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Behavioural quiescence reduces the penetration and toxicity of exogenous compounds in second-stage juveniles of *Heterodera glycines*

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Summary – Inactivity in nematodes is often correlated with survival of adverse environments. The non-feeding second-stage juvenile (J2) of *Heterodera glycines* must survive in a soil environment that may contain numerous toxins. In this report, we show that quiescent J2 of *H. glycines* survived higher concentrations of both ethanol and the plant-derived compound, allyl isothiocyanate, compared with actively moving nematodes. The mechanism for this quiescence-mediated resistance was investigated using fluorescein isothiocyanate (FITC). There was a reduction in the penetration of FITC in quiescent J2 of *H. glycines* compared with that in actively moving non-feeding J2. Furthermore, exposure of quiescent nematodes to octopamine, an invertebrate neurotransmitter, induced activity and a subsequent increase in FITC penetration compared with quiescent nematodes exposed to FITC alone. These data demonstrate that behavioural quiescence is correlated with exclusion of the compound from the body of the nematode. Finally, the entry point of FITC into the nematode was examined by the application of a veterinary cyanoacrylate adhesive to occlude either the cephalic or caudal openings of the nematodes. Nematodes glued at the anterior end showed a significant reduction in fluorescence compared with nematodes glued on the posterior end and non-glued nematodes. Thus, the entry of FITC is primarily through openings in the cephalic region. This research is the first report of behavioural quiescence correlated with reduced sensitivity to toxins in a plant-parasitic nematode, and provides insight into how these important organisms cope with stress due to exogenous toxins.

Keywords – chemical uptake, hypobiosis, soybean cyst nematode, survival, xenobiotic.

The non-feeding infective second-stage juvenile (J2) of the soybean cyst nematode, *Heterodera glycines*, lives in a soil environment that may contain compounds toxic to nematodes, including secondary metabolites of plants and microorganisms and man-made pesticides. Previous research has shown that a wide variety of compounds penetrate into the non-feeding infective stage of plant-parasitic nematodes (Marks *et al*., 1968; Bird, 1979; Djian & Pijarowski, 1996; Winter *et al*., 2002; Schroeder & MacGuidwin, 2007). Nematodes can escape the effects of toxins by either moving away from the source of the compound or by using endogenous mechanisms to tolerate the toxins.

Inactivity in nematodes is often correlated with survival of adverse environments. The various forms of inactivity in nematodes have been described as hypobiosis, cryptobiosis, quiescence, dormancy and diapause. The specific definitions for these terms are debated in the literature (Van Gundy, 1965; Evans & Perry, 1976; Demeure & Freckman, 1981; Womersley *et al*., 1998; Wharton, 2002). Quiescence is typically defined as a temporary state of inactivity induced by any of several environmental conditions including adverse temperature, osmotic stress and dehydration. However, it has been observed that plant-parasitic nematodes are often found in a quiescent state without any obvious cause and this suggests that quiescence in nematodes may be due to a lack of sensory stimulation or sensory habituation (Croll, 1970). Here, we define quiescence as inactivity, immediately reversible upon a stimulating change in conditions.

Previous data have indirectly associated quiescence with survival to toxins in non-feeding parasitic nematodes. Thomason and McKenry (1974) showed a four- to nine-fold reduction in sensitivity to two fumigant ne-

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maticides for *Meloidogyne javanica* at 5°C compared with sensitivity at 25°C. More recent work with the pine-wilt nematode, *Bursaphelenchus xylophilus*, demonstrated that 50% more methyl iodide was necessary to kill 100% of the population at 10-15°C compared with the kill at 25°C, even after accounting for the increased volatility of the product at higher temperatures (Soma *et al*., 2005). In these latter two studies, no confirmation of quiescence was made, although it must be assumed that 5°C would induce quiescence in *M. javanica*. Many animal-parasitic nematodes in an arrested state are less affected by anthelmintics given to the host (Schad & Page, 1982; Prichard, 1988; Sargison *et al*., 2007).

Freckman *et al*. (1980) directly correlated quiescence with survival to exogenous toxins for the mycophagous nematode, *Aphelenchus avenae*. Anhydrobiotic *A. avenae* were approximately five times less sensitive to methyl bromide than active nematodes. The authors proposed three alternatives to explain the increased resistance to methyl bromide seen in anhydrobiotic *A. avenae*: *i*) reduced penetration of the compound into the body when in an anhydrobiotic state; *ii*) reduced efficacy due to a lowered metabolism in the anhydrobiotic nematodes; *iii*) reduced solubility of methyl bromide in the absence of free water (Freckman *et al*., 1980).

The first objective of the current study was to determine if quiescence affects the sensitivity of J2 *H. glycines* to toxins. Quiescence was induced for this objective by exposing nematodes to both reduced temperatures and increased carbon dioxide. Ethanol, toxic to nematodes at appropriate concentrations, and allyl isothiocyanate (AITC), a plant-derived compound found in the Brassicaceae that is considered as an alternative to synthetic nematicides (Lazzeri *et al*., 1993; Buskov *et al*., 2002; Zasada & Ferris, 2004; Matthiessen & Kirkegaard, 2006), were the toxins used in our experiments.

The second objective was to test if quiescence is correlated with reduced penetration of an exogenous compound in J2 *H. glycines*. For this objective, we used fluorescein isothiocyanate (FITC), a non-toxic fluorescent compound. Fluorescein compounds are known to penetrate the non-feeding stage of plant-parasitic nematodes (Bird, 1979; Winter *et al*., 2002; Schroeder & MacGuidwin, 2007). To correlate behaviour with chemical penetration further, we examined the penetration of FITC into nematodes shifted from a quiescent to an active state by means of neurotransmitters.

As an ancillary objective, we used the FITC assay to test empirically how exogenous compounds enter into non-feeding J2 of *H. glycines* by occluding the caudal and cephalic regions of active J2 with a non-toxic cyanoacrylate adhesive.

Materials and methods

Nematodes

Heterodera glycines, collected from a soybean field in southeastern Wisconsin USA, was maintained in a growth chamber on susceptible soybean cv. McCall at 28°C and 12 h photoperiod. Cysts were extracted using a procedure modified from Jenkins (1964). J2 of *H. glycines* were obtained by incubating eggs on 25 μm pore filters (Sefar American, Depew, NY, USA) in 3 mM $ZnCl_2$ to induce hatch (Wong *et al.*, 1993). Nematodes were used within 1 week after hatching unless noted.

Lethality assays – temperature induced quiescence

Ethanol and AITC were purchased commercially (Sigma, St Louis, MO, USA). Specific concentrations were chosen based on preliminary data and previous published research regarding the toxicity of these compounds (Zasada & Ferris, 2004). The AITC was initially diluted with methanol and subsequently dissolved in 1.5% agarose to achieve the desired concentrations. At the highest concentrations of AITC, a slight precipitate formed in the agarose. Active J2 of *H. glycines* (40 $\pm$ 4 per treatment) were placed in 1 ml water for 3 h at room temperature to maintain activity or on ice to induce quiescence. Confirmation of quiescence and the ability to recover was made for nematodes incubated on ice. Nematodes in a quiescent state were transferred to AITC-containing plates stored at 4°C, whilst actively moving nematodes were transferred to AITC at room temperature (20-22°C). After 3 h exposure to AITC, nematodes were washed onto a 25 μm pore sieve and rinsed thoroughly with water. Nematodes were stored overnight to recover from exposure to AITC. The next day, nematodes were evaluated for movement by a researcher blind to the treatment identity. Nematodes were considered alive if they moved spontaneously or after being mechanically stimulated with a thin wire. This experiment was conducted four times. Probit analysis was used to estimate the LD_{50} and LD_{95} $\pm$ 95% CI. Statistical comparison of probit curves was made using the *Z*-test. This and all subsequent data were analysed using Minitab Statistical Software (Minitab, State Col-

lege, PA, USA). Ethanol lethality was examined similarly to AITC lethality. However, ethanol was diluted with deionised H_2O to achieve the desired concentrations and nematodes were exposed directly to this solution. Experiments with ethanol were conducted twice.

Lethality assays – CO_2-induced quiescence

Gaseous CO_2 was conducted from a flask to an enclosed experimental chamber *via* Tygon tubing. Temperature was recorded within the CO_2 experimental chamber to ensure that it did not deviate from ambient conditions. Active J2 of *H. glycines* (96 ± 3 per treatment) were incubated in 1 ml water for 3 h either in ambient conditions to maintain activity or within the enclosed CO_2 chamber to induce quiescence. A subsample of J2 was taken from the CO_2 chamber to confirm quiescence and the ability to recover. Nematodes were exposed to AITC for 3 h in ambient conditions or within the CO_2 chamber. Subsequent washing and examination was performed identically to the temperature induced quiescence experiment. This experiment was conducted twice.

FITC exposure and fluorescent measurements

Fluorescein isothiocyanate was purchased commercially (Sigma). Stock solutions of 5 mg ml^{-1} FITC in dimethylformamide (DMF) were stored at 4°C and used within 1 week. The final exposure concentration was 0.1 mg ml^{-1} FITC with 2% DMF in deionised water with a 3 h exposure time. Nematodes not exposed to FITC were included in all experiments as negative controls for fluorescence measurements. After exposure to FITC, all nematodes were washed repeatedly in deionised water. Fluorescence was measured as described previously (Schroeder & MacGuidwin, 2007). Briefly, after exposure to FITC, nematodes were mounted and examined at $\times 200$ magnification using a fluorescent compound microscope with mercury vapour lamp and Olympus NIB filter (excitation 480 ± 10 nm, emission 515 nm long pass). Both brightfield and fluorescent images were taken of individual nematodes at equivalent focal planes and magnification. All fluorescent images for a given experiment were taken with equivalent exposure times. Images were then analysed using the imaging software WCIF-ImageJ, version 1.37 (http://www.uhnres.utoronto.ca/facilities/wcif/imagej/) to calculate the percentage of an individual's body that fluoresced beyond the negative control (Schroeder & MacGuidwin, 2007). Statistical comparisons were made using Student's *t*-test or, where appropriate, ANOVA with Fisher's LSD for comparison between means.

Environmental and endogenous factors affecting FITC penetration

Temperature effects on FITC penetration were examined by comparing J2 of *H. glycines* (5-9 per treatment) in 0.1 mg ml^{-1} FITC at 4, 16, 32 or 36°C with nematodes incubated at 25°C in FITC. Originally actively moving nematodes were incubated at these temperatures for 1 h prior to and during the incubation with FITC. Each comparison with nematodes at 25°C was made in separate experiments. Each temperature was tested at least twice with data pooled from each experiment.

Mechanically hatched J2 were obtained by gently opening eggs with a wire pick. Six mechanically hatched J2, without any obvious morphological defects, were incubated in FITC and compared with actively moving individuals hatched in 3 mM $ZnCl_2$. This experiment was conducted twice with data pooled from both experiments.

Quiescent J2 from the laboratory were obtained by hatching *H. glycines* in 3 mM $ZnCl_2$. Hatched J2 were then stored at 4°C for approximately 1 month. Morphologically normal individuals were separated into groups of (4-9 per treatment) active and quiescent nematodes and then exposed to FITC. Prior to imaging, quiescent nematodes were mechanically stimulated with a wire pick to confirm viability. This experiment was repeated twice with data pooled from each experiment.

Quiescent J2 from the field were recovered from an infested soybean field at the Hancock Agricultural Research Station (Hancock, WI, USA) using centrifugation in a sucrose solution to obtain both cysts and vermiform J2 (Jenkins, 1964). Cysts were placed in hatching chambers with 3 mM $ZnCl_2$ to induce hatch. Five nematodes recovered from hatching chambers served as actively moving positive controls and were compared for penetration of FITC with 11 morphologically normal but non-moving vermiform J2 extracted directly from the soil. Between exposure to FITC and imaging, quiescent nematodes were mechanically stimulated with a wire probe to confirm viability. This experiment was repeated once with data pooled from each experiment.

Effect of biogenic amines on FITC penetration

Active J2 of *H. glycines* (5-10 per treatment) were exposed to 10 mM serotonin or octopamine for 1 h prior to and during a 3 h exposure to FITC. Concentrations were

chosen based upon previous work with biogenic amines in plant-parasitic nematodes (Twomey *et al*., 2000; Urwin *et al*., 2002; Masler, 2007). Nematodes exposed to each compound were compared with active nematodes exposed to FITC alone. This experiment was conducted three times. The effect of octopamine on behavioural quiescence was determined by exposing quiescent J2 (15-30 per treatment) obtained from a population hatched 1 month earlier to either M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 85.5 mM NaCl, 1 mM $MgCl_2$) with 50 mM octopamine or M9 buffer alone. Nematodes were incubated for 3 h at room temperature and then examined for spontaneous movement. This experiment was repeated twice with data pooled from each experiment. Treatments with non-overlapping 95% confidence intervals, determined using the one-tailed Fisher's exact test, were considered significantly different. To examine if octopamine increases the penetration of FITC into quiescent nematodes, inactive nematodes (10-15 per treatment) obtained from a population hatched 1 month earlier and stored at 4°C, were placed in 50 mM octopamine with 10% M9 buffer or 10% M9 buffer alone and incubated at room temperature for 3 h followed by exposure to FITC. Prior to image analysis, nematodes were mechanically stimulated to confirm viability. This experiment was conducted three times.

Route of chemical entry

Active nematodes were temporarily immobilised by placing them on 1 ml agar pads chilled on ice. The anterior or posterior openings of J2 of *H. glycines* (4-7 per treatment) were blocked by applying a drop of Nexaband Topical Tissue Adhesive (Henry Schein, Melville, NY, USA) to either the head or tail. Adhesive was applied using capillary tubes fashioned from Pasteur pipettes. Once the adhesive dried, nematodes were exposed to FITC at room temperature. Active nematodes not glued were also exposed to FITC. After exposure, nematodes were washed with water. The adhesive was then carefully removed from the nematodes using endodontic files (Henry Schein) followed by additional washing in H_2O. Only nematodes that survived this procedure were examined for fluorescence. An additional group of nematodes with no adhesive was included as a positive control. This experiment was conducted twice with data pooled from each experiment.

Results

Comparison of sensitivity of active and quiescent J2 of *Heterodera glycines* to exogenous toxins

Quiescent J2 of *H. glycines* were less sensitive ($P < 0.001$) to exogenous toxins than active nematodes (Table 1; Fig. 1). Nematodes that were quiescent due to chilling required a 2.5× greater concentration of AITC to achieve the LD_{50} compared with actively moving nematodes. All nematodes exposed to chilling were in a quiescent state but recovered within 10 min when returned to ambient temperatures (data not shown). Nematodes that were quiescent due to CO_2 exposure required a 3.5× greater concentration of AITC to kill 50% of the individuals compared with actively moving nematodes. All nematodes exposed to CO_2 for 3 h were in a quiescent state but resumed activity within 30 min upon return to ambient conditions (data not shown). Nematodes quiescent due to temperature required 78% more ethanol to achieve the LD_{50} compared with actively moving nematodes. Both AITC and ethanol inhibited movement during the exposure period, even at doses below the LD_{50}. Obvious inter-

Table 1. *Effect of quiescence on the sensitivity of J2 of* Heterodera glycines *to allyl isothiocyanate and ethanol.*

Status	Compound	LD_{50}	LD_{95}	*P*-value*
Cold-induced quiescence	AITC	4.68 ± 0.35 mM	6.18 ± 0.51 mM	<0.001
Active		1.79 ± 0.34 mM	3.29 ± 0.51 mM	
CO_2-induced quiescence	AITC	6.19 ± 0.78 mM	10.34 ± 1.26 mM	<0.001
Active		1.76 ± 0.62 mM	5.91 ± 1.12 mM	
Cold induced quiescence	Ethanol	25.6 ± 2.04%	28.4 ± 2.85%	<0.001
Active		15.5 ± 1.48%	18.3 ± 2.33%	

AITC, allyl isothiocyanate; LD_{50}, LD_{95}, concentration required to kill 50 and 95%, respectively of the test group after a 3 h exposure ± 95% CI as determined by probit analysis.

* Comparison of active and quiescence probit curves.

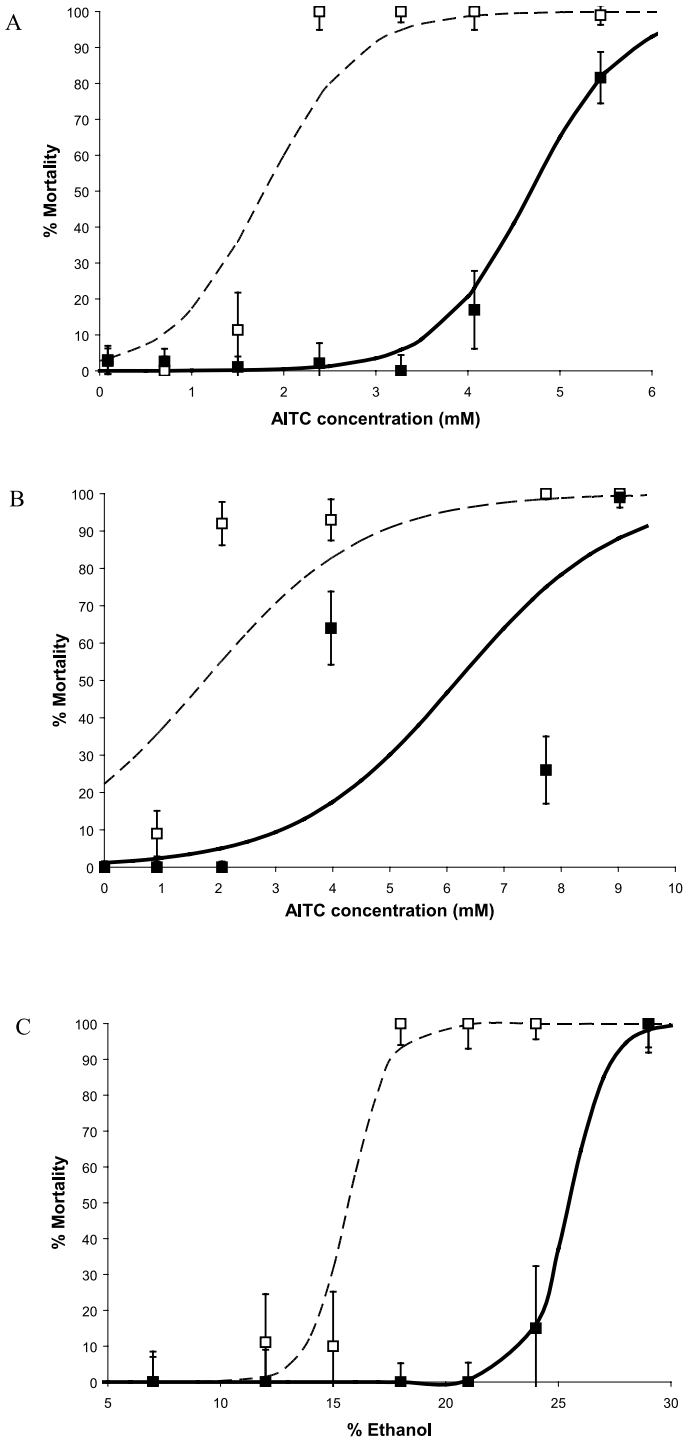

Fig. 1. *Dose-response curves for exposure of active (□) or quiescent (■) J2 of* Heterodera glycines *to toxins. A: Exposure to AITC with quiescence induced by chilling; B: Exposure to AITC with quiescence induced by CO_2; C: Exposure to ethanol with quiescence induced by chilling. Error bars represent 95% CI as determined by the exact method. Curves were fit using probit analysis to determine the LD_{50} and LD_{95} (see Table 1).*

nal morphological damage occurred following exposure to high concentrationsof both compounds.

QUIESCENCE AND THE PENETRATION OF FITC INTO *HETERODERA GLYCINES* J2

Between 4 and 25°C there was a positive correlation between temperature and FITC penetration as measured by fluorescence (Fig. 2). All nematodes exposed to 4°C were in a quiescent state. Nematodes exposed to 4°C showed 92% less fluorescence after exposure to FITC compared with nematodes actively moving at 25°C ($P < 0.001$). Nematodes exposed to 16°C, a typical soil temperature for soybean germination in the field, were either in a quiescent state or, if active, appeared more sluggish than individuals kept at 25°C. Nematodes exposed to 16°C showed 32% less fluorescence compared with nematodes at 25°C ($P = 0.015$). Nematodes exposed to 32 and 36°C showed no difference in fluorescence compared with nematodes at 25°C ($P = 0.228$ and $P = 0.891$, respectively) even though 36°C did induce quiescence in J2. However, nematodes exposed to FITC at 36°C showed an altered distribution of fluorescence with more cuticular and less intestinal staining than other treatments (Fig. 3).

Mechanically hatched inactive J2 had 57% less fluorescence following exposure to FITC compared with actively moving J2 ($P = 0.035$) (Fig. 4). Nematodes hatched in the laboratory and in a non-induced quiescent state showed 59% less fluorescence after exposure to FITC compared with actively moving J2 from the same hatching cohort ($P < 0.001$). Quiescent nematodes recovered from an infested soybean field had 65% less fluorescence

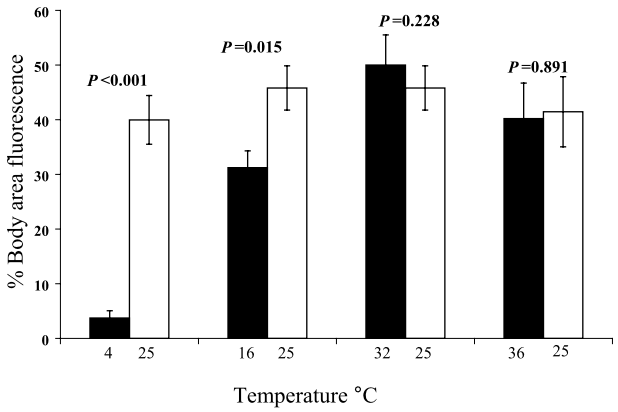

Fig. 2. *The effect of temperature-induced quiescence on penetration of FITC into J2 of* Heterodera glycines. *Active nematodes were incubated at varying temperatures prior to and during exposure to 0.1 mg m^{-1} FITC. Each pair-wise comparison represents a separate experiment. Black bars represent the test temperature listed on the* x*-axis and white bars represent the positive control tested at 25°C. Fluorescence was calculated as the percentage of the nematode's body that fluoresced beyond the negative control nematodes not exposed to FITC.* P*-values were calculated using Student's* t*-test.*

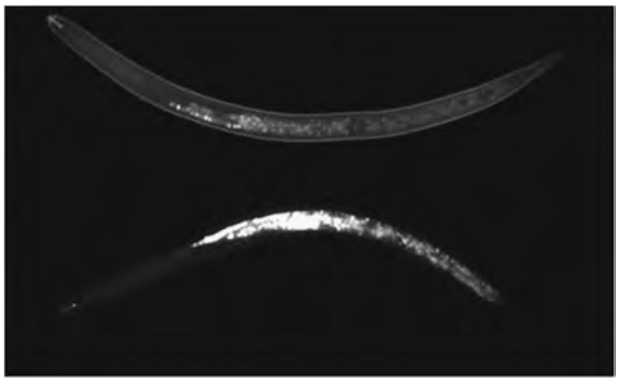

Fig. 3. *Fluorescent micrographs of J2 of* Heterodera glycines *incubated at 36°C (top) or 25°C (bottom) for 1 h prior to and during exposure to 0.1 mg ml^{-1} FITC. Nematodes exposed to FITC at 36°C show more cuticular staining and less intestinal staining than nematodes exposed to FITC at 25°C. Images were taken at ×200 magnification with equivalent exposure times.*

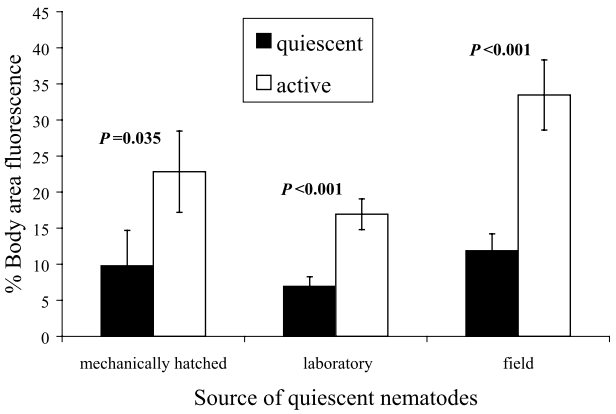

Fig. 4. *The effect of non-induced quiescence on FITC penetration in J2 of* Heterodera glycines. *Quiescent nematodes were obtained by mechanically opening eggs containing J2 of* H. glycines *(mechanically hatched), selecting non-moving but viable individuals from a population hatched 1 month prior to FITC exposure (laboratory), or recovering non-moving but viable individuals from an infested soybean field (field). These nematodes were compared with actively moving J2 for fluorescence after exposure to FITC. Each pair-wise comparison is shown by black bars representing quiescent nematodes and white bars representing active nematodes.* P*-values were calculated using Student's* t*-test.*

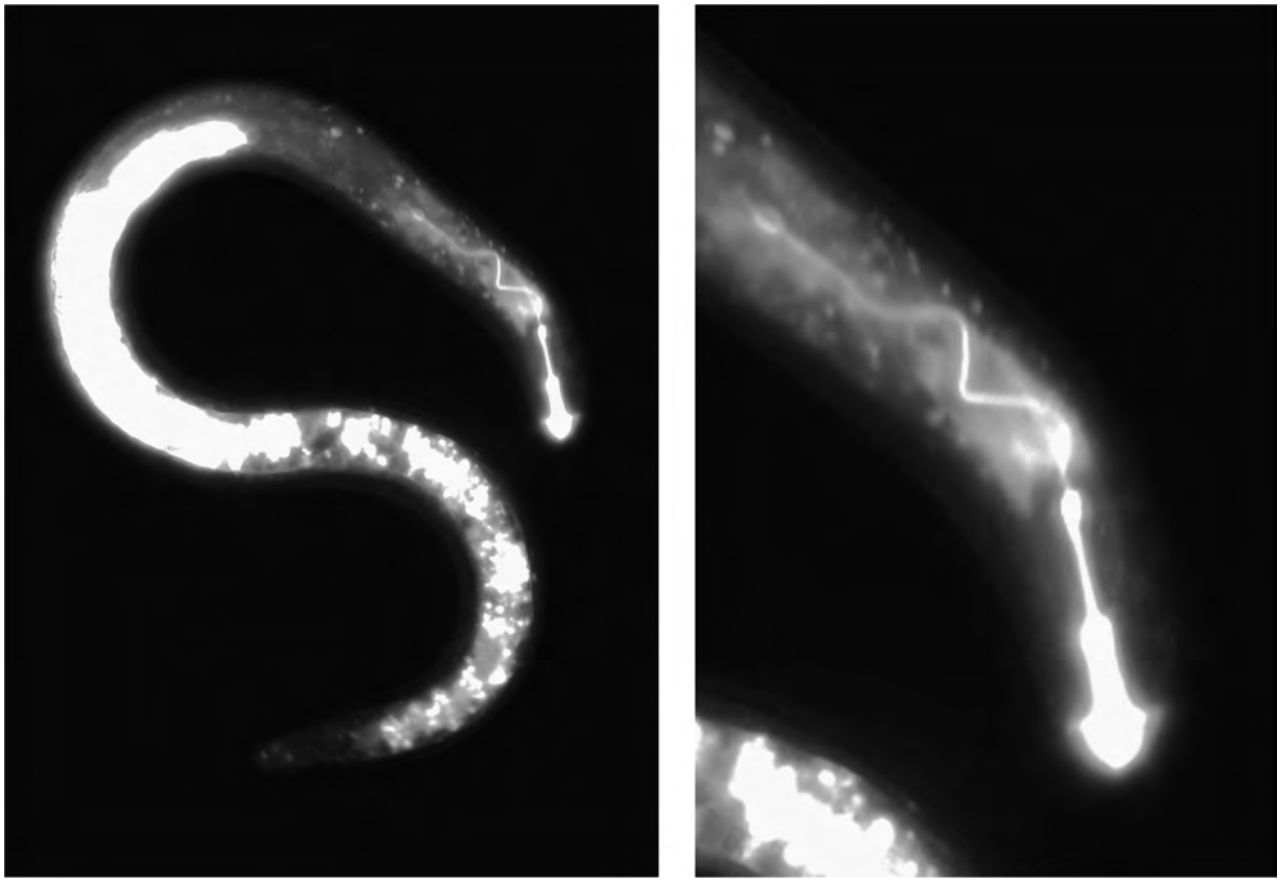

Fig. 5. *Incorporation of FITC in the pharyngeal lumen of J2 of* Heterodera glycines. *Fluorescent micrograph of J2 at ×200 (left) and ×400 magnification exposed to 0.1 mg ml^{-1} FITC and 10 mM serotonin showing distinct fluorescence in the pharyngeal lumen.*

after FITC exposure compared with actively moving individuals obtained from the same field ($P < 0.001$).

Exposure of active nematodes to either octopamine or serotonin and FITC caused no change in the total fluorescence compared with active nematodes exposed to FITC alone (data not shown). Approximately 18% (3/16) of active J2 exposed to 10 mM serotonin in combination with FITC showed obvious fluorescence in the pharyngeal lumen (Fig. 5). None of the active nematodes exposed to octopamine and FITC showed fluorescence in the pharyngeal lumen or excretory pore. We observed that octopamine increased the activity of J2 with many individuals exhibiting uncoordinated movement, whilst serotonin exposure induced stylet thrusting behaviour. Approximately 95% of nematodes originally in a quiescent state moved spontaneously after a 3 h exposure to octopamine compared with 15% in buffer alone (Table 2). Exposure of quiescent nematodes to octopamine and FITC caused an increase in fluorescence compared with fluorescence of quiescent nematodes exposed to FITC alone (Fig. 6).

Table 2. *Effect of octopamine on revival from quiescence in J2 of* Heterodera glycines.

Treatment	% active
M9 ($n = 83$)	14.4 (7.7-23.9)
M9 + 50 mM octopamine ($n = 48$)	95.8 (85.7-99.5)

% active, percentage (95% CI) of J2 of *H. glycines* showing spontaneous movement after 3 h incubation at room temperature. All nematodes selected were originally in a non-induced quiescent state. Data pooled from three independent trials. Confidence intervals based on Fisher's Exact test.

Route of FITC entry

Nematodes with their cephalic region occluded with a veterinary cyanoacrylate adhesive showed significantly

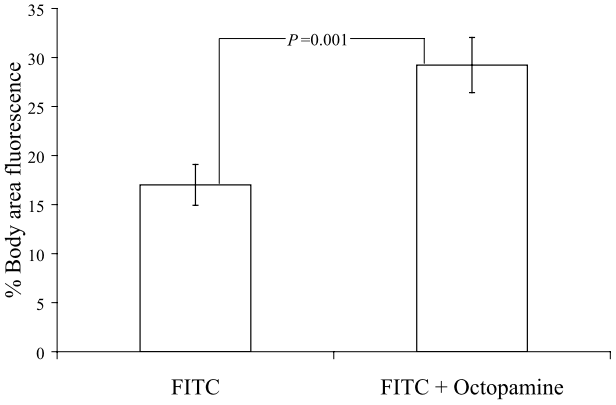

Fig. 6. *The effect of octopamine on the incorporation of FITC in quiescent J2 of* Heterodera glycines. *Quiescent nematodes (12 ± 1) were incubated in 10 mM octopamine with 10% M9 buffer or 10% M9 buffer alone at room temperature for 3 h followed by the addition of FITC. Following the incubation, images of nematodes were taken with both brightfield and fluorescent light at ×200 magnification. Image analysis was completed to determine the percentage of pixels in a given image that show fluorescence greater than negative control nematodes not exposed to FITC. The* P*-value was determined using Student's* t*-test.*

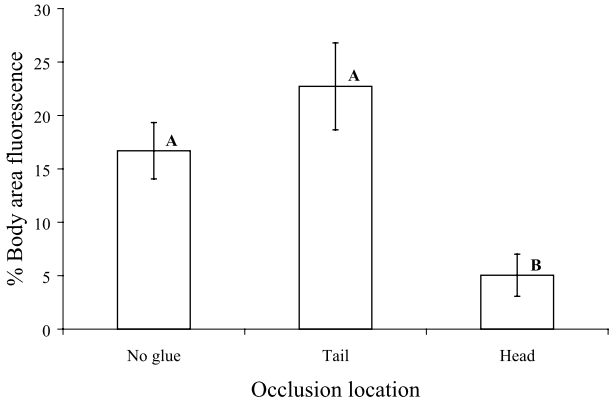

Fig. 7. *Penetration of FITC is inhibited by occlusion of the cephalic openings in J2 of* Heterodera glycines. *Active J2 were glued on the head, tail, or not at all, and exposed to 0.1 mg ml^{-1} FITC for 3 h followed by removal of adhesive and image analysis. Bars with different letters were statistically different based on ANOVA and Fisher's LSD for multiple comparisons $\alpha = 0.05$.*

less fluorescence after exposure to FITC than nematodes with their caudal region blocked or non-glued nematodes (ANOVA $P = 0.01$, Fisher's LSD $\alpha = 0.05$) (Fig. 7).

Discussion

Needham (1743) was the first to record nematodes in an inactive state. Numerous records since show nematodes undergo extreme physiological and/or morphological changes in association with survival to environmental stress. This report shows that short-term behavioural quiescence in a plant-parasitic nematode reduced its sensitivity to exogenous toxins. These results suggest that a temporary resting state not only allows for conservation of energy but also provides protection from external toxins.

Quiescent J2 of *H. glycines* were less sensitive to toxins than active nematodes. Two dissimilar compounds, ethanol and AITC, were tested. Both temperature and CO_2 levels were used to induce quiescence in order to confirm that the effects seen were not simply due to physico-chemical changes to the toxin. The difference in sensitivity between active and quiescent nematodes depended on the compound tested and the method used to induce quiescence. These differences between active and quiescent J2 of *H. glycines* were less than the five-fold difference found by Freckman *et al.* (1980) when they compared active and anhydrobiotic *A. avenae* for resistance to methyl bromide. The discrepancy may be due to factors inherent in the specific nematodes and chemicals tested as well as the more extreme hypobiotic state associated with anhydrobiosis. Neither Freckman *et al*. (1980) nor our study directly measured metabolism. However, it is known that anhydrobiosis often involves significant structural changes not observed in quiescent J2 of *H. glycines* (Bird & Buttrose, 1974; Wharton, 1996; Wharton & Lemmon, 1998).

The non-toxic fluorescent compound, FITC, previously demonstrated to be incorporated by plant-parasitic nematodes, was chosen to test the hypothesis that penetration of exogenous compounds is reduced in quiescent J2 of *H. glycines* when compared with active J2. FITC was chosen based on its use in previous research (Urwin *et al*., 2002; Winter *et al*., 2002; Schroeder & MacGuidwin, 2007). Temperature-induced quiescent nematodes were significantly less fluorescent than active nematodes following exposure to FITC. These results differ from those of Marks *et al.* (1968) who found no difference in chemical penetration for nematodes exposed to cold temperatures, but are similar to the findings of Djian and Pijarowski (1996) who found that a reduction in temperature reduced the penetration of radiolabelled dicarboxylic acids in *Meloidogyne arenaria*. Our work suggests that behavioural activity facilitates the penetration of exoge-

nous compounds into non-feeding nematodes. Temperature may affect chemical dynamics, so we obtained quiescent nematodes using three additional methods. *Heterodera glycines* develops to the J2 stage within the egg and can remain for years in a state of dormancy prior to hatching (Slack *et al.*, 1972). J2 of *H. glycines* that have not hatched can be released with slight mechanical probing. These mechanically hatched nematodes are morphologically normal but in an inactive state. Blair *et al.* (1999) found that mechanically hatched J2 of *Globodera rostochiensis* showed less nuclear staining after exposure to acridine orange compared with nematodes that hatched in tomato root diffusate. They attributed this to less transcription occurring when the nematodes did not hatch naturally. Our results support an alternative hypothesis, which Blair *et al.* (1999) recognised, where mechanically hatched nematodes in a dormant state are less apt to incorporate exogenous compounds. Morphologically normal, hatched, quiescent J2 of *H. glycines* are often recovered from the field in early spring (Schroeder & MacGuidwin, unpubl.). Quiescent nematodes from both lab and field populations showed reduced fluorescence following FITC exposure compared with active nematodes from the same populations, supporting a role for quiescence in mitigating chemical penetration.

Octopamine was previously shown to increase the penetration of FITC into cyst nematodes, as demonstrated by fluorescence in the pharyngeal lumen and excretory pore (Urwin *et al*., 2002). Previous researchers have demonstrated an increase in nematode activity following octopamine exposure (Horvitz *et al*., 1982; Masler, 2007). Indeed, our behavioural data show that octopamine can revive J2 of *H. glycines* from a quiescent state. Given that activity is correlated with increased chemical penetration, we were not surprised to find that quiescent nematodes exposed to octopamine and FITC showed greater fluorescence compared with quiescent nematodes exposed to FITC alone. Originally active nematodes did not show any change in fluorescence upon biogenic amine exposure. This pharmacological data further supports the hypothesis that quiescence inhibits chemical penetration in J2 of *H. glycines*. Although not completed in this study, it would be interesting to examine if octopamine would have success as a chemical adjuvant by increasing the toxicity of nematicides applied to quiescent nematodes.

The occlusion of the cephalic region led to the greatest decrease in FITC incorporation, suggesting that either amphid or stoma openings, rather than the cuticle, are the major entry point for FITC. The reduction of fluorescence shown in nematodes glued on the head was not complete. This may be due to absorption of FITC through the adhesive or limited cuticular penetration. Previous studies have shown neuronal staining of FITC by plant-parasitic nematodes (Winter *et al*., 2002; Schroeder & MacGuidwin, 2007). However, neuronal staining was not necessary for staining of the intestinal region. Penetration of FITC through the stoma of plant-parasitic nematodes has been shown, but only when applied in tandem with an additional compound such as octopamine (Urwin *et al*., 2002; Bakhetia *et al*., 2005, 2007; Sukno *et al*., 2007). Due to the difficulty in handling the adhesive, we cannot empirically distinguish between amphidial and stomal penetration. Our data differs significantly from work done with animal-parasitic nematodes in the order Ascaridida, which generally show trans-cuticular penetration of various compounds (Trim, 1949; Fleming & Fetterer, 1984; Fetterer, 1986; Ho *et al*., 1992). However, our data is similar to results with the animal-parasitic nematode, *Pseudoterranova decipiens*, in which ligation of the mouth caused a significant decrease of inulin penetration (Fuse *et al*., 1993).

Research regarding survival ecophysiology of plant-parasitic nematodes has primarily focused on adaptations to temperature and anhydrobiotic stress (Wharton, 2002). As soil-borne organisms, plant-parasitic nematodes are exposed to a range of potentially harmful toxins. This is the first report that demonstrates conclusively that a plant-parasitic nematode can survive normally lethal concentrations of exogenous toxins when in a quiescent state. Our data with FITC support the hypothesis that quiescence is associated with reduced penetration of an exogenous compound. However, caution must be used in relating the FITC data regarding chemical penetration to data regarding the toxicity of AITC and ethanol. While FITC and AITC share a reactive isothiocyanate group, FITC is substantially larger (MW FITC = 389, MW AITC = 99, MW ethanol = 46) and much more hydrophobic (log P FITC = 5.25, log P AITC = 2.21, log P ethanol = 0.06, calculated using prediction software Molinspiration Cheminformatics, Slovensky Grob, Slovak Republic) than AITC or ethanol. These chemical differences may affect the incorporation results. Future work should directly test the incorporation of known toxins in quiescent *versus* active nematodes using microautoradiography. An additional hypothesis, not explored in this study, is that the exogenous compounds enter the nematode at the same rate in quiescent and active nematodes, but that an upregulation of detoxification mechanisms occurs during quiescence.

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