emodepside on
376 brood size and development varied across the wild strains (**Figures 3 and 4,**
377 **Supplementary Files 3 and 4**) and was correlated with protein-coding variation in
378 the resistance candidate gene *slo-1*. Importantly, we found that low doses of
379 emodepside have a hormetic effect on brood size in *C. elegans*. Hormesis was
380 observed in the N2 laboratory strain and eight out of nine wild strains, regardless of
381 their susceptibility to emodepside (**Figure 4, Supplementary File 4**). This consistent
382 hormetic effect across the wild *C. elegans* strains included in our study suggests that
383 emodepside might also cause a hormetic effect in parasitic nematodes. This study
384 shows the power of using natural variation in *C. elegans* to study emodepside
385 responses.
386
387 **4.1 High-throughput assays across wild strains show the effects of**
388 **emodepside on development and reproduction**
389 In the present study, development and reproductive success in the presence
390 of emodepside was measured for the N2 strain and a set of wild *C. elegans* strains
391 using a HTA. Previously, reproduction was measured on agar plates (Bull et al.,
392 2007), where both the timing and the quantity of egg laying was measured. Using the
393 HTA, brood size is assayed as the overall reproductive success of three L4 larvae
394 over a 96-hour period. Results from agar-based assays and HTA are highly
395 correlated, and HTA intra- and inter-assay correlations are substantially greater
396 compared to agar plate-based methods (Andersen et al., 2015). On agar plates,
397 emodepside prevents egg laying, and the animals are bloated with embryos at

398 higher drug concentrations (20 nM - 500 nM) (Bull et al., 2007). In the HTA,
399 reproduction was inhibited for the N2 strain as well as the wild strains at
400 concentrations similar to previous studies (19.6 μM to 312.5 nM), confirming that
401 brood size in response to emodepside can be measured reproducibly with both
402 assays. Agar-based developmental rate, based on the percentage of eggs that hatch
403 and reach different larval stages in increasing concentrations of emodepside, is
404 delayed (Bull et al., 2007). The HTA measures animal length and optical density of a
405 population established by three L4 larvae over a 96-hour period. The two lowest
406 concentrations of emodepside, 9.8 nM and 19.8 nM, have either no effect on
407 development or a hormetic effect. Higher concentrations (39.1 μM to 312.5 nM),
408 which overlap with the effective concentrations from the agar-based development
409 phenotypes, negatively affect animal length and optical density (**Figure 4**). The
410 results from both the agar-based and HTA methods indicate that emodepside inhibits
411 reproduction at lower concentrations than development. Emodepside inhibited
412 reproduction from approximately 20 nM and up, compared to approximately 40 nM
413 and up for development. The agar-based study did not find a hormetic effect, but our
414 results suggest that such an effect is likely to be present at concentrations below the
415 range tested on agar plates. Our results show that the HTA provides a platform to
416 screen hundreds of strains efficiently and that the different measures of reproduction
417 and development are similarly affected across assay platforms.
418

419 **4.2 Natural variation affects development and brood size in the presence of**
420 **emodepside**

421 The response of *C. elegans* to emodepside is affected by natural genetic
422 variation (**Figure 4, Supplementary File 4**). Our results showed that all strains are

423 affected by emodepside, and that higher doses inhibit development, as measured by
424 animal length and optical density, and reproduction, as measured by brood size
425 (**Figure 4, Supplementary File 4**). For brood size, strain-specific differences were
426 correlated with variation in *slo-1* where strains with predicted deleterious variation
427 were more resistant to emodepside treatments. However, strains with higher brood
428 sizes at lower concentrations do not have variation in *slo-1*, suggesting that the
429 hormetic effect is not mediated by *slo-1*. It will be informative to introduce *slo-1*
430 variation in the wild strains with higher fitness using CRISPR-Cas9 genome editing
431 to test if *slo-1* variation reduces brood size in a more resistant background. Our
432 results show that reproduction and development are inhibited by higher
433 concentrations of emodepside, and that natural variation affects the extent of this
434 response. Future measurements of emodepside responses across additional wild
435 isolates will improve the power to detect candidate resistance genes across the
436 species using genome-wide association studies.
437

438 **4.3 Hormetic effects of emodepside suggest a potential risk of treatment failure**
439 **at suboptimal doses**

440 Nine of ten strains showed a hormetic response in reproduction when treated
441 with a low emodepside concentration (**Figure 4 A-C, Figure 5, Supplementary File**
442 **4**). The presence of hormetic responses across strains illustrates that hormesis is a
443 common response to low concentrations of emodepside across *C. elegans* strains. If
444 this response is shared with parasitic nematodes, then experimental designs to
445 calculate EC_{50} values in parasites need to account for this effect. A low EC50
446 estimate caused by a hormetic effect can lead to recommended treatment doses that
447 will be insufficient to treat parasitic nematode infections. Underdosing is a known risk

448 factor for the selection of resistance against all anthelmintics (Sangster et al., 2018;
449 Silvestre et al., 2001; Smith et al., 1999). If hormetic doses are administered, either
450 because of erroneous EC_{50} calculations or as a result of other treatment factors like
451 ineffective drug delivery (Sangster et al., 2018), the infection might be intensified
452 rather than treated. Our results also imply that low doses of emodepside are
453 beneficial rather than detrimental for nematode growth. To prevent hormesis from
454 rendering emodepside treatment ineffective, it is essential to investigate hormetic
455 effects and adjust treatment recommendations accordingly.
456

457 **4.4 Natural variation in *C. elegans* can facilitate the study of anthelmintic**
458 **resistance**

459 The free-living nematode *C. elegans* is a long standing model to study
460 anthelmintic mode of action and resistance of parasitic nematodes (Geary and
461 Thompson, 2001; Hahnel et al., 2020; Holden-Dye et al., 2014; Wit et al., 2020). The
462 suitability of *C. elegans* as a model is the result of a range of attributes, including the
463 phylogenetic relationship of *C. elegans* with many parasitic nematodes of human and
464 veterinary importance, its short and direct life cycle, a wide range of genome-editing
465 tools, and its high-quality reference genome and gene models. Additionally, larval
466 stages of many parasitic nematodes occupy the same niches as *C. elegans*
467 (Crombie et al., 2019; Frézal and Félix, 2015). Similar environmental stressors,
468 including naturally occuring precursors of anthelmintics (Alivisatos et al., 1962;
469 Campbell, 2012, 2005), can cause similar selective pressures for both species to
470 evolve resistance.

471 Previous studies on the emodepside resistance candidate gene *lat-1*, showed
472 that putative *lat-1* null mutants were resistant in reproduction and pharyngeal

473 pumping assays, but sensitive in locomotion assays (Guest et al., 2007), and that
474 putative *slo-1* null mutants are resistant in all assays. Here, we show that although
475 putative *slo-1* null mutants are resistant to emodepside treatment, *slo-1* variation is
476 not the only determinant of resistance across wild strains. These results imply that
477 multiple genes likely affect the response to emodepside. To identify these genes,
478 genetic variation across wild strains can be correlated with phenotypic responses to
479 emodepside. Genes identified based on population-wide variation are more likely to
480 translate to other species than genes identified based on one genetic background.
481 After identification of candidate genes, genetic variation in these genes should be
482 tested in a controlled genetic background by introducing specific mutations using
483 CRISPR-Cas9 genome editing (Dilks et al., 2020).
484

485 **Declaration of competing interests**

486 The authors have no competing financial or personal interests that impacted the
487 work presented in this manuscript.
488

489 **Acknowledgements**

490 We would like to thank members of the Andersen laboratory for their technical
491 assistance and helpful comments on the manuscript. This work was supported by
492 R01 AI153088 to E.C.A. We would also like to thank Wormbase and the *C. elegans*
493 Natural Diversity Resource (CeNDR) for data and tools critical for our analyses of
494 natural variation (NSF CSBR 1930382). The *slo-1* mutant strains (BZ142 and
495 NM1968) were provided by the *Caenorhabditis* Genetics Center, which is funded by
496 NIH Office of Research Infrastructure Programs (P40 OD010440).

497 **References**

498 Alivisatos, S.G., Lamantia, L., Matijevitch, B.L., 1962. Imidazolytic processes. VI.
499 Enzymic formation of benzimidazole and 5,6-dimethylbenzimidazole containing
500 dinucleotides. Biochim. Biophys. Acta 58, 209–217.
501 Altreuther, G., Buch, J., Charles, S.D., Davis, W.L., Krieger, K.J., Radeloff, I., 2005.
502 Field evaluation of the efficacy and safety of emodepside/praziquantel spot-on
503 solution against naturally acquired nematode and cestode infections in domestic
504 cats. Parasitol. Res. 97 Suppl 1, S58–S64.
505 Andersen, E.C., Bloom, J.S., Gerke, J.P., Kruglyak, L., 2014. A variant in the
506 neuropeptide receptor npr-1 is a major determinant of Caenorhabditis elegans
507 growth and physiology. PLoS Genet. 10, e1004156.
508 Andersen, E.C., Shimko, T.C., Crissman, J.R., Ghosh, R., Bloom, J.S., Seidel, H.S.,
509 Gerke, J.P., Kruglyak, L., 2015. A Powerful New Quantitative Genetics Platform,
510 Combining Caenorhabditis elegans High-Throughput Fitness Assays with a
511 Large Collection of Recombinant Strains. G3 5, 911–920.
512 Boyd, W.A., Smith, M.V., Freedman, J.H., 2012. Caenorhabditis elegans as a Model
513 in Developmental Toxicology. Methods in Molecular Biology.
514 https://doi.org/10.1007/978-1-61779-867-2_3
515 Brady, S.C., Zdraljevic, S., Bisaga, K.W., Tanny, R.E., Cook, D.E., Lee, D., Wang,
516 Y., Andersen, E.C., 2019. A Novel Gene Underlies Bleomycin-Response
517 Variation in Caenorhabditis elegans. Genetics 212, 1453–1468.
518 Bukowski, J.A., Lewis, R.J., 2000. Hormesis and health: a little of what you fancy
519 may be good for you. South. Med. J. 93, 371–374.
520 Bull, K., Cook, A., Hopper, N.A., Harder, A., Holden-Dye, L., Walker, R.J., 2007.
521 Effects of the novel anthelmintic emodepside on the locomotion, egg-laying

522 behaviour and development of Caenorhabditis elegans. Int. J. Parasitol. 37,
523 627–636.
524 Bürglin, T.R., Lobos, E., Blaxter, M.L., 1998. Caenorhabditis elegans as a model for
525 parasitic nematodes. International Journal for Parasitology.
526 https://doi.org/10.1016/s0020-7519(97)00208-7
527 Campbell, W.C., 2012. History of avermectin and ivermectin, with notes on the
528 history of other macrocyclic lactone antiparasitic agents. Curr. Pharm.
529 Biotechnol. 13, 853–865.
530 Campbell, W.C., 2005. Serendipity and new drugs for infectious disease. ILAR J. 46,
531 352–356.
532 Cook, D.E., Zdraljevic, S., Roberts, J.P., Andersen, E.C., 2017. CeNDR, the
533 Caenorhabditis elegans natural diversity resource. Nucleic Acids Res. 45,
534 D650–D657.
535 Crombie, T.A., Zdraljevic, S., Cook, D.E., Tanny, R.E., Brady, S.C., Wang, Y.,
536 Evans, K.S., Hahnel, S., Lee, D., Rodriguez, B.C., Zhang, G., van der Zwagg,
537 J., Kiontke, K., Andersen, E.C., 2019. Deep sampling of Hawaiian
538 Caenorhabditis elegans reveals high genetic diversity and admixture with global
539 populations. Elife 8, e50465.
540 Davies, A.G., Pierce-Shimomura, J.T., Kim, H., VanHoven, M.K., Thiele, T.R., Bonci,
541 A., Bargmann, C.I., McIntire, S.L., 2003. A Central Role of the BK Potassium
542 Channel in Behavioral Responses to Ethanol in C. elegans. Cell.
543 https://doi.org/10.1016/s0092-8674(03)00979-6
544 Dilks, C.M., Hahnel, S., Sheng, Q., Long, L., McGrath, P.T., Andersen, E.C., 2020.
545 Quantitative benzimidazole resistance and fitness effects of parasitic nematode
546 beta-tubulin alleles. International Journal for parasitology: Drugs and Drug

547 Resistance 14, 28–36.
548 Epe, C., Kaminsky, R., 2013. New advancement in anthelmintic drugs in veterinary
549 medicine. Trends Parasitol. 29, 129–134.
550 Evans, K.S., Andersen, E.C., 2020. The Gene scb-1 Underlies Variation in
551 Caenorhabditis elegans Chemotherapeutic Responses. G3 .
552 https://doi.org/10.1534/g3.120.401310
553 Evans, K.S., Brady, S.C., Bloom, J.S., Tanny, R.E., Cook, D.E., Giuliani, S.E.,
554 Hippleheuser, S.W., Zamanian, M., Andersen, E.C., 2018. Shared Genomic
555 Regions Underlie Natural Variation in Diverse Toxin Responses. Genetics.
556 https://doi.org/10.1534/genetics.118.301311
557 Evans, K.S., Zdraljevic, S., Stevens, L., Collins, K., Tanny, R.E., Andersen, E.C.,
558 2020. Natural variation in the sequestosome-related gene, sqst-5, underlies zinc
559 homeostasis in Caenorhabditis elegans. PLoS Genet. 16, e1008986.
560 Frézal, L., Félix, M.-A., 2015. C. elegans outside the Petri dish. Elife 4.
561 https://doi.org/10.7554/eLife.05849
562 García-González, A.P., Ritter, A.D., Shrestha, S., Andersen, E.C., Safak Yilmaz, L.,
563 Walhout, A.J.M., 2017. Bacterial Metabolism Affects the C. elegans Response
564 to Cancer Chemotherapeutics. Cell. https://doi.org/10.1016/j.cell.2017.03.046
565 Geary, T.G., Thompson, D.P., 2001. Caenorhabditis elegans: how good a model for
566 veterinary parasites? Veterinary Parasitology. https://doi.org/10.1016/s0304-
567 4017(01)00562-3
568 Guest, M., Bull, K., Walker, R.J., Amliwala, K., O'Connor, V., Harder, A., Holden-
569 Dye, L., Hopper, N.A., 2007. The calcium-activated potassium channel, SLO-1,
570 is required for the action of the novel cyclo-octadepsipeptide anthelmintic,
571 emodepside, in Caenorhabditis elegans. Int. J. Parasitol. 37, 1577–1588.

572 Hahnel, S.R., Dilks, C.M., Heisler, I., Andersen, E.C., Kulke, D., 2020.
573 Caenorhabditis elegans in anthelmintic research – Old model, new perspectives.
574 International Journal for Parasitology: Drugs and Drug Resistance.
575 https://doi.org/10.1016/j.ijpddr.2020.09.005
576 Hahnel, S.R., Zdraljevic, S., Rodriguez, B.C., Zhao, Y., McGrath, P.T., Andersen,
577 E.C., 2018. Extreme allelic heterogeneity at a Caenorhabditis elegans beta-
578 tubulin locus explains natural resistance to benzimidazoles. PLoS Pathog. 14,
579 e1007226.
580 Harder, A., Holden-Dye, L., Walker, R., Wunderlich, F., 2005. Mechanisms of action
581 of emodepside. Parasitol. Res. 97 Suppl 1, S1–S10.
582 Harder, A., Schmitt-Wrede, H.-P., Krücken, J., Marinovski, P., Wunderlich, F.,
583 Willson, J., Amliwala, K., Holden-Dye, L., Walker, R., 2003.
584 Cyclooctadepsipeptides—an anthelmintically active class of compounds
585 exhibiting a novel mode of action. Int. J. Antimicrob. Agents 22, 318–331.
586 Harder, A., von Samson-Himmelstjerna, G., 2001. Activity of the cyclic depsipeptide
587 emodepside (BAY 44-4400) against larval and adult stages of nematodes in
588 rodents and the influence on worm survival. Parasitol. Res. 87, 924–928.
589 Henikoff, S., Henikoff, J.G., 1992. Amino acid substitution matrices from protein
590 blocks. Proc. Natl. Acad. Sci. U. S. A. 89, 10915–10919.
591 Holden-Dye, L., Southampton Neuroscience Group (SoNG), Centre for Biological
592 Sciences, University of Southampton, 1bj, S.S., UK., Walker, R.J., 2014.
593 Anthelmintic drugs and nematicides: studies in Caenorhabditis elegans.
594 WormBook. https://doi.org/10.1895/wormbook.1.143.2
595 Jeschke, R., Iinuma, K., Harder, A., Schindler, M., Murakami, T., 2005. Influence of
596 the cyclooctadepsipeptides PF1022A and PF1022E as natural products on the

design of semi-synthetic anthelmintics such as emodepside. Parasitol. Res. 97 Suppl 1, S11–S16.

Kassambara, A., 2020. rstatix: pipe-friendly framework for basic statistical tests. R package version 0.4. 0.

Kotze, A.C., Prichard, R.K., 2016. Anthelmintic Resistance in Haemonchus contortus: History, Mechanisms and Diagnosis. Adv. Parasitol. 93, 397–428.

Kulke, D., von Samson-Himmelstjerna, G., Miltsch, S.M., Wolstenholme, A.J., Jex, A.R., Gasser, R.B., Ballesteros, C., Geary, T.G., Keiser, J., Townson, S., Harder, A., Krücken, J., 2014. Characterization of the Ca2+-gated and voltage-dependent K+-channel Slo-1 of nematodes and its interaction with emodepside. PLoS Negl. Trop. Dis. 8, e3401.

McKellar, Q.A., Jackson, F., 2004. Veterinary anthelmintics: old and new. Trends Parasitol. 20, 456–461.

Mühlfeld, S., Schmitt-Wrede, H.-P., Harder, A., Wunderlich, F., 2009. FMRFamide-like neuropeptides as putative ligands of the latrophilin-like HC110-R from Haemonchus contortus. Mol. Biochem. Parasitol. 164, 162–164.

Prichard, R.K., 2017. Drug Resistance in Nematodes, in: Mayers, D.L., Sobel, J.D., Ouellette, M., Kaye, K.S., Marchaim, D. (Eds.), Antimicrobial Drug Resistance: Mechanisms of Drug Resistance, Volume 1. Springer International Publishing, Cham, pp. 689–704.

Saeger, B., Schmitt-Wrede, H.P., Dehnhardt, M., Benten, W.P., Krücken, J., Harder, A., Von Samson-Himmelstjerna, G., Wiegand, H., Wunderlich, F., 2001. Latrophilin-like receptor from the parasitic nematode Haemonchus contortus as target for the anthelmintic depsipeptide PF1022A. FASEB J. 15, 1332–1334.

Sangster, N.C., Cowling, A., Woodgate, R.G., 2018. Ten Events That Defined

622 Anthelmintic Resistance Research. Trends Parasitol. 34, 553–563.
623 Shimko, T.C., Andersen, E.C., 2014. COPASutils: an R package for reading,
624 processing, and visualizing data from COPAS large-particle flow cytometers.
625 PLoS One 9, e111090.
626 Silvestre, A., Cabaret, J., Humbert, J.F., 2001. Effect of benzimidazole under-dosing
627 on the resistant allele frequency in Teladorsagia circumcincta (Nematoda).
628 Parasitology 123, 103–111.
629 Smith, G., Grenfell, B.T., Isham, V., Cornell, S., 1999. Anthelmintic resistance
630 revisited: under-dosing, chemoprophylactic strategies, and mating probabilities.
631 Int. J. Parasitol. 29, 77–91; discussion 93–4.
632 Sterken, M.G., Snoek, L.B., Kammenga, J.E., Andersen, E.C., 2015. The laboratory
633 domestication of Caenorhabditis elegans. Trends Genet. 31, 224–231.
634 von Samson-Himmelstjerna, G., Harder, A., Sangster, N.C., Coles, G.C., 2005.
635 Efficacy of two cyclooctadepsipeptides, PF1022A and emodepside, against
636 anthelmintic-resistant nematodes in sheep and cattle. Parasitology 130, 343–
637 347.
638 Welz, C., Harder, A., Schnieder, T., Hoglund, J., von Samson-Himmelstjerna, G.,
639 2005. Putative G protein-coupled receptors in parasitic nematodes--potential
640 targets for the new anthelmintic class cyclooctadepsipeptides? Parasitol. Res.
641 97 Suppl 1, S22–S32.
642 Willson, J., Amliwala, K., Davis, A., Cook, A., Cuttle, M.F., Kriek, N., Hopper, N.A.,
643 O'Connor, V., Harder, A., Walker, R.J., Holden-Dye, L., 2004. Latrotoxin
644 Receptor Signaling Engages the UNC-13-Dependent Vesicle-Priming Pathway
645 in C. elegans. Current Biology. https://doi.org/10.1016/j.cub.2004.07.056
646 Willson, J., Amliwala, K., Harder, A., Holden-Dye, L., Walker, R.J., 2003. The effect

647 of the anthelmintic emodepside at the neuromuscular junction of the parasitic
648 nematode Ascaris suum. Parasitology.
649 https://doi.org/10.1017/s0031182002002639
650 Willson J, Holden-Dye L, Harder A, Walker RJ, 2001. A possible mechanism for the
651 action of the novel anthelmintic emodepside, using Ascaris suum body wall
652 muscle preparations. J. Physiol. 536, 132P–133P.
653 Wit, J., Dilks, C., Andersen, E., 2020. Complementary Approaches to Understand
654 Anthelmintic Resistance Using Free-Living and Parasitic Nematodes. Trends in
655 Parasitology. https://doi.org/10.20944/preprints202008.0313.v1
656 Zahner, H., Taubert, A., Harder, A., von Samson-Himmelstjerna, G., 2001. Filaricidal
657 efficacy of anthelmintically active cyclodepsipeptides. Int. J. Parasitol. 31, 1515–
658 1522.
659 Zamanian, M., Cook, D.E., Zdraljevic, S., Brady, S.C., Lee, D., Lee, J., Andersen,
660 E.C., 2018. Discovery of genomic intervals that underlie nematode responses to
661 benzimidazoles. PLoS Negl. Trop. Dis. 12, e0006368.
662 Zdraljevic, S., Fox, B.W., Strand, C., Panda, O., Tenjo, F.J., Brady, S.C., Crombie,
663 T.A., Doench, J.G., Schroeder, F.C., Andersen, E.C., 2019. Natural variation in
664 C. elegans arsenic toxicity is explained by differences in branched chain amino
665 acid metabolism. Elife 8. https://doi.org/10.7554/eLife.40260
666 Zdraljevic, S., Strand, C., Seidel, H.S., Cook, D.E., Doench, J.G., Andersen, E.C.,
667 2017. Natural variation in a single amino acid substitution underlies
668 physiological responses to topoisomerase II poisons. PLoS Genet. 13,
669 e1006891.