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Effects of insect host chemical secretions on the entomopathogenic nematode *Steinernema carpocapsae*

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Research Paper

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Abstract

Given the threat presented by parasites and pathogens, insects employ various defences to protect themselves against infection, including chemical secretions. The red flour beetle *Tribolium castaneum* releases a secretion containing the benzoquinones methyl-1,4-benzoquinone (MBQ) and ethyl-1,4-benzoquinone (EBQ) into the environment. These compounds have known antimicrobial effects; however, their role in defence against macroparasites is not known. Entomopathogenic nematodes, such as *Steinernema carpocapsae*, present a serious threat to insects, with successful infection leading to death. Thus, quinone-containing secretions may also aid in host defence. We tested how exposure to the individual components of this quinone secretion, as well as a mix at naturally-occurring proportions, affected the survival and thrashing behaviour of *S. carpocapsae*, as well as their virulence to a model host (*Galleria mellonella*). Exposure to high concentrations of MBQ and EBQ, as well as the quinone mix, significantly increased nematode death but did not consistently reduce thrashing, which would otherwise be expected given their toxicity. Rather, quinones may act as a host cue to *S. carpocapsae* by triggering increased activity. We found that exposure to quinones for 24 or 72 hours did not reduce nematode virulence, and surviving nematodes remained infective after non-lethal exposure. Our results indicate that quinone secretions likely serve as a defence against multiple infection threats by reducing *S. carpocapsae* survival, but further research is required to contextualize their roles by testing against other nematodes, as well as other helminths using insects as hosts.

Introduction

Infection by parasites and pathogens represents a constant and severe threat, with host costs ranging from fitness reductions to death (Alexander & Antonovics 1988). As such, hosts have evolved a wide suite of anti-parasite defences to reduce the potential risk or costs of infection (Hart 1990). These can be broadly categorized as resistance and tolerance, which are pre- or post-infection defenses, respectively (Amoroso 2021; Råberg *et al.* 2008). Defences against parasites and pathogens (hereafter collectively referred to as parasites) can involve chemicals, either through compounds produced by the host or in materials collected in their environment (Li *et al.* 2013; Singer *et al.* 2004). These defensive compounds can be used against parasites internally through host ingestion of compounds or externally by applying them topically or throughout the host's environment (Li *et al.* 2013; Singer *et al.* 2004).

Chemical defences against parasites range in specificity – some extend to other natural enemies such as predators, some are effective against multiple parasite taxa, and some are more limited to a particular parasitic group. One example includes the spotless starling *Sturnus unicolor*, which selectively forages for plant materials containing volatile compounds and essential oils (Ruiz-Castellano *et al.* 2016). These materials are used for nest building and reduce the risk of bacterial disease in bird embryos (Clark & Mason 1985), but they have not been tested against other parasitic threats. Newts in the genus *Tarichia* produce tetrodotoxin, a potent neurotoxin that decreases the risk of fungal infection and is associated with reduced parasite richness compared to newt species that do not produce toxins (Johnson *et al.* 2018). However, this toxin also serves as a defence against predators (Reimche *et al.* 2020). Insects also use chemical defences against parasites, such as ant species that cultivate bacterial biofilms on their exoskeletons (Oh *et al.* 2008). By producing antimicrobial compounds like dentigerumycin, this biofilm has been shown to inhibit fungal pathogens as well as harmful bacteria and therefore increases the odds of ant survival (Batey *et al.* 2020).

In order to defend themselves against natural enemies, many arthropods produce chemicals in the form of various quinones. For instance, quinone secretions are produced by millipedes, harvestmen, and coleopterans, including tenebrionids like the red flour beetle *Tribolium castaneum* (Blum & Crain 1961; Eisner *et al.* 1978; Pedrini *et al.* 2015; Rocha *et al.* 2013). These

compounds have been demonstrated to be effective antimicrobial defences and inhibit the growth of pathogenic bacteria, including multiple members from the *Bacillus* genus and fungi such as *Beauveria bassiana* (Pedrini *et al.* 2015; Yezerski *et al.* 2007). The toxic effects of quinones mainly come from the generation of reactive oxygen species (ROS) via a redox reaction, which can cause harmful cascade reactions (Monks *et al.* 1992). These cascades can result in widespread damage to cellular components, including proteins, lipids, and DNA (Bergamini *et al.* 2004). Quinones have also been linked to DNA damage – in particular, through alkylation (Foti *et al.* 2012). Additionally, there is evidence they may decrease insect palatability and thus deter potential predators as well (Eisner & Meinwald 1966).

In *T. castaneum*, quinone secretions are made up of 3 main components: methyl-1,4-benzoquinone, ethyl-1,4-benzoquinone, and 1-pentadecene (Loconti & Roth 1953; Villaverde *et al.* 2007). The two quinones are the active antimicrobials, whereas 1-pentadecene plays a role in social modulation in these beetles (Đukić *et al.* 2021). By acting as a semiochemical, *T. castaneum* can regulate their population densities via attractant or repellent effects when 1-pentadecene concentrations are low or high, respectively (Đukić *et al.* 2021). In this way, 1-pentadecene reduces the risk of self-harm that could be caused by high quinone concentrations in the environment (Yezerski *et al.* 2004). Although the roles of quinone secretions have been established in terms of their antimicrobial properties (Pedrini *et al.* 2015; Yezerski *et al.* 2007), it is not clear how they may be involved in defence against other parasitic groups, such as nematodes. Given the critical role of defenses in host–parasite interactions, it is important to understand how quinone secretions, including their individual components, may serve in broad protection (i.e., against macroparasites) as well.

When it comes to the risk posed by macroparasites to insects, entomopathogenic nematodes (EPNs) are a common threat and can harm natural insect populations (Campos-Herrera *et al.* 2012; Peters 1996). However, they also have important uses in agriculture as a biocontrol strategy against insect pests because they must kill their host to complete their life cycle (Keshari *et al.* 2019). EPNs comprise over 120 known nematode species found in two families, Steinernematidae and Heterorhabditidae (Bhat *et al.* 2020). These nematodes are generalists, capable of infecting (and killing) a range of potential host species (Denno *et al.* 2008). Both families of nematode have similar life cycles, with free-living juvenile infectious stages living in soil (Wright & Perry 2002). After contact with a potential host, the nematode will attach itself to the host's exterior and enter through various openings (Dowds & Peters 2002). Once inside, juveniles begin feeding on host hemolymph and then release toxins and bacterial symbionts that kill their host (Ciche *et al.* 2006). Feeding also triggers the juveniles to develop into adults (Ciche *et al.* 2006). After host death, adult nematodes continue to feed and reproduce within the cadaver until all resources have been depleted (Blanco-Pérez *et al.* 2017), which results in new free-living juveniles that leave the cadaver to begin the cycle over again (Greenwood *et al.* 2011).

Although we know that insect quinone secretions are effective antimicrobials (anti-fungal and anti-bacterial), it is unknown whether these host secretions represent a specific or generalized chemical defence. Understanding how quinones affect EPNs is valuable because these parasites are a major threat to insects; therefore, it is important to study the extent to which such host chemical defences may be effective against a range of infection threats. In addition, if quinone secretions serve as a chemical defence against EPNs, it will be critical to contextualize the role of the individual components by considering how they affect nematode survival, activity, and virulence. This work will also lead to a better understanding of how pest insects may defend themselves against commonly used biocontrol tactics, such as the fungus *Beauveria bassiana* or EPNs.

One prominent EPN is represented by the species *Steinernema carpocapsae*, which can be found on all continents except Antarctica (Bhat *et al.* 2020). This species is commonly used to control pest insects, including lepidopterans (e.g., webworms, cutworms, and armyworms) and coleopterans like weevils and wood-boring beetles (Dembilio *et al.* 2010; Levine & Oloumi-Sadeghi 1992; Marannino *et al.* 2003; Park *et al.* 2004; Viteri *et al.* 2018). *S. carpocapsae* is also capable of infecting tenebrionid beetles such mealworms (*Tenebrio molitor*), the larger black flour beetle (*Cynaeus angustus*), and the red flour beetle (*Tribolium castaneum*) (Erdoğuş 2021; Nansen *et al.* 2013; Prabowo *et al.* 2019). Notably, *T. castaneum* is a common pest globally and feeds on stored flour, grains, and bean products (Daglish 2005; Rafter *et al.* 2016). Given the importance of EPNs to insects, it would be very useful to know whether host quinone secretions could potentially act as a chemical defence against these lethal and ubiquitous parasites.

Materials and methods

Organisms and chemicals

We used infective juveniles of the EPN species *Steinernema carpocapsae* for this study (Capsanem© obtained from Koppert Biological Systems). For the nematode virulence assay, we used live waxworms of the species *Galleria mellonella* that were purchased from a local PetSmart®. This species was selected because it is commonly used as an experimental host for EPN research (Grewal *et al.* 1999; Krishnayya & Grewal 2002). The following chemicals were used to create the experimental treatments to which the nematodes were exposed: methyl-1,4-benzoquinone (MBQ) (Sigma-Aldrich 160 CAS 553-97-9), ethyl-1,4-benzoquinone (EBQ) (Toronto Research Chemicals CAS 4754-26-1), and 1-pentadecene (1P) (Sigma-Aldrich CAS 13360-61-7).

Experimental treatments

Nematodes were exposed to one of eight treatments (see Table 1). We chose these treatments to include a mixture of the key individual components found in natural *T. castaneum* secretions that represented the average ratio (75% EBQ, 15% MBQ, and 10% 1P) reported by other studies (Loconti & Roth 1953; Villaverde *et al.* 2007), as well as treatments with individual secretion components, including the two benzoquinones (EBQ and MBQ) at different concentrations. These high and low benzoquinone concentrations

Table 1. Experimental conditions and their corresponding concentrations used throughout the experiment. The benzoquinone mix treatment reflects the total concentration at naturally occurring proportions (75% EBQ, 15% MBQ, and 10% 1P) in *Tribolium castaneum* secretions

Treatments	Concentration 1	Concentration 2
Methy-1,4-benzoquinone (MBQ)	1.6 mg/mL	0.4 mg/mL
Ethy-1,4-benzoquinone (EBQ)	1.6 mg/mL	0.4 mg/mL
Benzoquinone mix	1.6 mg/mL[1]	–
1-Pentadecene (1P)	0.16 mg/mL	–
Ethanol solvent control	4%	
Distilled water control	n/a	

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were used to test potential effects on *S. carpocapsae* based on what may be secreted by a relatively small or large group of adult *T. castaneum*, given that each individual beetle can internally store up to 45 µg of quinones (Unruh *et al.* 1998). We did not test different concentrations of 1P. Rather, the 1P treatment represents the total quantity of this compound present in the mixed benzoquinone treatment. This was done to examine if 1P at a level found in naturally occurring *T. castaneum* secretions could contribute to survival-, behavioural-, or virulence-related changes in nematodes, as this compound is not known to be toxic. Because the benzoquinones used here do not dissolve in water, we included a solvent control treatment (4% ethanol to reflect its level in the high concentration conditions), as well as distilled water as a negative control.

Survival Assay

We tested the effects of nematode exposure to chemicals found in *T. castaneum* secretions using a nematode toxicity procedure modified from Barua *et al.* (2020). A single well of a 96-well tissue culture plate constituted a replicate in this experiment. Working solutions for each treatment (see Table 1) were prepared in 12-well plates and aliquoted into each replicate. Each well was first filled with 288 µL of distilled water before adding 2592 µL of the solution corresponding to its assigned treatment, with the final concentrations for each matching those described in Table 1. 200 µL aliquots of each treatment were randomly pipetted into wells of 96-well tissue culture plates, with each well containing 2–7 infective juvenile nematodes. The plates were kept at 22 °C in an incubator (Yamato® 37 L benchtop, model 1C-103C) between assessments of survival that occurred 0, 4, 8, 24, 72, and 96 hours after exposure began. We chose these time points because previous toxicology studies using *S. carpocapsae* measured survival from 2 days (Oliveira *et al.* 2019) to 4 days (Barua *et al.* 2020). Here, earlier time points were added to capture possible short-term toxicity, and observations were extended to 5 days as preliminary data showed changes to survival past the fourth day of exposure to the treatments.

Nematodes were prodded for up to 5 minutes at each time point under a dissecting microscope with a brush that consisted of a short piece of fishing line to confirm whether each was alive. A nematode was considered dead if it showed no movement and had gone rigidly straight rather than showing a curved position – a commonly used indicator of death in other studies (Barua *et al.* 2020; Molina *et al.* 2007). At the conclusion of this experiment, we then calculated the proportion of nematodes surviving in each well. The experiment was conducted with two observers that were responsible for three replicates each and was repeated 7 days later. Each treatment was thus ultimately represented by a total of twelve replicates.

Thrashing assay

The procedure for this assay was also modified from Barua *et al.* (2020); however, we decided to use individual nematodes in each well instead of groups to better see their fine movements. Each replicate was an individual well of a 96-well tissue culture plate that contained a single infective juvenile nematode. Nematodes were pipetted into plate wells containing 20 µL of distilled water before adding 180 µL of a treatment to create the concentrations outlined in Table 1. The location for each treatment replicate on the plate was randomly assigned. Thrashing was defined as a bend in the center of the nematode – in one direction and then back and the number of thrashes was counted for 30 seconds at each timepoint. Thrashing was measured after 0, 15, and 30 minutes of nematode exposure to treatment, as well as after 1, 4, 8, 24, and 48 hours. Behavioural observations were conducted by observing the individual wells under a dissecting microscope; otherwise, the plates were kept at 22 °C in an incubator (Yamato® 37 L benchtop, model 1C-103C) between time points. This assay was conducted in three runs, with each run consisting of three replicates from each treatment. The first week contained one run conducted by a single observer, whereas the final two runs were simultaneously conducted by different observers 1 week later. The total number of replicates for each treatment was a minimum of 14 to account for nematode death before the last timepoint. However, the two controls each had 18 replicates because three replicates for 1P treatment failed due to a solvent issue; therefore, a fourth round was required.

Virulence assay

Nematode virulence was assessed using a procedure modified from Grewal *et al.* (1999). Using a 48-well tissue culture plate, infective juvenile nematodes were exposed for 24 or 72 hours in groups of 24–38 to 400 µL of treatment; this represents one of the treatments found in Table 1. After this exposure period, the same treatment solution was pipetted into single wells in a 12-well plate with 800 µL of distilled water immediately added to each. Individual live nematodes were then pipetted into randomly assigned wells of 24-well plates lined with filter paper to which 30 µL of distilled water had been added. After each transfer, pipettes were rinsed with water to ensure no nematodes remained. After the nematode transfer was complete, a single late instar of *Galleria mellonella* was added to each well. In addition to testing how the treatments in Table 1 affected nematode virulence, we added another *G. mellonella* condition in which each instar received 30 µL of water with no nematode. This was done to confirm that waxworm death in the other conditions was due to nematode infection rather than background host death. The plates were then kept at 22 °C in an incubator (Yamato® 37 L benchtop, model 1C-103C) for 72 hours before counting the proportion of dead larvae for each treatment and exposure period. Each treatment had a total of 20 *G. mellonella* larvae used for both exposure periods (40 total per chemical treatment).

Statistical analysis

We conducted separate analyses for each assay using a generalized linear mixed model (GLMM) procedure. For each assay, nonsignificant main effects and interactions were removed one at a time in order of least significance, and the analysis was re-run until either a significant overall model containing only significant fixed effects remained or a significant overall model could not be achieved. For models with significant main effects, we used Tukey LSD post hoc tests to examine within-effect differences.

For the nematode survival assay, we first used Q-Q plots to determine if the data (proportion of nematodes surviving at each time point) met the assumption of normal distribution. This assumption was not met, so we used a GLMM with a gamma distribution and log link. We included the fixed effects of treatment and time point, as well as their interaction. The random effects of week, plate, and observer were also included. The data for the thrashing assay (number of thrashes during the observation period

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for each time point) also did not meet the assumption of a normal distribution. We used a repeated-measures GLMM with a Poisson distribution and log link given that we conducted observations of the same nematodes at different time points. The same fixed and random effects were included as for the survival assay.

Lastly, we used GLMMs with a binomial distribution and logit link for the virulence assay due to the binary nature of the data collected (i.e., the host was alive or dead). We included the fixed effects of nematode treatment and length of treatment exposure, as well as their interaction, along with the random effects of plate and well position. We conducted two analyses for this experiment. The first compared waxworm survival between the water-exposed nematode and no-nematode conditions in order to establish that host death was largely attributable to parasitism rather than a high background mortality rate. We then ran a second GLMM containing all conditions except for no-nematode in order to examine the effect of the secretion components on nematode virulence. All statistical analyses were conducted using SPSS 28.0.1.1.

Results

Survival

Our analysis of nematode survival resulted in an overall significant model ($F_{47, 527} = 3.625$, $P < 0.001$) that included the significant fixed effects of treatment ($F_{7,527} = 8.251$, $P < 0.001$) and time ($F_{5,527} = 19.634$, $P < 0.001$), as well as a significant interaction between these ($F_{35,527} = 2.093$, $P < 0.001$). Nematode survival decreased with time (Figure 1), with post hoc comparisons indicating a significant overall reduction in survival across treatments after 8 hours from the onset of exposure ($P = 0.008$). Post hoc tests for treatment indicated no significant difference in nematode survival between the water and solvent control ($P = 0.763$), nor was survival in the 1P treatment different from either control (all $P \geq 0.528$). Nematode survival was lowest in the quinone mixture treatment and significantly differed from that in all other treatments (all $P < 0.007$). Survival in the HMBQ and HEBQ treatments was also significantly lower than that of nematodes in both control treatments (all $P \leq 0.012$). Lastly, while nematodes in the LMBQ condition had significantly reduced survival compared to those only exposed to water ($P = 0.031$), this showed only a marginally insignificant difference compared to those in the ethanol solvent control treatment ($P = 0.062$).

Thrashing

The GLMM for this assay generated a significant overall model ($F_{14, 983} = 9.316$, $P < 0.001$) that included the significant fixed effects of treatment ($F_{7,983} = 4.454$, $P < 0.001$) and time ($F_{7,983} = 14.270$, $P < 0.001$). Thrashing generally decreased over time across treatments (Figure 2), with post hoc tests indicating that a significant reduction began after 1 hour of exposure compared to the initial timepoint ($P = 0.037$). As seen for the survival assay, nematodes in the water and ethanol control treatments showed no difference in their thrashing ($P = 0.57$), and thrashing by those exposed to 1P also did not differ from those in the water ($P = 0.959$) or ethanol ($P = 0.609$) treatments. Thrashing was significantly higher in LEBQ compared to HMBQ ($P = 0.047$), which was the only significant difference seen for any of the quinone-related treatments. However, there was a tendency towards more thrashing in nematodes exposed to LEBQ compared to ethanol ($P = 0.072$), as well as compared to the water ($P = 0.057$) and 1P treatments ($P = 0.059$). Nematode thrashing in the quinone mix also tended to be higher than that seen in the water ($P = 0.072$) and 1P ($P = 0.075$) treatments and showed a marginally insignificant increase compared to HMBQ ($P = 0.055$).

Virulence

Our first analysis for this assay only considered whether the survival of *G. mellonella* (waxworm) larvae differed depending on whether they were uninfected or infected by water-exposed nematodes; this

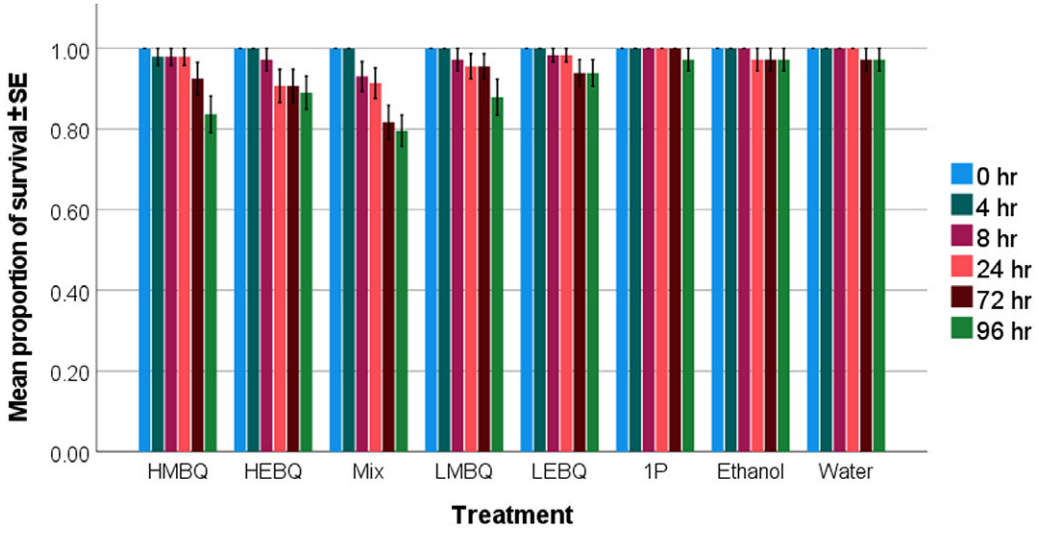

Figure 1. Mean (±SE) proportion of survival of *Steinernema carpocapsae* infective juveniles exposed to high (1.6 mg/mL) and low (0.4 mg/mL) concentrations of methyl-1,4-benzoquinone (MBQ), ethyl-1,4-benzoquinone (EBQ), 1-pentadecene (1P), and a mix of the conditions at naturally occurring proportions (see Table 1). Distilled water and ethanol served as controls. Proportion of surviving nematodes is shown at 0, 4, 8, 24, 72, and 96 hours post-exposure to the conditions. Abbreviated treatments: HMBQ – high methyl-1,4-benzoquinone; HEBQ – high ethyl-1,4-benzoquinone; mix – high mix of quinone secretion (75% EBQ, 15% MBQ, and 10% 1P); LMBQ – low methyl-1,4-benzoquinone; LEBQ – low ethyl-1,4-benzoquinone.

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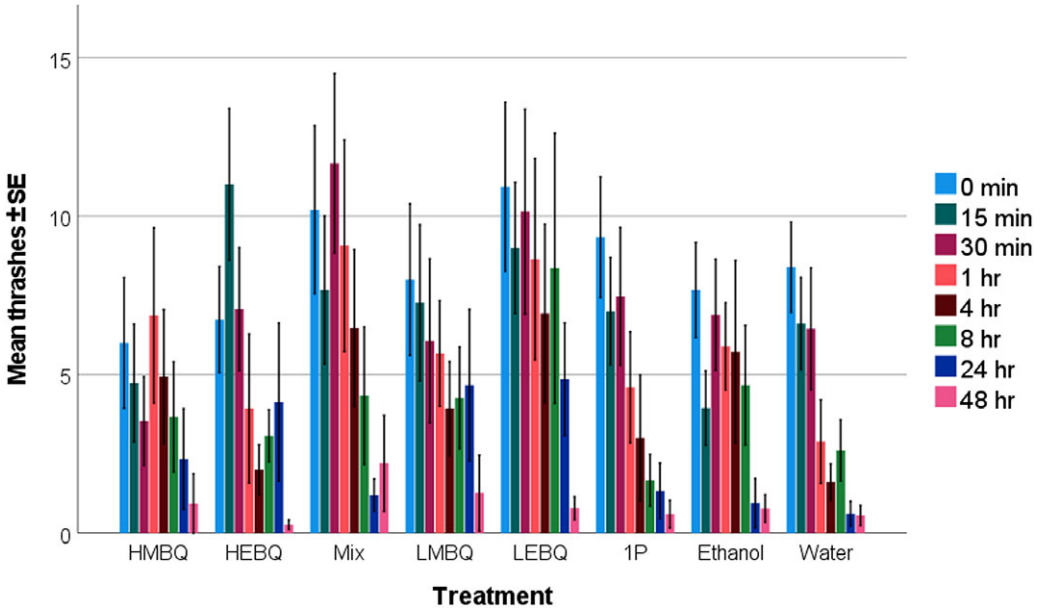

Figure 2. Mean (±SE) thrashing of *Steinernema carpocapsae* infective juveniles exposed to high (1.6 mg/mL) and low (0.4 mg/mL) concentrations of methyl-1,4-benzoquinone (MBQ), ethyl-1,4-benzoquinone (EBQ), 1-pentadecene (1P), and a quinone mix of the 3 compounds (see Table 1). Distilled water and ethanol served as controls. The number of thrashes in a 30-second interval was counted at the following timepoints (0, 15, 30, 60 minutes, 4, 8, 24, and 48 hours) after exposure to each treatment. Abbreviated treatments: HMBQ – high methyl-1,4-benzoquinone; HEBQ – high ethyl-1,4-benzoquinone; mix – high mix of quinone secretion (75% EBQ, 15% MBQ and 10% 1P); LMBQ – low methyl-1,4-benzoquinone; LEBQ – low ethyl-1,4-benzoquinone.

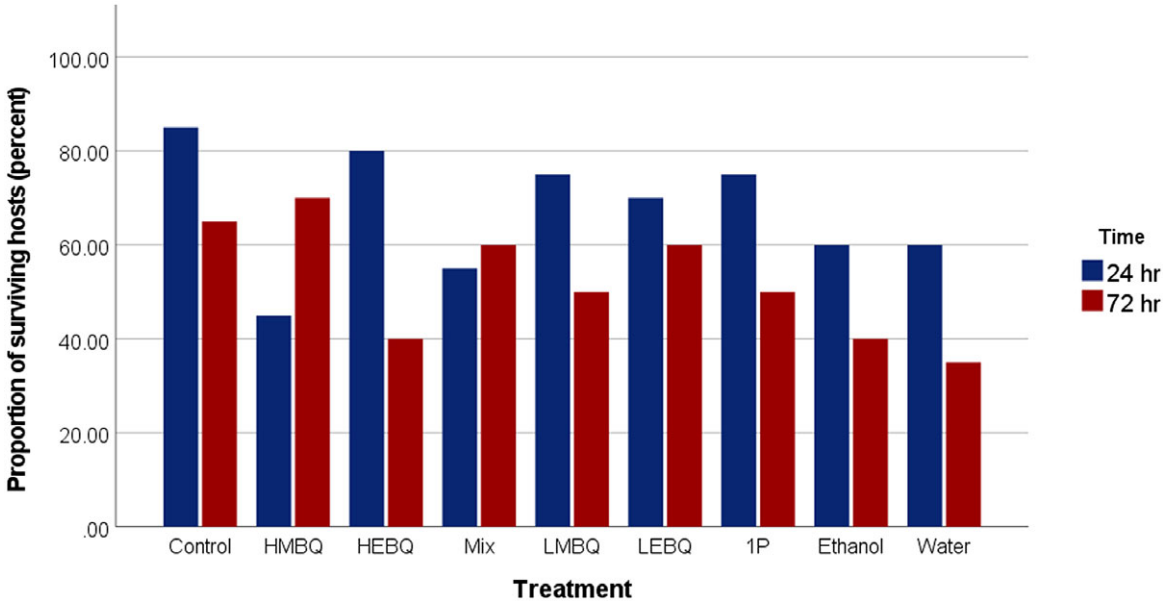

Figure 3. Proportion of surviving larvae of the host species *Galleria mellonella* exposed to *Steinernema carpocapsae* infective juveniles exposed to high (1.6 mg/mL) and low (0.4 mg/mL) concentrations of methyl-1,4-benzoquinone (MBQ), ethyl-1,4-benzoquinone (EBQ), 1-pentadecene (1P), and a mix of the 3 compounds for either 24 or 72 hours. Distilled water and ethanol served as treatment controls, whereas control hosts were not exposed to nematodes. Abbreviated treatments: HMBQ – high methyl-1,4-benzoquinone; HEBQ – high ethyl-1,4-benzoquinone; mix – high mix of quinone secretion (75% EBQ, 15% MBQ and 10% 1P); LMBQ – low methyl-1,4-benzoquinone; LEBQ – low ethyl-1,4-benzoquinone.

was used to establish the extent to which host death in the primary virulence assay could be attributed to nematode infection rather than high baseline mortality. This GLMM resulted in a significant overall model ($F_{2,77} = 5.218$, $P = 0.007$) that included the significant fixed effects of infection ($F_{1,77} = 7.018$, $P = 0.01$) and time ($F_{1,77} = 4.835$, $P = 0.031$). Nematode infection significantly reduced *G. mellonella* survival, and waxworm survival was generally lower in the 72-hour treatment group.

A second GLMM that excluded the no-nematode condition was thus used to test for the effect of nematode exposure to beetle quinone secretion components on their virulence to *G. mellonella*, having established that host mortality was largely attributable to infection. This GLMM resulted in a significant overall model ($F_{1,318} = 7.539$, $P = 0.006$), but time was the only significant fixed effect as hosts generally showed reduced survival if infected by nematodes exposed to treatments for 72 hours compared to 24 hours (Figure 3).

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Discussion

By exposing the entomopathogenic nematode *S. carpocapsae* to benzoquinones, we quantified how these known antimicrobial compounds that are found in insect secretions affect nematode survival, thrashing behaviour, and virulence, thus potentially also serving as a defence against macroparasites. Our findings indicate that benzoquinones have varied effects on *S. carpocapsae* as a whole. Exposure to high concentrations of benzoquinones consistently reduced nematode survival with time; however, there were less consistent outcomes for nematode thrashing. The quinone mix and LEBQ were the only two treatments to significantly increase thrashing, indicating that the toxic effects of these compounds do not interfere with nematode movement. Additionally, non-lethal exposure to benzoquinones does not appear to affect the virulence of *S. carpocapsae* to a model host (*G. mellonella*). Our results therefore indicate that benzoquinones could serve as an effective defence against a wider range of parasites than previously observed, but also that *S. carpocapsae* is relatively resistant to short-term exposure.

We observed reduced nematode survival in the quinone-containing treatments (HMBQ, HEBQ, and quinone mix), especially at the higher concentrations tested. Although quinone compounds from whole millipede secretions increased mortality in a free-living nematode (Gasch *et al.* 2013), this is the first study to show that quinones may serve as an effective defence against EPNs as well as to identify how the individual compounds from arthropod quinone secretions affect parasite survival. Despite a number of nematodes surviving quinone exposure in our experiment, we posit that there would still be defensive benefits primarily driven by reducing the number of living nematodes in the vicinity of the potential host, thus lowering the risk of infection by reducing encounter. Although quinones are known to be effective against various microparasites (Pedrini *et al.* 2015; Yezerski *et al.* 2007), our results show they could affect parasite–host relationships more broadly than previously known. Although both MBQ and EBQ were toxic here, that nematode survival was reduced even at the lower MBQ concentration could indicate that *S. carpocapsae* is more sensitive to this particular compound. Thus, future studies should investigate the specific roles of both compounds in parasite defence.

When comparing the relative toxicity of MBQ and EBQ, the free-living nematode *C. elegans* seems more sensitive than *S. carpocapsae*, as Gasch *et al.* (2013) found that 48 hours of exposure at concentrations lower than our highest (approximately 0.51 mg/mL) killed most individuals. This difference could be due to greater resistance by EPNs to oxidative stress, but infective juveniles of EPNs also represent a dauer stage (Wright & Perry 2002). The infective juveniles retaining this cuticle of their previous life stage which increases the overall thickness of the cuticle in a manner that provides increased protection against environmental harm, including desiccation and exposure to chemicals (Glazer 2002). Related to this, disruptions to cuticle-maintaining genes have been shown to significantly reduce the ability of *C. elegans* to withstand toxicity from the herbicide paraquat (Sandhu *et al.* 2021). However, to cause toxic effects, quinones must be able to penetrate the cuticle, and some have speculated that volatile compounds (like benzoquinones) may accomplish this via diffusion (Barua *et al.* 2020). Some individual EPNs in this study seemed to have high resistance to quinone exposure while others are more sensitive; this could be caused by intraspecific variation within the population but requires further study. Although a thicker cuticle is the most likely reason for the higher benzoquinone tolerance that we observed with juvenile *S. carpocapsae* vs. previous results with adult *C. elegans*, larval EPNs also have an arrested development stage during which their metabolism is reduced, but their resistance to oxidative stress increases (Anderson 1982; Vanfleteren & de Vreese 1996). Future investigations should thus explore the role of the cuticle in protecting nematodes against the toxic effects of benzoquinones, as well their mechanism of action.

Although we found evidence that quinones had some effect on thrashing behaviour, it seemed to be limited; we observed increased thrashing in the LMBQ and quinone mix, but HMBQ appeared to cause a slight reduction. Because there was no consistent effect of any quinone-related treatment on thrashing, more studies are needed to understand the potential of quinones to affect EPN behaviour given that various chemicals are known to affect their activity. Interestingly, various toxicological studies have specifically reported reduced thrashing in *C. elegans* as a result of ROS damage (Li *et al.* 2012; Wu *et al.* 2012). Quinones did not consistently reduce locomotion in *S. carpocapsae* in our study, which is inconsistent with negative ROS effects on behaviour. Increased activity could be indicative of quinones acting as chemical irritants and triggering nematode escape behaviour. However, to the best of our knowledge, there are no studies at this time which have reported that chemical irritants increase thrashing in nematodes. Alternatively, the increased thrashing in some of the quinone treatments could indicate that these compounds are actually excitatory by signalling the presence of a nearby host because a wide range of chemicals originating from both plants and insects act as EPN attractants (Zhang *et al.* 2021). These compounds could be used to identify precise host locations (chemotaxis), but a general increase in nematode activity (kinesis) may also make host encounter more likely even without directed movement. Additionally, the free-living infectious stages of nematodes and other parasites vary in their mobility. The extent to which such stages are stationary could impact how long they are exposed to host secretions, thereby affecting the effectiveness of the latter against particular parasites.

Although MBQ and EBQ have not previously been tested with respect to EPN toxicity or behaviour, other investigations have examined the role of 1,4-benzoquinone in terms of nematode chemotaxis. These have shown that some species of EPN, including *S. carpocapsae,* respond to 1,4-benzoquinone as an attractant and move towards it during choice tests (Dillman *et al.* 2012). Exposure to 1,4-benzoquinone at concentrations less than those used in our experiment also increased the rate of jumping in *S. carpocapsae*, which is likely an important behaviour used to attach to potential hosts (Campbell & Kaya 1999). This could indicate that the behaviours we observed in our thrashing assay were a response to the presence of host chemical cues, with resultant changes in movement being a form of host-searching. Interestingly, at the concentrations we used, 1P did not increase *S. carpocapsae* thrashing. Thus, it is unlikely to be used as a host presence or location cue despite the fact that insects such as *Tribolium* beetles use it as a semiochemical to sense one another (Đukić *et al.* 2021).

Our results demonstrate that quinone exposure did not significantly affect the ability of *S. carpocapsae* to infect and kill larvae of the moth *G. mellonella* compared to unexposed nematodes. Although days-long exposure to quinones was lethal, it seems that surviving nematodes were not less virulent. This would indicate that there was a range in sensitivity to benzoquinones observed in the nematodes, with the most sensitive dying as a result of exposure. This was likely driven by variation among individual nematodes when it comes to the toxic effects of quinones, potentially driven by differences in genetics, condition, age, or other factors. Because those that survived quinone exposure were also still infective, they

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thus demonstrated high resistance to quinones overall. There may be intraspecific variation for such resistance and maintenance of virulence, but further study would be needed to quantify this on a population level.

Whereas our study is the first to examine MBQ and EBQ as a defence against EPNs, others have demonstrated that quinone derivatives can be toxic to nematodes, especially those infecting plants. For example, the application of hydroquinones to tomato plants reduced the survival of their root-knot nematodes (*Meloidogyne incognita*) (Oliveira *et al.* 2019). Other nematode toxicology assays that tested quinone derivatives include that of chlorinated benzoquinones on *C. elegans* and that of anthraquinones on *Meloidogyne incognita* (Mayer 1995). The general mechanism of toxicity for these other quinone compounds seems to be the same and involves ROS generation that causes oxidative damage to cells (Chen *et al.* 2015; Kanvah & Schuster 2006). Interestingly, plants use many non-quinone chemical compounds in defence, such as hydrogen peroxide with peroxidase (Zacheo *et al.* 1982) and alpha-terthienyl (Faizi *et al.* 2011), which also have toxic effects against nematodes by generating ROS. This supports the need for further investigation into the mechanisms by which EBQ and MBQ reduced *S. carpocapsae* survival here.

Our results demonstrate the potential of benzoquinones to be used as a defence by insects against entomopathogenic nematodes, and our study is the first to demonstrate toxic effects of MBQ and EBQ against EPNs to our knowledge. This indicates that benzoquinones play a more generalized role in parasite and pathogen defence than previously observed, given their known effects on bacteria and fungi. Although this work demonstrates the effects of benzoquinones on *S. carpocapsae*, more study is needed to fully contextualize the role of these compounds in insect defence by further testing how quinone secretions and 1-pentadecene may impact EPNs at different concentrations, as well as introducing quinones in a more natural setting to see if this changes how they interact with nematode pathogens. Quinones should also be tested using other species of EPNs, as well as other helminths using arthropod hosts (e.g., tapeworms). Further research is also required with respect to the mechanism(s) of quinone toxicity against EPNs, as well as exploring the effects of benzoquinones on their behaviour. Given the threat that parasites and pathogens pose to insects, as well as their use in biocontrol, it is important to understand how such hosts can defend themselves through chemical means.

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