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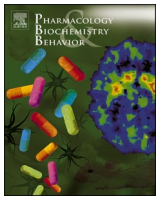

The inhibitory effect of nicotine on *Lumbriculus variegatus* stereotypical movements and locomotor activity

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ABSTRACT

Nicotine has been shown to induce profound physiological and behavioural responses in invertebrate model organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster*. *Lumbriculus variegatus* is an aquatic oligochaete worm which we have previously demonstrated has application within pharmacological research. Herein, we demonstrate the presence of endogenous acetylcholine and cholinesterase activity within *L. variegatus* and show the time-dependent effects on the sensitivity of *L. variegatus* to nicotine. We describe the effects of a broad range of concentrations of nicotine (1 μM – 1 mM) on *L. variegatus* response to tactile stimulation and locomotor activity following acute (10-min) and chronic (24-h) exposure. Here, we show that 10 min of exposure to ≥0.1 mM nicotine reversibly reduces the ability of tactile stimulation to elicit stereotypical movements of body reversal and helical swimming, and locomotor activity in *L. variegatus*. We also demonstrate that exposure to ≥0.1 mM nicotine for 24 h was toxic to *L. variegatus*. Chronic low-dose nicotine ≥25 μM similarly inhibits *L. variegatus* behaviours with 50 μM causing irreversible inhibition of movement. Thus, *L. variegatus* presents a model for studying the effects of nicotine and further demonstrates the application of the *in vivo* model *L. variegatus* for behavioural pharmacology research.

1. Introduction

Lumbriculus variegatus (Annelida: Clitellata: Lumbriculidae), more commonly known as the blackworm, are aquatic annelid worms inhabiting shallow freshwater ponds, lakes and marshes (Drewes, 1999) which have been widely used as an ecological indicator species to test the toxicity of various metals (Colombo et al., 2016; O'Gara et al., 2004), pesticides (Sardo and Soares, 2010), synthetic compounds (Aikins et al., 2023; Vought and Wang, 2018) and microplastics (Silva et al., 2021).

These worms display quantifiable locomotor behaviours of body reversal and helical swimming following tactile stimulation and unstimulated locomotor activity (Seeley et al., 2021, 2024). Tactile stimulation of the anterior segments of *L. variegatus* evokes body reversal, whereby stereotypical bending movements reverse the head and tail positions, while stimulation of the posterior region evokes helical swimming movements characterised by rapid helical body bends (Drewes, 1999; Seeley et al., 2021). These behaviours are analogous to the behaviours observed in the extensively characterised *C. elegans* model, which displays quantifiable body bending and locomotor activity (Parida, 2022).

L. variegatus is increasingly being used as a model organism in studies of pharmacologically active compounds including; the selective-serotonin reuptake inhibitor, fluoxetine (Nentwig, 2007), non-steroid anti-inflammatory drugs such as diclofenac (Karlsson et al., 2016), the channel-blockers dantrolene, quinine and lidocaine (Seeley et al., 2021), anti-histamines such as mepyramine and loratadine (Carriere et al., 2023) and ethanol (Seeley et al., 2024). These studies have utilised behavioural parameters such as locomotor activity, the response to tactile stimulation to elicit stereotypical behaviours of body reversal and helical swimming, feeding, and reproduction.

Previously, the effects of nicotine in *L. variegatus* have been shown to affect pulsation of the dorsal blood vessel (Lesiuk and Drewes, 1999). This study observed the dose-dependent effects of ≤1 mM nicotine on *L. variegatus* pulse rates and found that 0.25 mM nicotine resulted in increased pulsations while 1 mM nicotine resulted in complete inhibition of pulsation rates, suggesting the presence of nicotinic receptors within *L. variegatus*. Furthermore, work by Martinez et al. (2008) and Lesiuk and Drewes (2001) utilised 0.25 mM nicotine as a paralytic agent

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in *L. variegatus* to study regeneration, further suggesting the presence of nicotinic receptors in this worm and demonstrating that nicotine can affect the locomotor activity of *L. variegatus*.

Nicotine, the main addictive component of tobacco, exerts its effects primarily through the family of pentameric nicotinic acetylcholine receptors (nAChRs), which are ligand-gated ion channels (Benowitz, 2009; Champtiaux and Changeux, 2004). The binding of the endogenous agonist, acetylcholine, or exogenous agonists, such as nicotine, promotes channel opening to enable the entry of sodium and calcium cations (Benowitz, 2009). In mammals, nAChRs are found in both the peripheral and central nervous system where the composition of the five receptor subunits mediates nicotine responses. As reviewed by Benowitz (2009), the differential expression of the nAChR subunits regulates the behavioural and cardiovascular effects of nicotine, as well as nicotine sensitivity, dependence and tolerance. nAChRs are also implicated in rapid synaptic transmission and may play a role in learning and memory (Levin et al., 1999). Studies in the invertebrate models *C. elegans* (Polli et al., 2015) and *Drosophila melanogaster* (Korona et al., 2022) have demonstrated the presence of nicotinic receptors in these organisms with over forty putative nicotinic acetylcholine receptor subunits identified within *C. elegans* (Bargmann, 1998) and ten receptors with homology to mammalian nAChRs identified in *Drosophila* (Littleton and Ganetzky, 2000).

Nicotine effects are complex and studies often conflict, with locomotive effects shown to vary according to both dose and time, with nicotine administration being both stimulatory and inhibitory (Matta et al., 2007). Previously, nicotine has been shown to modulate the locomotor activity of *C. elegans* (Feng et al., 2006; Gottschalk et al., 2005; Sobkowiak et al., 2011) and *Drosophila* (Velazquez-Ulloa, 2017). Nicotine can exert these effects rapidly, having been shown to inhibit locomotion and negative geotaxis within 2–10 min in *C. elegans* and *Drosophila* (Feng et al., 2006; Sobkowiak et al., 2011; Velazquez-Ulloa, 2017). The effects of nicotine are dose-dependent, with *C. elegans* exposed to 0.001–0.1 mM nicotine displaying a "locomotion-stimulation phase" when exposed for 3–50 min (Feng et al., 2006; Sobkowiak et al., 2011). Higher doses of nicotine decrease the locomotion rate in a dose-dependent manner (Sobkowiak et al., 2011) with 20–30 mM nicotine inducing paralysis (Matta et al., 2007). *Drosophila* exposed to volatilised nicotine exhibit locomotor hyperactivity and induction of spasmodic movements at low doses, while hypokinesis, decreased locomotor activity and paralysis were seen at higher doses (Bainton et al., 2000). Moreover, *C. elegans* display a withdrawal response phenotype following nicotine exposure and removal to nicotine-free plates, whereby there is a locomotion-stimulation phase when removed from nicotine, analogous to the effects seen following acute nicotine exposure (Feng et al., 2006).

While cholinergic systems have been well studied in *C. elegans* and *Drosophila*, examination of the cholinergic system in annelid worms represents a paucity in the scientific literature. Genomic identification of nicotinic receptor homologues in *L. variegatus* has not yet been done given the limited genomic information and lack of a full genomic sequence (Gustafsson et al., 2009; Martinez Acosta et al., 2021). However, muscle regulation in annelids has been suggested to be mediated by acetylcholine with a "nicotinic-like" receptor within the annelid body wall muscle (Walker et al., 1993) and, recently, muscarinic subtypes have been identified in the terrestrial annelid *Lumbricus terrestris* (Nurullin and Volkov, 2022). As such, cholinergic transmission in *L. variegatus* has remained a long-standing question (Lesiuk and Drewes, 1999; Martinez Acosta et al., 2021).

We have previously demonstrated that *L. variegatus* display concentration- and time-dependent locomotor behavioural changes when exposed to ethanol by examining the effects of tactile stimulation to elicit stereotypical behaviours and the effects on locomotor activity (Seeley et al., 2024). Locomotor behaviours may be more sensitive to toxicants which act through neuromuscular targets such as the cholinergic agonist nicotine (Sobkowiak et al., 2011) and we sought to examine the concentration- and time-dependent locomotor behaviours of *L. variegatus* exposed to nicotine. Here, we provide quantification of *L. variegatus* endogenous acetylcholine and cholinesterase activity indicating the presence of the previously hypothesised cholinergic system within *L. variegatus* (Lesiuk and Drewes, 1999; Martinez Acosta et al., 2021). Using nicotine concentrations previously investigated by Lesiuk and Drewes (1999) in their study on pulsation rates, we describe the behavioural effects of nicotine exposure on *L. variegatus* responses to tactile stimulation to elicit the stereotypical behaviours of body reversal and helical swimming, and the effects on locomotor activity. Further, we describe the toxicity and behavioural responses of *L. variegatus* when exposed to nicotine for 24 h.

2. Material & methods

2.1. Lumbriculus variegatus culture

L. variegatus were purchased from Alfa Fish Foods and cultured in artificial pond water, composed of 1 mM NaCl; 13 μM KCl, 4 μM Ca $(NO_3)\cdot 4H_2O$; 17 μM $Mg(SO_4)\cdot 7\ H_2O$; 71 μM HEPES buffer in UV-treated deionised water produced by Elix® Essential 3 UV Water Purification System, as previously described (O'Gara et al., 2004; Seeley et al., 2021; Seeley et al., 2024). Cultures were subject to a 16:8-h light-dark cycle and fed TetraMin flakes and 10 mg/L spirulina weekly and maintained at room temperature (18–21 °C) with continuous aeration and water filtration using commercial air stones and aquarium filters, respectively. *L. variegatus* populations were maintained for a minimum of three months before experimentation to ensure *L. variegatus* acclimatisation and expansion of *L. variegatus* cultures (O'Gara et al., 2004; Seeley et al., 2021). Individual worms used in experiments were randomly selected, lacked any obvious morphological defects, and ranged from 2 to 8 cm in length as per previous studies (O'Gara et al., 2004; Seeley et al., 2021; Seeley et al., 2024).

2.2. Materials

Nicotine (≥99 %) was obtained from Sigma-Aldrich (Dorset, United Kingdom) and diluted in artificial pond water. Artificial pond water was used as a vehicle control.

2.3. Acetylcholine & cholinesterase quantification assays

Acetylcholine levels were quantified using an established methodology (Deng et al., 2019) by a commercial acetylcholine quantification colourimetric assay (MAK056, Sigma-Aldrich, Dorset, United Kingdom). To determine acetylcholine levels, the amount of free choline in *L. variegatus* homogenate was determined by a coupled enzyme reaction which produces a colourimetric product (570 nm). Matched homogenate samples were treated with acetylcholinesterase, which hydrolyses acetylcholine to choline and acetate, and total choline levels were determined. Acetylcholine levels were determined by subtraction of free choline levels from total choline levels and the concentration of acetylcholine in *L. variegatus* was determined according to manufacturer's instructions.

Cholinesterase activity within *L. variegatus* was determined with a commercial colourimetric assay (ab138871, Abcam, Cambridge, United Kingdom) based on Ellman's method (Ellman et al., 1961) which uses 5,5-dithio-bis-(2-nitobenzoic acid) (DTNB) to quantify the thiocholine produced from the hydrolysis of acetylthiocholine by cholinesterase. The absorption intensity of the DTNB adduct (410 nm) is used to measure the amount of thiocholine formed, which is proportional to the cholinesterase activity.

18–24 h before experimentation, twenty *L. variegatus* per assay were added to a CellStar® 6-well plate (Greiner Bio-One). After the acclimatisation period, *L. variegatus* were transferred to a 1.5 mL Eppendorf tube on ice and artificial pond water was removed. *L. variegatus* were homogenised in 100 μL of choline assay buffer for acetylcholine

quantification or 100 µL of the manufacturer's lysis buffer for cholinesterase activity quantification using a Cole-Parmer® motorised pestle mixer. Homogenates were centrifuged at 16.1 RCF for 15 min and 25 µL of each sample was added into a 96-well plate and quantification was conducted as per manufacturer's instructions. Plates were mixed well using a horizontal shaker at room temperature for 30 min or 10 min, respectively, and the absorbance was measured at 570 nm, for acetylcholine quantification, or 410 nm, for cholinesterase activity quantification, using a FLUOstar Omega microplate reader (BMG Labtech). The absorbances obtained were from the three experimental repeats ran in duplicate, or five experimental repeats ran in triplicate, respectively. Data is reported per µL of assay buffer.

2.4. Stereotypical movement and locomotor activity assays

The effects of nicotine on tactile stimulation to elicit stereotypical behaviours of body reversal and helical swimming were conducted as previously described (Carriere et al., 2023; Seeley et al., 2021, 2024). Briefly, 18–24 h before experimentation, individual *L. variegatus* was placed in individual wells of a Cellstar® 6-well plate (Greiner Bio-One) containing 4 mL of artificial pond water and kept at room temperature, subject to a 16:8-h light-dark. After this acclimation period, the artificial pond water was replaced and the baseline ability of the worm to respond to tactile stimulation of the anterior or posterior was alternately tested using a 20–200 µL plastic pipette tip. The artificial pond water was then removed and immediately replaced with the vehicle control (artificial pond water only) or 0.01–1 mM nicotine for acute 10-min exposure or 1–50 µM nicotine for chronic 24-h exposure. After nicotine exposure, *L. variegatus* were retested using the same procedure. Solutions were aspirated from the well, and to remove any latent nicotine, fresh artificial pond water was added, immediately aspirated and replaced with fresh artificial pond water. *L. variegatus* were then retested 10 min and 24 h after exposure (Recovery (10 min) and Recovery (24 h), respectively). Data were expressed as a ratio of the movement score relative to baseline, from eight experimental repeats with a single *L. variegatus* exposed to each concentration per experimental repeat.

The effect of nicotine on *L. variegatus* locomotor activity were conducted as previously described (Seeley et al., 2021, 2024) with the same acclimatisation period as outlined above. After the acclimation period, artificial pond water was replaced with 2 mL fresh artificial pond water to limit movement in the *z*-axis, and baseline locomotor activity was recorded by rapid, sequential image collection with a 13-megapixel camera at a rate of one image per second for 50 s. Artificial pond water was then removed and immediately replaced with 2 mL of 0.01–1 mM nicotine for acute 10-min exposure, 1–50 µM nicotine for chronic 24-h exposure or with the vehicle control (artificial pond water only). Images were then collected after acute (10 min) or chronic (24 h) exposure to nicotine. After nicotine exposure, images were taken as described above and solutions were aspirated from the well. Latent nicotine was removed by washing with fresh artificial pond water and 2 mL of artificial pond water was added to the wells. Images were collected 10 min and 24 h after incubation in artificial pond water only (Recovery (10 min) and Recovery (24 h), respectively).

Locomotor activity was determined by analysis of the collected images in ImageJ as described in Seeley et al. (2021). Analysis was conducted by superimposing the images for each time point and calibration of ImageJ to an area of known distance within each image to establish the pixels per centimetre within each superimposed image set. To determine the area covered by each worm, the foreground and background were separated using the thresholding functionality of ImageJ to separate the pixels activated by *L. variegatus* from those activated by the 6-well plate. The total area covered by the *L. variegatus* before nicotine exposure, after 10 min or 24-h nicotine exposure, and both recovery time points were then determined based on the calibration of pixels/cm within ImageJ. Data were expressed as a percentage of the area covered by *L. variegatus* compared to baseline conditions, from eight experimental repeats with a single *L. variegatus* exposed to each concentration per experimental repeat.

2.5. Onset and offset of action assay

18–24 h before experimentation, one *L. variegatus* was placed in each well of a Cellstar® 6-well plate (Greiner Bio-One) containing artificial pond water only. After this acclimation period, artificial pond water was aspirated and replaced with fresh artificial pond water. Locomotor activity before exposure (0 mins) with nicotine was recorded by rapid, sequential image collection using a 13-megapixel camera at a rate of one image per second for 50 s and analysed as described above. Image collection was repeated at two-minute intervals for 10 min following the removal of artificial pond water and immediately replacing artificial pond water with nicotine (0.01–1 mM) or vehicle control (artificial pond water only). After 10 min of nicotine exposure, wells were washed once with artificial pond water to remove any latent nicotine or vehicle residue, and artificial pond water only was added. Image collection was then repeated at two-minute intervals for a total of 10 min. Images were analysed for locomotor activity as previously described (Seeley et al., 2021) with data expressed as a percentage of the area covered by *L. variegatus* at 0 mins from eight experimental repeats with a single *L. variegatus* exposed to each concentration per experimental repeat.

2.6. Nicotine toxicity

18–24 h before experimentation, five *L. variegatus* were placed in each well of a Cellstar® 6-well plate (Greiner Bio-One) containing artificial pond water only. After this acclimation period, artificial pond water was aspirated and replaced with 0–1 mM nicotine or vehicle control. 24 h after exposure, surviving *L. variegatus* were counted. Data is expressed as a percentage of *L. variegatus* surviving nicotine exposure from six experimental repeats with five *L. variegatus* exposed to each concentration per experimental repeat.

2.7. Statistical analysis

The sample size for each assay and exposure was $\geq$3 experimental repeats. Data is displayed as the mean ± standard error of the mean (SEM) for each data set. Stereotypical movement and locomotor activity data is relative to the untreated, baseline control condition. Values for each behavioural measurement were compared to the untreated control conditions for each *L. variegatus* per condition. Nicotine exposure conditions were compared to baseline conditions by paired non-parametric two-tailed *t*-tests for stereotypical movement assays and paired parametric two-tailed *t*-tests for locomotor activity. A two-way ANOVA with Dunnett's post-test was used to analyse 10-min and 24-h recovery time points compared to baseline conditions for *L. variegatus* exposed to nicotine for 10 min. The onset and offset of nicotine effects were determined for each time point by a one-way ANOVA comparing each tested concentration to its corresponding baseline control. The lethal dose of 50 % (LD_{50}) was determined using GraphPad Prism 10. $p < .05$ was the threshold for statistical significance. Statistical analysis was performed in GraphPad Prism 10.

3. Results

3.1. Quantification of acetylcholine & cholinesterase in Lumbriculus variegatus

First, we sought to determine if *L. variegatus* had quantifiable levels of acetylcholine and cholinesterase. We determined that whole *L. variegatus* homogenate had detectable levels of both acetylcholine (3.65 ± 0.80 ng/µL of *L. variegatus* homogenate, $n = 3$ with twenty per replicate) and cholinesterase activity (81.96 ± 7.08 U/µL of *L. variegatus* homogenate, $n = 5$ with twenty *L. variegatus* per replicate).

3.2. Behavioural response to acute nicotine exposure

Having determined the presence of acetylcholine and cholinesterase activity, we sought to examine the effects of nicotine in *L. variegatus*. Acute exposure to ≥0.1 mM nicotine resulted in the inhibition of tactile stimulation to elicit *L. variegatus* body reversal ($p < .05$, Fig. 1A) and helical swimming ($p < .01$, Fig. 1B). While exposure to 0.1 mM nicotine inhibited both stereotypical movements ($p < .05$, Fig. 1A-B), the effects were readily reversible when *L. variegatus* were removed from nicotine and placed in artificial pond water only for 10 min where the ability to perform either movement was indistinguishable from the baseline ability to perform stereotypical movements ($p > .05$, Fig. 1C-D). Exposure to 0.25–1 mM nicotine significantly inhibited both body reversal ($p < .01$, Fig. 1A) and helical swimming ($p < .01$, Fig. 1B) movements following tactile stimulation. Unlike the response to 0.1 mM nicotine, after removal of 0.25–1 mM nicotine and incubation in artificial pond water only, the capacity of *L. variegatus* to respond to tactile stimulation remained significantly reduced after 10 min when compared to baseline responses ($p < .0001$, Fig. 1C-D). 24 h after removal of 0.25–1 mM nicotine and incubation in artificial pond water only, body reversal movements in response to tactile stimulation returned to baseline levels ($p > .05$, Fig. 1C). After the removal of nicotine, helical swimming returned to baseline levels for all test concentrations ($p > .05$, Fig. 1D) except for 0.5 mM which remained inhibited even 24 h after removal from nicotine ($p < .05$, Fig. 1D).

L. variegatus locomotor activity was reduced after 10 min of exposure to ≥0.1 mM nicotine (Fig. 1E) with 0.1 mM nicotine exposure significantly reducing locomotor activity to 9.01 ± 2.26 % ($p < .0001$, Fig. 1F). Locomotor activity was similarly reduced to 31.17 ± 4.77 % when exposed to 0.25 mM, 8.90 ± 0.80 % when exposed to 0.5 mM, and 7.66 ± 0.66 % when exposed to 1 mM, when compared to baseline conditions ($p < .0001$, Fig. 1F), respectively. Following removal of nicotine and incubation in artificial pond water only, *L. variegatus* locomotor activity remained inhibited at ≥0.1 mM nicotine with movement reduced to 9.73–49.90 % ($p < .0001$, Fig. 1G). 24 h after removal of 0.1 mM, 0.5 mM and 1 mM nicotine, *L. variegatus* locomotor activity had returned to levels indistinguishable from baseline conditions ($p > .05$, Fig. 1G). However, we observed that 24 h after the removal of 0.25 mM nicotine, locomotor activity remained reduced to 59.18 ± 11.23 % ($p = .001$, Fig. 1G).

3.3. Onset and offset of nicotine effects

Having determined that nicotine reduced the response to tactile stimulation and locomotor activity of *L. variegatus* following 10 min of nicotine exposure, we next sought to determine the time at which nicotine exerted its effects on *L. variegatus*.

Nicotine was shown to exert its locomotor inhibitory effects at 0.1, 0.5 and 1 mM after 2 min of exposure ($p < .05$, Fig. 2A) and ≥ 0.1 mM after 4 min ($p < .05$, Fig. 2A). After 2 min of nicotine exposure, we observed almost complete locomotor inhibition at 0.5 mM, with movement reduced to 15.56 ± 2.20 % ($p < .0001$, Fig. 2A), and 1 mM nicotine reduced to 10.47 ± 1.23 % compared to baseline measurements ($p < .0001$, Fig. 2A). After 10 min of exposure to nicotine, *L. variegatus* locomotor activity was reduced to 28.77 ± 10.62 % when exposed to 0.1 mM nicotine ($p = .0005$, Fig. 2A), 16.03 ± 5.54 % when exposed to 0.25 mM nicotine ($p = .001$, Fig. 2A), 31.52 ± 6.15 % when exposed to 0.5 mM nicotine ($p = .0007$, Fig. 2A) and 8.69 ± 1.29 % when exposed to 1 mM nicotine ($p < .0001$, Fig. 2A). However, no locomotor inhibitory effects were observed in *L. variegatus* exposed to 0.01 mM nicotine at any time point ($p > .05$, Fig. 2A).

Two minutes after the removal of nicotine, *L. variegatus* locomotor activity remained significantly reduced to 51.10 ± 7.85 % following removal of 0.1 mM nicotine ($p = .005$, Fig. 2B), 17.61 ± 3.38 % following removal of 0.25 mM nicotine ($p < .0001$, Fig. 2B), 30.92 ± 6.11 % following removal of 0.5 mM nicotine ($p = .0005$, Fig. 2B) and 10.55 ± 1.44 % following removal of 1 mM nicotine ($p < .0001$, Fig. 2B) and incubation in artificial pond water only. The effects of 0.1–0.25 mM nicotine was observed to be reversible, with the effects of 0.1 mM nicotine decreasing to levels indistinguishable from vehicle controls 4 min after the removal of nicotine ($p = .343$, Fig. 2B). In *L. variegatus* exposed to 0.25 mM nicotine, locomotor activity was recovered after 10 min of incubation in artificial pond water only, with movement indistinguishable from vehicle controls ($p = .09$, Fig. 2B). However, locomotor activity of *L. variegatus* removed from 0.5 mM and 1 mM nicotine remained significantly reduced to 50.72 ± 8.65 % and 14.40 ± 1.48 % ($p \leq .001$, Fig. 2B), respectively.

3.4. Toxicity of chronic nicotine exposure

We next sought to determine the effects of 24-h exposure to nicotine on *L. variegatus*. We observed that the lowest observed adverse effect level (LOAEL) for *L. variegatus* was 0.1 mM, whereby 83.33 ± 16.67 % of *L. variegatus* survived nicotine exposure (Fig. 3). At the highest tested concentration of 1 mM nicotine, 6.67 ± 6.67 % of *L. variegatus* survived nicotine exposure (Fig. 3). The no observed adverse effect level (NOAEL) for 24-h exposure to nicotine was determined to be 0.01 mM nicotine, where no *L. variegatus* expired. Moreover, we determined the lethal dose of 50 % (LD_{50}) to be 0.29 mM nicotine (95 % CI: 0.10–0.48 mM) in *L. variegatus*.

3.5. Behavioural response to chronic nicotine exposure

When *L. variegatus* were exposed to nicotine concentrations below the LOAEL (<0.1 mM) for 24 h, nicotine exposure had no significant effects on the response to tactile stimulation ($p > .05$, Fig. 4A-B). However, we observed that following removal of 25 μM nicotine and incubation in artificial pond water only, body reversal was inhibited after 10 min ($p = .03$, Fig. 4C). 24 h after removal of 25 μM nicotine and in incubation in artificial pond water only, body reversal responses were indistinguishable from baseline responses ($p > .05$, Fig. 4C). Comparatively, following the removal of 25 μM nicotine, helical swimming was significantly reduced after 24 h ($p = .007$, Fig. 4D). Helical swimming was similarly inhibited following the removal of 50 μM nicotine and incubation in artificial pond water only for 24 h ($p = .024$, Fig. 4D).

Exposure to ≤0.25 μM for 24 h did not affect *L. variegatus* locomotor activity ($p > .05$, Fig. 4E-F), but 24-h exposure to 50 μM nicotine decreased locomotor activity to 65.69 ± 10.83 % compared to baseline conditions ($p = .016$, Fig. 4F). When *L. variegatus* were removed from nicotine and placed in artificial pond water only, no significant effect was observed on locomotory activity 10 min after nicotine removal ($p > .05$, Fig. 4G). However, we did observe that locomotor activity in *L. variegatus* removed from 25 μM nicotine and incubated for 24 h in artificial pond water only was reduced to 65.29 ± 12.25 % ($p = .012$, Fig. 4G). Similarly, we observed a significant inhibition of locomotor activity in *L. variegatus* following 24-h removal from 50 μM nicotine, whereby locomotor activity was reduced to 50.63 ± 13.51 % compared to baseline conditions ($p = .008$, Fig. 4G).

4. Discussion

In our study, we demonstrate that *L. variegatus* display rapid locomotory behavioural changes when exposed to nicotine, with 10-min exposure to ≥0.1 mM nicotine resulting in reduced tactile stimulation responses and locomotor activity. Irreversible inhibition of locomotory behaviours occurs when *L. variegatus* are exposed to 50 μM nicotine for 24 h, and 24-h exposure to ≥0.1 mM nicotine results in toxic effects in *L. variegatus*. Furthermore, we demonstrate detectable and quantifiable levels of acetylcholine and cholinesterase activity in *L. variegatus*.

Previously, Lesiuk and Drewes (1999) evaluated the effects of 15 min of exposure to 0.1–1 mM nicotine on *L. variegatus* pulsation rates and described a biphasic response; ≤0.25 mM nicotine increased pulsation

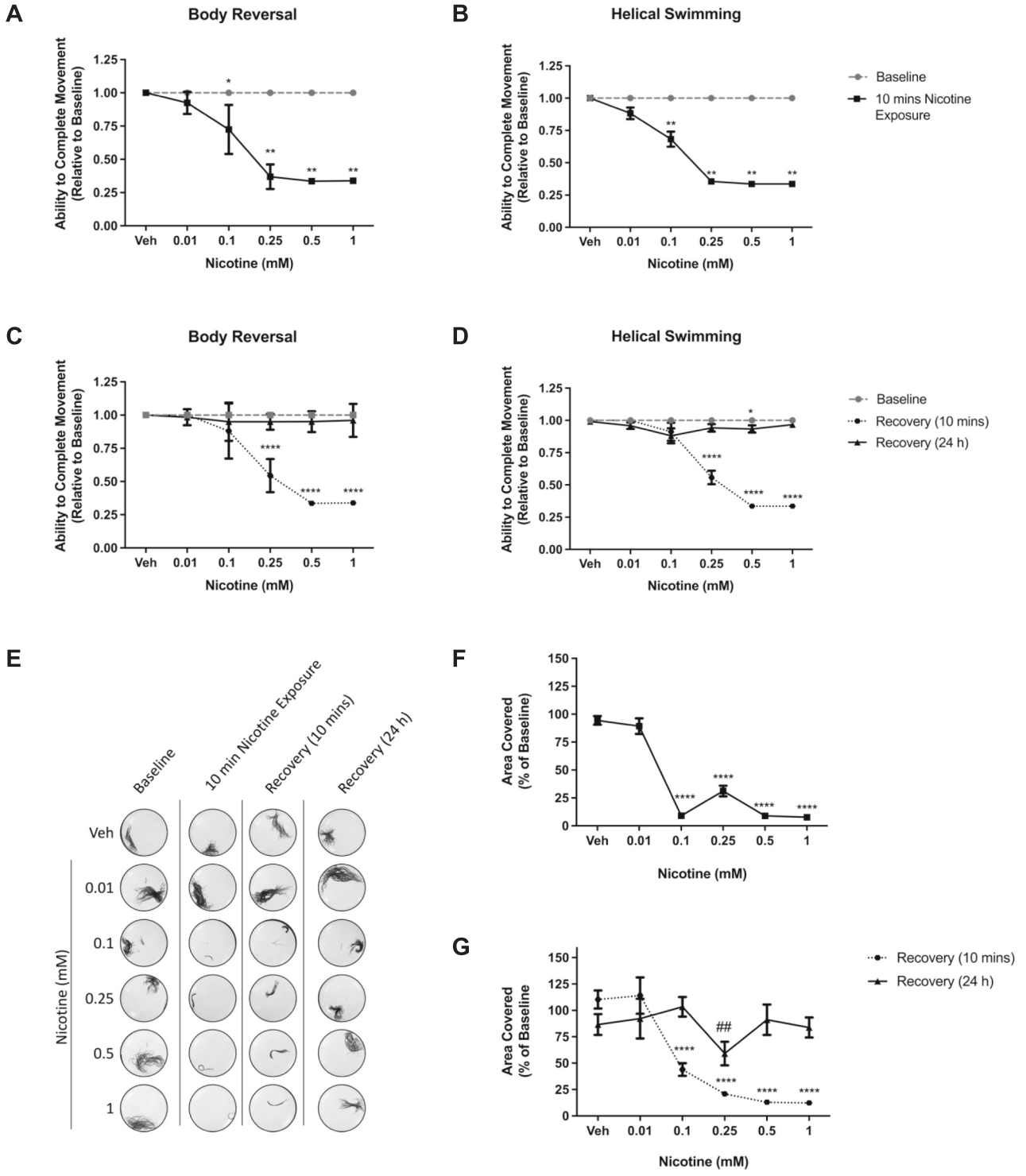

Fig. 1. The effect of acute nicotine exposure on *Lumbriculus variegatus* locomotory behaviours. *L. variegatus* were stimulated anteriorly and posteriorly to elicit body reversal and helical swimming movements, respectively, before nicotine exposure (Baseline). *L. variegatus* were then exposed to nicotine (0–1 mM) for 10 min and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. Nicotine was then removed and the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 min and 24 h. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. The effect of 10 min of exposure to nicotine on locomotor activity was measured before nicotine exposure (Baseline), after 10 min of exposure to 0–1 mM nicotine (10 min Nicotine Exposure), 10 min after nicotine removal (Recovery (10 mins)) and 24 h after nicotine removal (Recovery (24 h)). **(E)** Representative images showing the area covered by *L. variegatus* used to determine locomotor activity before nicotine exposure (Baseline), after 10 min of exposure to 0–1 mM nicotine (10 min Nicotine Exposure), 10 min after nicotine removal (Recovery (10 mins)) and 24 h after nicotine removal (Recovery (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** 10 min of exposure to 0–1 mM nicotine and **(G)** removal of nicotine for 10 min and 24 h are expressed as a percentage of the area covered at baseline. Error bars represent the standard error of the mean, $n = 8$. Veh: artificial pond water only. * $p < .05$, **/## $p < .01$; where * refers to statistical significance between Baseline and Nicotine Exposure or statistical significance between Baseline and Recovery (10 mins), # refers to statistical significance between Baseline and Recovery (24 h).

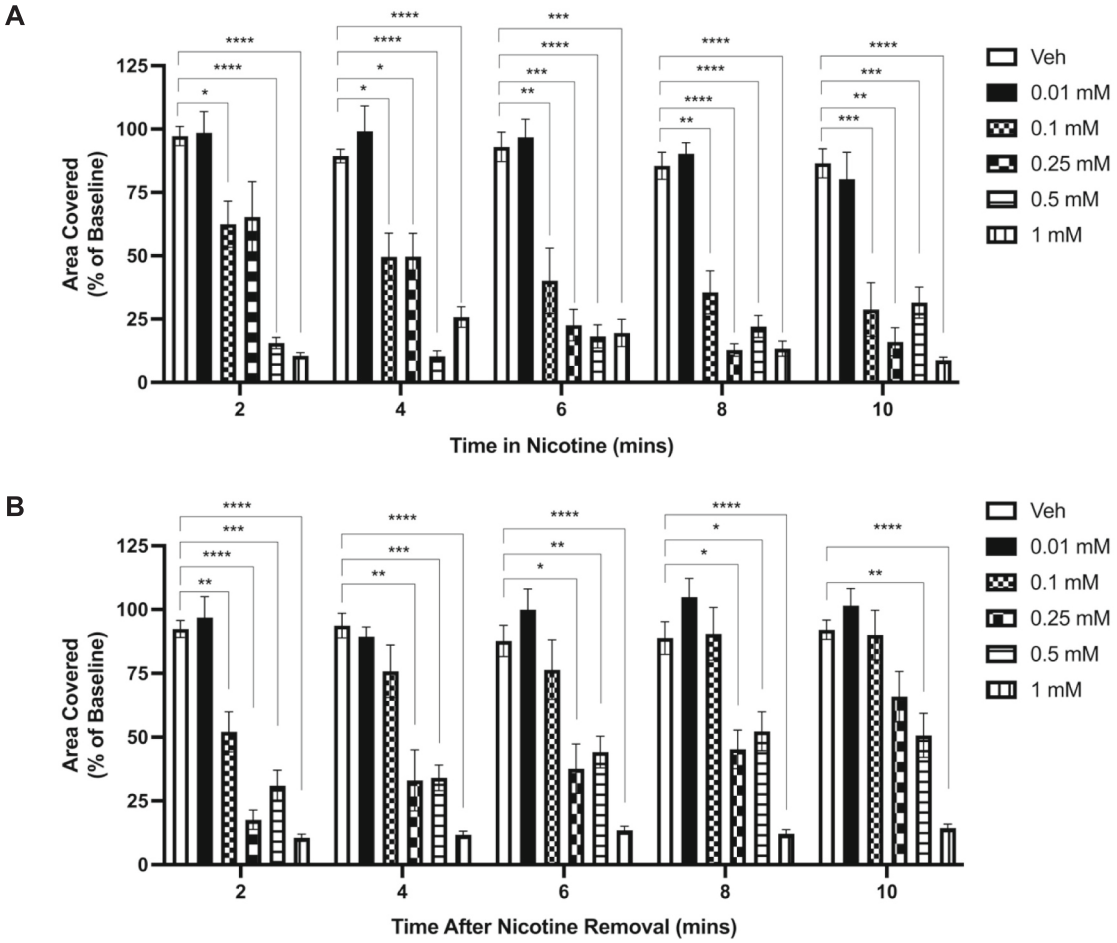

Fig. 2. The onset and offset of concentration-dependent inhibitory effects of nicotine on *Lumbriculus variegatus* locomotor activity. **(A)** *L. variegatus* were exposed to nicotine (0–1 mM) for 10 min and locomotor activity was measured before nicotine exposure and every 2 min for 10 min of exposure to 0–1 mM nicotine. **(B)** Nicotine was removed and the locomotor activity was assessed every 2 min for 10 min after exposure to 0–1 mM nicotine. Data are expressed as a percentage of the area covered before exposure (0 mins). Error bars represent the standard error of the mean, $n = 8$. Veh: artificial pond water only. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

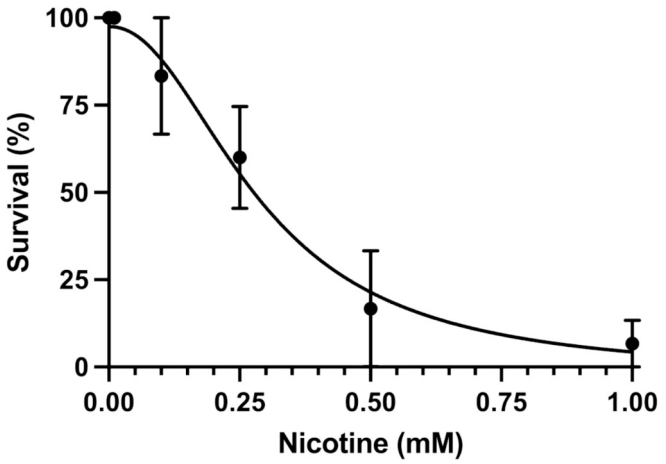

Fig. 3. *Lumbriculus variegatus* survival after 24-h exposure to nicotine. *L. variegatus* were exposed to nicotine (0–1 mM) for 24 h to observe signs of whole organism toxicity. After 24 h of exposure, surviving *L. variegatus* were counted and expressed as a percentage. Error bars represent the standard error of the mean, $n = 30$ per concentration.

rates, while 1 mM nicotine inhibited these. Subsequent work by Lesiuk and Drewes (2001) and Martinez et al. (2008) utilised 0.25 mM nicotine as a paralytic agent when studying *L. variegatus* regeneration. Together, these studies are indicative of the presence of nicotinic receptors within *L. variegatus* and formed the basis for our study presented here.

It has previously been suggested that muscle regulation in annelid worms is mediated by acetylcholine with a "nicotinic-like" receptor within the annelid body wall muscle (Walker et al., 1993) and acetylcholine receptors have been identified in the terrestrial annelid *Lumbricus terrestris* (Nurullin and Volkov, 2022). In our study, we detect the presence of acetylcholine and cholinesterase activity within *L. variegatus* homogenate which provides further evidence of the presence of a cholinergic system within this organism.

When *L. variegatus* were exposed to nicotine concentrations matching those used in the study by Lesiuk and Drewes (1999), we observed that 0.1–1 mM nicotine resulted in concentration-dependent inhibitory effects in *L. variegatus* responses to tactile stimulation (Fig. 1A-B) and locomotor activity (Fig. 1E-G). Here, we observed no biphasic responses to these concentrations as Lesiuk and Drewes (1999) described when studying pulsation rates. Additionally, in this study, *L. variegatus* were exposed for a shorter duration of 10 min (Fig. 1–2), while Lesiuk and Drewes (1999) evaluated the effects of nicotine after 15 min. We have previously demonstrated that 10-min exposure is effective for observing acute effects on stimulated behaviours and locomotor activity of *L. variegatus* (Carriere et al., 2023; Seeley et al., 2021, 2024). Our

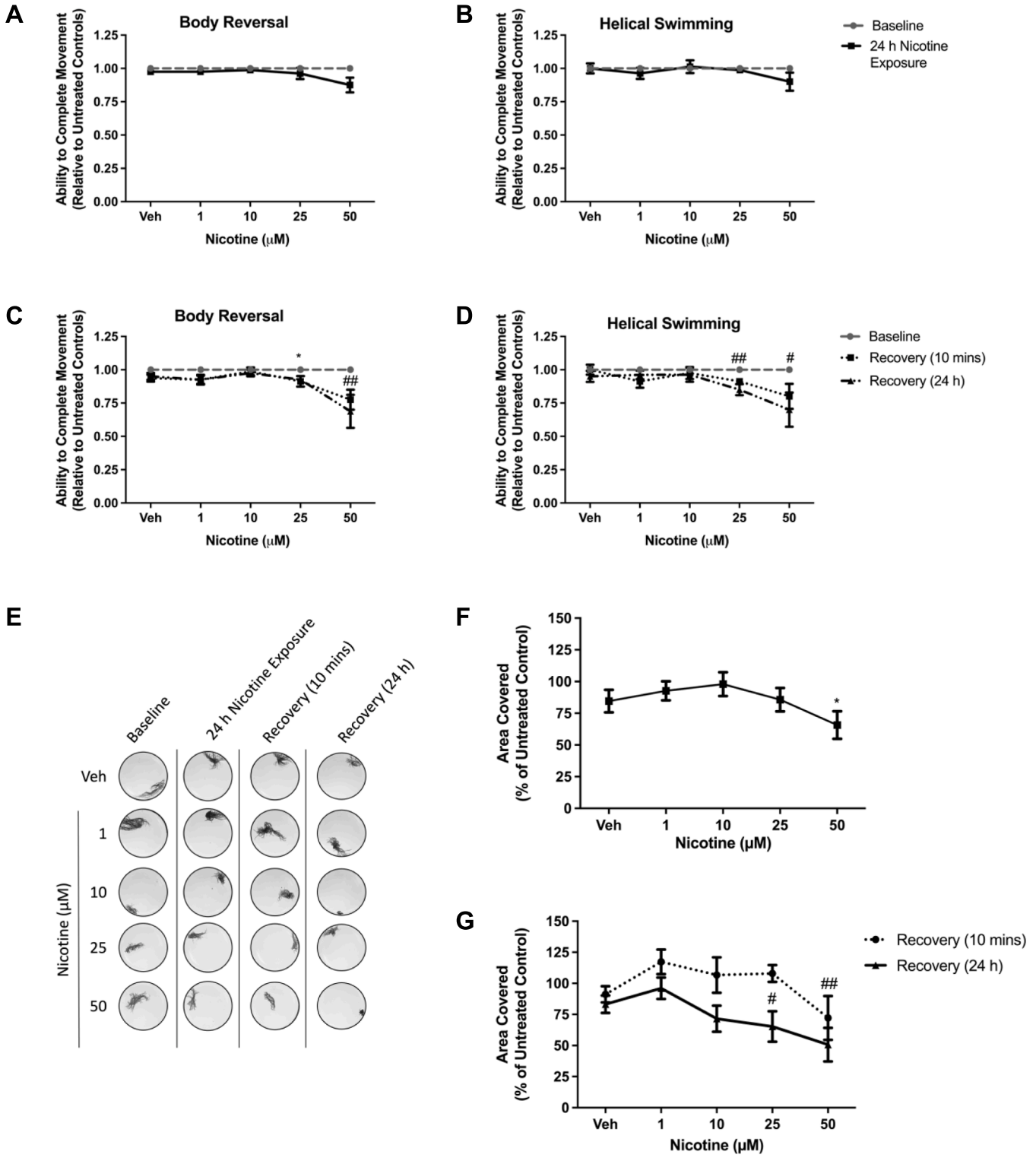

Fig. 4. The effect of 24-h chronic nicotine exposure on *Lumbriculus variegatus* locomotory behaviours. *L. variegatus* were stimulated anteriorly and posteriorly to elicit body reversal and helical swimming movements, respectively, before nicotine exposure (Baseline). *L. variegatus* were exposed to nicotine (0–50 μM) for 24 h minutes and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. Nicotine was then removed and the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 min and 24 h. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** Representative images showing the area covered by *L. variegatus* used to determine locomotor activity before nicotine exposure (Baseline), after 24 h of exposure to 0–50 μM nicotine (24 h Nicotine Exposure), 10 min after nicotine removal (Recovery (10 mins)) and 24 h after nicotine removal (Recovery (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** 24 h of exposure to 0–50 μM nicotine and **(G)** removal of nicotine for 10 min and 24 h are expressed as a percentage of the area covered at baseline. Error bars represent the standard error of the mean, $n = 8$. Veh: artificial pond water only. * $p < .05$, **/## $p < .01$; where * refers to statistical significance between Baseline and Nicotine Exposure or statistical significance between Baseline and Recovery (10 mins), # refers to statistical significance between Baseline and Recovery (24 h).

observations on the concentration-dependent inhibitory effects of nicotine in *L. variegatus* contrast with nicotine responses in *C. elegans,* whereby 0.1 and 1 mM nicotine resulted in hyperkinesis of *C. elegans* when exposed for 10 min (Sobkowiak et al., 2011). However, it has previously been highlighted that nicotine exposure of a given concentration in solid nematode growth medium (NGM) plates, is not directly experimentally comparable to the same concentration in a liquid medium (Matta et al., 2007), as used in our study. These differences are likely due to differences in osmoregulation, cuticular permeability or total surface area in contact (Matta et al., 2007).

While previous studies have described the effects of acute (≤15 min) nicotine exposure on *L. variegates* (Lesiuk and Drewes, 1999, 2001), longer exposure times have not been previously studied to our knowledge. When *L. variegatus* were exposed to 1–50 μM nicotine for 24 h, we observed no effects on *L. variegatus* response to tactile stimulation (Fig. 4A-B) and ≤ 25 μM nicotine had no effects on locomotor activity, while 50 μM nicotine decreased locomotor activity. These findings resemble previous studies in *C. elegans* exposed to 19.5 μM and 61.7 μM nicotine after 24 h and allowed to acclimatise for 1 h, where *C. elegans* displayed decreased responses to tactile stimulation (Smith et al., 2013). Previously, Feng et al. (2006) and Sobkowiak et al. (2011) both observed a "locomotion-stimulation phase" in which nicotine increases locomotion in a dose-dependent manner in *C. elegans* at 1.0–1.5 μM. At similar concentrations of 1 μM nicotine, we observed no stimulatory effects in *L. variegatus*. However, it should be noted that these studies observed these effects within 3–30 min while we measured *L. variegatus* locomotory effects after 24 h. Further study of low-dose, acute nicotine exposure in *L. variegatus* may show comparable results and reveal locomotor responses akin to the locomotion-stimulation phase observed in *C. elegans*.

Acute 10-min exposure to ≥0.1 mM nicotine was not reversible after the removal of nicotine for 10 min (Fig. 1C-D, G). While 0.25 mM nicotine has previously been used as a paralytic agent in *L. variegatus* by Lesiuk and Drewes (2001) and Martinez et al. (2008), the Lesiuk and Drewes study noted that these effects occurred within 15 min, but this study does not describe a time frame for *L. variegatus* recovery following nicotine exposure. Chronic exposure to nicotine has been more extensively studied in *C. elegans* and Feng et al. (2006) observed that *C. elegans* chronically treated (≥16 h) with 1.5 μM nicotine increase their locomotion speed at ~4 min after nicotine withdrawal. At a comparable timeframe of 10 min after nicotine removal, we observed no significant increase in locomotor activity of *L. variegatus*. Smith et al. (2013) previously described a "no response to touch" in *C. elegans* exposed to 6.17–194.5 μM nicotine for 24 h and removal for 1 h, and we observed similar decreased responses to tactile stimulation (Fig. 4C-D) and locomotor activity (Fig. 4G) once nicotine was removed. The effects observed in *L. variegatus* likely occur due to muscle hyper-contraction paralysis (akinesia) through over-stimulation of nicotinic receptors, mirroring the effects observed in *C. elegans* exposed to higher doses of nicotine in liquid culture (Matta et al., 2007).

We also observed that nicotine had a rapid onset of locomotor inhibitory effects in *L. variegatus* at ≥0.1 mM from 4 min (Fig. 2A) with *L. variegatus* exposed to ≤0.5 mM displaying a rapid recovery from locomotor inhibitory effects within 10 min of nicotine removal (Fig. 2B). This aligns with previous findings in *L. variegatus* where 0.25 mM nicotine was used as a paralytic agent for 15 min of exposure (Lesiuk and Drewes, 2001). The onset of action of nicotine in *L. variegatus* closely replicates those observed in *C. elegans* and *Drosophila* where acute exposure to nicotine has also been shown to inhibit locomotion and inhibit negative geotaxis within 2–10 min (Feng et al., 2006; Sobkowiak et al., 2011; Velazquez-Ulloa, 2017). Reduced movements following nicotine exposure are akin to those seen in higher organisms, whereby nicotine decreases ambulatory activity (Kita et al., 1988; Umezu, 2012).

Furthermore, *L. variegatus* appear to have increased toxicity to nicotine compared to *C. elegans* as we show that ≥100 μM resulted in lethal effects in *L. variegatus* (Fig. 3). Comparatively, *C. elegans* are capable of surviving >194.5 μM in liquid culture (Smith et al., 2013) and > 500 μM on solid NGM plates (Kanteti et al., 2015) for 24 h. It has been shown that blood vessel pulsation waves are abolished following acute exposure to 1 mM (Lesiuk and Drewes, 1999) and the lethality following chronic exposure to nicotine is likely due to insufficient blood vessel pulsations, thereby preventing adequate tissue perfusion by erythrocruorin (Ryan and Elwess, 2017), the haemoglobin-like pigment found within *L. variegatus*. This increased toxicity of nicotine in *L. variegatus* may be due to increased nicotine permeation through the cuticle, with *L. variegatus* having a thinner cuticle compared to *C. elegans* (Pakarinen et al., 2011; Peixoto et al., 1997).

A key limitation of the work presented here is the limited genetic knowledge of *L. variegatus* (Anderson et al., 2017; Gustafsson et al., 2009; Tellez-Garcia et al., 2021). Previously, Gustafsson et al. (2009) observed the chromosome number of *L. variegatus* and suggested that clade I specimens were highly polyploid while clade II individuals were diploid. In this study, the ploidy level of the *L. variegatus* is unknown, and while recent studies did not describe any morphological differences between the two clades (Zhou et al., 2023), these results presented in this study may not hold for all populations of *L. variegatus*.

5. Conclusion

Our results indicate that behavioural responses of *L. variegatus* are not dissimilar to those observed in conventional invertebrate models, such as *C. elegans* and *Drosophila*, or those seen in higher organisms. While it remains unknown if *L. variegatus* express "nicotinic-like" receptors (Walker et al., 1993), this study demonstrates that nicotine exposure results in time- and concentration-dependent inhibition of tactile stimulation to elicit stereotypical behaviours and inhibitory effects on *L. variegatus* locomotor activity. Additionally, we demonstrate quantifiable levels of acetylcholine and cholinesterase activity. Together, our findings are suggestive of the presence of nicotinic receptors within *L. variegatus* while demonstrating the applicability of *L. variegatus* for wider use within biomedical research.

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CRediT authorship contribution statement

Nia A. Davies: Writing – original draft, Supervision, Project administration, Conceptualization. **Julanta J. Carriere:** Visualization, Investigation. **Aneesha Gopal:** Visualization, Investigation. **Annie Rajan:** Visualization, Investigation. **Melisa J. Wallace:** Writing – original draft, Supervision, Project administration, Conceptualization. **Aidan Seeley:** Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Data availability

Data will be made available on request.

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