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Title Page

Title: The behavioural, toxicological, and biochemical effects of caffeine on *Lumbriculus variegatus*.

Running Title: The effects of caffeine on *L. variegatus*.

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Abstract

Caffeine is an emerging contaminant of concern frequently detected in freshwater systems, yet the behavioural, toxicological and biochemical effects of caffeine in aquatic invertebrates remain poorly characterised. Here, we investigate the effects of caffeine exposure on survival, behaviour, locomotion, and energy stores in the freshwater annelid *Lumbriculus variegatus*. Exposure to ≥5.0 mM caffeine for 10 minutes or ≥3.0 mM for 24 hours reduced stimulated behaviours, with locomotion suppressed at ≥5.0 mM (10 minutes) and ≥1.0 mM (24 hours) ($p < .05$, $n = 8$), which persisted 24 hours after exposure to 10 mM (10 minutes) or 3.5 mM (24 hours). A 24-hour LC_{50} of 4.7 mM (95% CI: 4.60–4.70 mM) was observed, with significant lethality after seven days at 4.5 mM ($p < .0001$). These findings provide the first characterisation of caffeine's effects in *L. variegatus* and inform environmental risk assessment of caffeine in freshwater systems.

Keywords: Caffeine, *L. variegatus*, *Lumbriculus variegatus,* behavioural toxicology, ecotoxicology

1. Introduction

Caffeine is the most widely consumed psychoactive stimulant worldwide (Reddy et al., 2024), with the main sources being coffee beans and tea leaves (Heckman et al., 2010), as well as chocolate and soft drinks (Lorist and Tops, 2003). In humans, caffeine acts as a non-selective antagonist at adenosine receptors, which, when activated by adenosine in the central nervous system, promote drowsiness (Reddy et al., 2024). Consumption of caffeine antagonises these effects, leading to temporary relief of drowsiness and increased alertness (Reddy et al., 2024). Additionally, caffeine has also been shown to affect energy metabolism and energy stores, namely glycaemic metabolism, carbohydrate oxidation and lipid turnover (Acheson et al., 2004; Reis et al., 2018; Sinha et al., 2014; Yeo et al., 2005), with effects observed in human (Acheson et al., 2004; Reis et al., 2018; Yeo et al., 2005), rodent (Du et al., 2018; Sinha et al., 2014), and invertebrate studies (Du et al., 2018; Naing et al., 2025).

Due to the widespread consumption of caffeine, its high water solubility and long half-life (Vieira et al., 2022), caffeine has become ubiquitous in aquatic environments and is now recognised as an environmental contaminant of emerging concern (Li et al., 2020; Vieira et al., 2022; Wilkinson et al., 2022). Caffeine residues have been detected in groundwater, rainwater and drinking water worldwide (Li et al., 2020; Wilkinson et al., 2022), and its continuous release through domestic and industrial effluents, combined with its pharmacological activity, highlights the need to understand its impact on aquatic species.

Caffeine can be passively absorbed through integumental surfaces (Rodrigues et al., 2025a) and has been shown to induce interspecies differences including negligible responses in filter-feeders such as *Magallana gigas and* oxidative stress in gastropods such as *Littorina littorea* when exposed to nanomolar concentrations of caffeine (Baracchini et al., 2023), delays hatching of *Palaemonetes*

pugio at ≈0.1 mM (Garcia et al., 2014), shows acute mortality and growth affects at ≈3.3 mM and ≈0.42 mM, respectively, in *Chironomus riparius* (Rodrigues et al., 2025a), impaired reproduction in *Ceriodaphnia dubia* exposed to ≈0.23 mM, with an observed LC_{50} of ≈0.31 mM (Moore et al., 2008), an LC_{50} of ≈6.3 mM in *Chironomus dilutes* (Moore et al., 2008), and ≈8.6 mM causing complete lethality in *Daphnia magna* (Rodrigues et al., 2025b).

While previous studies have shown that caffeine exposure increases the dorsal blood vessel pulsation rates in *Lumbriculus variegatus* (Lesiuk and Drewes, 1999; Ryan and Elwess, 2017), the behavioural, toxicological and biochemical effects of caffeine exposure on *L. variegatus* behaviours are not yet characterised.

L. variegatus is a freshwater oligochaete worm found in shallow ponds, lakes and marshes (Drewes, 1999; Seeley et al., 2021) which have been utilised extensively as a model organism in ecological studies of pollutants (Aikins et al., 2023; Colombo et al., 2016; O'Gara et al., 2004; Sardo et al., 2011; Sardo and Soares, 2010; Silva et al., 2021; Vought and Wang, 2018; Wang et al., 2023). Increasingly, they are being used to study the effects of biologically active compounds, as γ-aminobutyric acid (GABA) (Seeley et al., 2025) and histamine (Carriere et al., 2023), as well pharmacologically active compounds including cannabidiol (Williams et al., 2025), dantrolene, lidocaine, quinine (Seeley et al., 2021), dehydroepiandrosterone (Frank et al., 2025), diclofenac (Karlsson et al., 2016), ethanol (Seeley et al., 2024), ethinylestradiol (Wang and Wang, 2021), fluoxetine (Karlsson et al., 2016; Nentwig, 2007), ivermectin (Ding et al., 2001; Egeler et al., 2010), loratadine, mepyramine (Carriere et al., 2023), metoprolol (Buchberger et al., 2018), and nicotine (Davies et al., 2025).

L. variegatus display stereotyped escape behaviours which can be elicited by tactile stimulation of the anterior segments to evoke a rapid body reversal response, characterised by bending movements that invert head and tail orientation, whereas stimulation of posterior segments produces helical

swimming, consisting of rapid, repetitive body bends (Drewes, 1999). The effects of exposure to biologically and pharmacologically active compounds on these stereotyped behaviours have previously been evaluated and have also been described for use in practical pharmacology education (Carriere et al., 2023; Davies et al., 2025; Ding et al., 2001; Seeley et al., 2021, 2024, 2025; Williams et al., 2025).

In this study, we examined the effects of 10-minute and 24-hour exposure to caffeine at concentrations established in previous studies of *L. variegatus (Lesiuk and Drewes, 1999)* and akin to previous ecotoxicological studies of other freshwater species conducted in the millimolar range (Garcia et al., 2014; Moore et al., 2008; Rodrigues et al., 2025a, 2025b). We assessed the effects of exposure on stereotypical responses to tactile stimulation, locomotor activity, lethality, survival and evaluated the effects of caffeine on energy stores in *L. variegatus*. Finally, we explored the reproducibility of these results by replicating behavioural assays in undergraduate pharmacology practical classes across three institutions using established pedagogical methodology (Carriere et al., 2023; Seeley et al., 2021, 2025). This work is the first integrated characterisation of caffeine's effects in *L. variegatus,* providing insights into caffeine's effects in this organism and highlighting the need for further studies of caffeine's ecotoxicological impact.

2. Methods

2.1 *Lumbriculus variegatus* Culture

L. variegatus were purchased from Fish Mania Aquatics Ltd and cultured using established protocols (Carriere et al., 2023; Davies et al., 2025; Seeley et al., 2021, 2024; Williams et al., 2025). Briefly, worms were maintained in artificial pond water (1 mM sodium chloride, 13 µM potassium chloride, 4 µM calcium nitrate tetrahydrate, 17 µM magnesium sulphate heptahydrate, 71 µM 4-(2-

hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in UV-treated deionised water) with continuous aeration and filtration, at 18 – 21 °C and a 16:8-hour light-dark cycle. Cultures were fed TetraMin® flakes and 10 mg / L spirulina weekly, and cultures were maintained for a minimum of three months before experimentation.

As per previous studies (Carriere et al., 2023; Davies et al., 2025; Seeley et al., 2021, 2024; Williams et al., 2025), *L. variegatus* selected for experimentation were randomly selected, ranged from 2 – 8 cm in length, lacked any obvious morphological defects and were euthanised by rapid submersion in 70% ethanol at experimental endpoints.

All experiments adhere to the applicable ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) reporting guidelines (Sert et al., 2020). As invertebrates, *L. variegatus* are not covered under the Animal (Scientific Procedures) Act 1986 and, therefore, ethical approval was not required for this work.

2.2 Materials

Caffeine (Sigma-Aldrich, Dorset, United Kingdom) solutions were prepared in artificial pond water on the day of use.

2.3 Behavioural assays

2.3.1 Stereotypical responses to tactile stimulation

Stereotypical behaviours of body reversal and helical swimming were assessed as per Seeley *et al*. (2021). Briefly, individual *L. variegatus* were acclimated for 18 - 24 hours in six well plates containing 4 mL of artificial pond water before experimentation. Baseline measurements of stereotyped behaviours were evaluated by alternate stimulation of anterior and posterior ends using a pipette tip for a total of five stimuli per end, with 5 – 10 second inter-stimulus intervals. Responses to stimuli

were objectively scored as 1 = no movement, 2 = incomplete stereotypical movement, and 3 = full stereotypical movement (Seeley et al., 2021).

Following baseline assessment, artificial pond water was replaced with an experimental control (artificial pond water only) and either 0.01 – 10 mM caffeine for 10 minutes or 0.5 – 3.5 mM caffeine for 24 hours. Stereotypical behaviours in response to tactile stimulation were reassessed after caffeine exposure for either 10 minutes or 24 hours, after which wells were washed with artificial pond water to remove any latent solution, and replaced with artificial pond water only. Recovery of stereotypical behaviours in response to tactile stimulation was reassessed 10 minutes (10 min recovery) and 24 hours (24 hr recovery) after incubation in artificial pond water only. Data are expressed as the ratio of exposure or recovery scores relative to baseline responses, from eight experimental repeats with a single *L. variegatus* exposed to each concentration per experimental repeat (Seeley et al., 2021).

2.3.2 Locomotor activity

Locomotor activity was quantified as per Seeley *et al*. (2021). Following acclimation, as above, artificial pond water was replaced with 2 mL of fresh artificial pond water to limit vertical movement. Baseline locomotor activity was determined by sequential image collection with a 13-megapixel camera at 1 frame/s for 50 seconds. Artificial pond water was then removed and replaced with experimental controls or caffeine solutions, as above, and images were acquired 10 minutes or 24 hours after caffeine exposure. Recovery of locomotor activity was assessed in the same manner as above, with image acquisition 10 minutes (10 min recovery) and 24 hours (24 hr recovery) after incubation in artificial pond water only.

Collected images were analysed in ImageJ, whereby sequential images were superimposed, spatially calibrated to an area of known distance within each image, and thresholded to isolate the total area

covered by individual *L. variegatus* at baseline, during caffeine exposure, and at both recovery time points. Data are expressed as a percentage of the locomotor activity by *L. variegatus* compared to baseline conditions, from eight experimental repeats with a single *L. variegatus* exposed to each concentration per experimental repeat (Seeley et al., 2021).

2.4 Lethality and survival assays

To determine the lethality of caffeine, *L. variegatus* were acclimated for 18 – 24 hours in 24-well plates containing artificial pond water only and then exposed to either an experimental control (artificial pond water only) or 0.01 – 10 mM caffeine for 24 hours. 24 hours after exposure, surviving *L. variegatus* were counted.

For survival assays, *L. variegatus* were acclimated as above, then exposed to either an experimental control (artificial pond water only) or 0.5 – 4.5 mM caffeine for seven days, with surviving *L. variegatus* counted daily.

For both assays, lethality was determined by daily visual inspection for decomposition (Davies et al., 2025; Williams et al., 2025). Data is expressed as the percentage of survival relative to experimental controls.

2.5 Quantification of energy stores

To examine potential effects of caffeine exposure on energy stores, analysis of total energy available by quantification of protein, carbohydrate and lipid levels in *L. variegatus* homogenate was conducted as per Williams *et al.* (2025).

Briefly, ten *L. variegatus* per condition were transferred to a 30 mL specimen bottle and acclimated for 18 – 24 hours. After the acclimation period, *L. variegatus* were exposed to either an experimental control (artificial pond water only) or caffeine (1.0 – 3.5 mM) for 24 hours. *L. variegatus* were then

dried on filter paper, weighed, and homogenised in ice-cold artificial pond water. An aliquot of total homogenate was removed for lipid quantification, with the remaining homogenate centrifuged at 16.1 RCF at 4 ^{o}C for 15 minutes and two aliquots were removed for protein and carbohydrate quantification.

Proteins were quantified using the Bradford method (1976), using bovine serum albumin as the quantification standard, carbohydrates were quantified using the phenol-sulphuric acid method (DuBois et al., 1956), using glucose as the standard for quantification, and lipids, following extraction using the Bligh & Dyer method (1959), were quantified using the sulpho-phospho-vanillin technique (Men et al., 2019), with triolein as the quantification standard (2025).

Absorbances were measured, in triplicate, using the FLUOstar Omega Microplate Reader (BMG Labtech) at 595 nm, 492 nm and 530 nm, respectively, and energetic equivalents calculated according to De Coen & Janssen (1997). Total energy available (E_a) was calculated as the sum of energy from protein ($E_{Protein}$), energy from carbohydrates ($E_{Carbohydrate}$) and energy from lipids (E_{Lipid}), expressed as mJ / mg of *L. variegatus* (Williams et al., 2025).

2.6 Practical Classes using *Lumbriculus variegatus*

Three practical classes using *L. variegatus* were conducted at Swansea University, University College Cork, and Strathclyde University and conducted by students enrolled on undergraduate courses within the biomedical science discipline during academic years 2018 – 2019, 2022 – 2023 and 2023 – 2024, respectively. *L. variegatus* purchased for use during the Swansea University practical were purchased from Alfa Fish Food and cultured as outlined above. *L. variegatus* used during the practical class at University College Cork were purchased from Seahorse Aquariums Ltd and from Fish Mania Aquatics Ltd for the practical class at the University of Strathclyde, with both cultures maintained in artificial pond water before use.

All institutions followed the same experimental protocol and evaluated the same caffeine concentrations. 18 - 24 hours before the practical class, individual *L. variegatus* were transferred to each well of a 6-well plate containing approximately 4 mL of artificial pond water at room temperature. During the practical class, the pond water was replaced, and the baseline ability of *L. variegatus* to respond to tactile stimulation was tested as described above. The artificial pond water was then removed and immediately replaced with either an experimental control (artificial pond water only) or 0.01 – 10 mM caffeine. *L. variegatus* responses to tactile stimulation to elicit body reversal or helical swimming were then tested after 10 minutes of exposure to caffeine. At practical class endpoints, *L. variegatus* were euthanised by rapid submersion in 70% ethanol or freezing for ≥48 hours.

Data are expressed as a ratio of the movement score during or after caffeine exposure relative to baseline, calculated as described above. As per Carriere et al. (2023), students were blinded to the identity of the compound being tested while conducting the behavioural assessments, with treatment identity disclosed only after data collection was completed.

2.7 Statistical analysis

For behavioural assays, responses following caffeine exposure were compared with baseline responses using paired, two-tailed non-parametric tests for stereotypical movement assays, and paired, two-tailed parametric tests for locomotor activity. 10-minute and 24-hour recovery timepoints were analysed by two-way ANOVA followed by Dunnett's multiple comparisons test versus baseline.

The LC_{50} for the lethality assay was determined using GraphPad Prism 10. Survival assays were analysed using Kaplan-Meier curves and were compared using the log-rank (Mantel-Cox) test. A one-

way ANOVA with Dunnett's post-test was used to compare energy stores and total energy available to experimental controls.

For comparison of *L. variegatus* stereotypical movement responses from practical classes, a one-way ANOVA with Tukey's post-test was used to compare exposure conditions to their corresponding baseline controls within each institution, and to compare responses between institutions.

Data are expressed as mean ± standard error of the mean (SEM) with $p < .05$ being the threshold for statistical significance. Data were analysed using GraphPad Prism 10.

3. Results

3.1 Behavioural response to 10-minute caffeine exposure

It was observed that *L. variegatus* exposed to 0 – 10 mM caffeine for 10 minutes similarly displayed reduced responses to tactile stimulation at ≥5 mM caffeine, with both body reversal and helical swimming responses being reduced ($p < .01$, Figure 1A and B, $n = 8$). Following removal of 5.0 mM caffeine for 10 minutes, *L. variegatus* regained responses to tactile stimuli for both movements, returning to responses indistinguishable from baseline conditions ($p > .05$, Figure 1C and D, $n = 8$). However, 10 minutes after removal of 10 mM caffeine, body reversal movements in response to tactile stimulation remained reduced ($p = .008$, Figure 1C, $n = 8$) but returned to levels indistinguishable from baseline 24 hours after caffeine exposure ($p > .05$, Figure 1C, $n = 8$). Helical swimming in response to tactile stimuli was similarly reduced following removal of 10 mM caffeine, with effects persisting for 24 hours after exposure ($p < .05$, Figure 1D, $n = 8$).

We also observed dose-dependent effects on *L. variegatus* locomotor activity, with ≥5.0 mM caffeine reducing locomotor activity. In *L. variegatus* exposed to 5.0 mM caffeine, movement reduced to 87.09 ± 3.98 %, compared to untreated baseline ($p = .001$, Figure 1E and F, $n = 8$), while 10 mM

caffeine decreased locomotor activity to 78.40 ± 3.83 % of baseline conditions (p = .0008, Figure 1E and F, *n* = 8). These effects persisted 24 hours after removal, with ≥5.0 mM caffeine resulting in decreased locomotor activity in *L. variegatus* (*p* < .05, Figure 1G).

3.2 Lethality of caffeine exposure

We next sought to determine the toxicological effects of ≥24-hour exposure to caffeine on *L. variegatus.* When exposed to 0 – 10 mM caffeine for 24 hours, we determined the LC_{50} to be 4.65 mM (95 % CI: 4.60 – 4.70 mM, Figure 2A, n ≥ 6). Next, we evaluated the effects of ≤4.5 mM caffeine on *L. variegatus* survival for a period of seven days (Figure 2B). We observed no lethality in *L. variegatus* exposed to ≤3.5 mM caffeine over the seven days, with 4.5 mM caffeine causing significant lethality when analysed by the log-rank (Mantel-Cox) tests (*p* < .0001, Figure 2B, *n* ≥ 6).

3.3 Behavioural response to 24-hour caffeine exposure

We then examined the impact of 24-hour exposure to caffeine on *L. variegatus* at sub-lethal concentrations. We observed that exposure to 3.0 – 3.5 mM caffeine for 24 hours reduced body reversal responses following tactile stimulation (*p* < .01, Figure 3A, *n* = 8), while ≥2.0 mM caffeine inhibited helical swimming responses (*p* < .05, Figure 3B, *n* = 8). Following caffeine removal, both responses to tactile stimulation returned to levels indistinguishable from baseline conditions in *L. variegatus* exposed to ≤3.0 mM (*p* > .05, Figure 3C and D, *n* = 8) while inhibition of both movements was observed in *L. variegatus* exposed to 3.5 mM caffeine 24 hours after caffeine removal (p < .01, Figure 3C and D, *n* = 8).

24 hours of caffeine (0 – 3.5 mM) exposure also had effects on the locomotor activity of *L. variegatus*. Concentrations of 1.0 mM, 2.0 mM and 3.5 mM decreased locomotor activity to 82.47 ± 6.04 %, 76.52 ± 5.72 % and 61.43 ± 7.14 %, respectively (*p* < .05, Figure 3E and F, *n* = 8). The effects of 1.0 mM and

2.0 mM caffeine were readily reversible 10 minutes after caffeine removal, with movement returning to levels indistinguishable from baseline ($p > .05$, Figure 3G, $n = 8$). However, 10 minutes after removal of caffeine, locomotion was reduced to 58.44 ± 19.77 % and 31.82 ± 14.29% in *L. variegatus* exposed to 3.0 mM and 3.5 mM caffeine, respectively ($p < .05$, Figure 3G, $n = 8$). In *L. variegatus* exposed to 3.5 mM caffeine, locomotor activity did not recover and remained inhibited 24 hours after removal of caffeine, with movement decreased to 41.68 ± 7.92 % ($p < .0001$, Figure 3G, $n = 8$).

3.4 Effects of caffeine exposure on energy stores and total energy available

We next quantified protein, carbohydrate and lipid levels in *L. variegatus* following 0 – 3.5 mM caffeine exposure for 24 hours (Figure 4A – C, $n = 6$). We observed no significant difference in energy stores ($p > .05$, Figure 4A – C, $n = 6$) nor E_a ($p > .05$, Table 1, $n = 6$) within *L. variegatus* homogenate for any of the caffeine exposure conditions.

3.5 Stereotypical movements of *L. variegatus* exposed to caffeine in undergraduate practical classes

When *L. variegatus* were exposed to 0 – 10 mM caffeine for 10 minutes during undergraduate practical classes, it was observed across all three institutions that 10 mM inhibited the tactile stimulation to elicit body reversal and helical swimming stereotypical movements compared to untreated baseline conditions ($p < .05$, Figure 5A and B, $n \geq 6$). Observations from both the University of Strathclyde and Swansea University observed that 5.0 mM was also able to inhibit both movements following tactile stimulation of *L. variegatus* ($p < .05$, Figure 5A and B, $n \geq 10$). Similar effects were observed from practical classes conducted at University College Cork, where 5.0 mM caffeine inhibited body reversal behaviours ($p = .0047$, Figure 5A, $n = 6$) but not helical swimming movements ($p = .343$, Figure 5B, $n = 6$).

Observations between the three institutions at all tested concentrations were compared by one-way ANOVA with Tukey's post-test, which showed no significant differences between the institutions in the ability of *L. variegatus* to respond to tactile stimulation ($p > .05$, Figure 5A and B, $n \geq 6$).

4. Discussion

In our study, we demonstrate the rapid behavioural changes in *L. variegatus* exposed to caffeine, with 10-minute exposure to ≥5.0 mM and 24-hour exposure to 3.5 mM caffeine resulting in reduced stereotypical movements in response to tactile stimulation and locomotor activity. Failure to recover behavioural function occurred following 10 mM exposure for 10 minutes and 3.5 mM exposure for 24 hours. We also demonstrate that ≥4.5 mM caffeine resulted in lethal effects in *L. variegatus*, but that this was not as a result of altered energy stores. Finally, we demonstrate that the behavioural observations are robust and reproducible across academic institutions using *L. variegatus* from different commercial sources.

In this study, we used equimolar maximum concentrations of caffeine to those used by Lesiuk & Drewes, who described 15 minutes exposure to 1 – 10 mM of caffeine increased DBV pulsations (Lesiuk and Drewes, 1999), and concentrations similar to those used in previous ecotoxicological studies of caffeine (Moore et al., 2008; Rodrigues et al., 2025a, 2025b). Herein, we examined locomotory behaviours for a shorter duration of exposure, namely 10 minutes, having previously demonstrated that 10 minutes of exposure is effective for observing acute effects on locomotory behaviours in *L. variegatus* (Carriere et al., 2023; Davies et al., 2025; Seeley et al., 2025, 2024, 2021; Williams et al., 2025).

We observed that 10-minute exposure to ≥5.0 mM caffeine decreased both stereotypical movements and free locomotion of *L. variegatus,* similar to findings observed in the marine invertebrate *Aurelia*

aurita, where 5 - 10 mM caffeine was shown to decrease spontaneous swimming. Studies using planarian flatworms have shown similar decreased locomotor activity when exposed to 10 mM caffeine for two minutes (Moustakas et al., 2015), while exposure to ≤0.1 mM for five minutes did not affect locomotor activity of planaria (Sacavage et al., 2008), which closely resembled the findings of this study. Conversely, caffeine has been reported to increase activity in terrestrial invertebrates, such as *Drosophila melanogaster, Apis mellifera, Vespa orientalis, Coccus viridis, Tribolium castaneum* and *Tribolium confusum* (Fernandes et al., 2012; Ishay and Paniry, 1979; Nakayama et al., 2012; Nishi et al., 2010; Shaw et al., 2000). It may be that aquatic invertebrates have increased sensitivity to caffeine exposure, with cellular stress via upregulation of Hsp70 and lysosomal membrane destabilisation in aquatic invertebrates having previously been documented at concentrations magnitudes lower than those used in this study (Gabriela V. Aguirre-Martínez et al., 2013; G. V. Aguirre-Martínez et al., 2013; del Rey et al., 2011).

Having characterised the effects of 10-minute caffeine exposure, we next examined the consequences of an extended 24-hour exposure, consistent with previous studies of pharmacologically active compounds in *L. variegatus* (Davies et al., 2025; Williams et al., 2025). Exposure to 5.0 mM caffeine resulted in 83.3 ± 16.7% lethality within 24 hours, while concentrations ≥6.0 mM caused complete lethality. Comparable 24-hour exposures in *Drosophila* have also demonstrated caffeine-induced toxicity, with ~2.6 mM and ~5.1 mM producing 28% and 43% lethality, respectively (Lin et al., 2010). In contrast, studies in *Caenorhabditis elegans* report increased lifespan following exposure to 5–15 mM caffeine, with reduced lifespan only observed at concentrations ≥30 mM (Bridi et al., 2015). When *Daphnia magna* were exposed to 0.139 mM – 15.45 mM caffeine for 48 hours, 100% lethality was observed at ≥8.59 mM (Rodrigues et al., 2025b), findings which closely resemble those presented here.

Next, we evaluated the behavioural effects of 24-hour exposure to caffeine and found that exposure to 3.5 mM caffeine failed to recover behavioural function for both body reversal and helical swimming, as well as locomotor activity, following the removal of caffeine and incubation in artificial pond water for 24 hours. This may be mechanistic, but the observed behavioural suppression likely reflects early toxicological effects rather than selective neuromodulatory action. The results presented here closely replicate those seen in *D. magna,* where 24-hour exposure to ≥0.45 mM caffeine resulted in immobilisation (Rodrigues et al., 2025b). However, studies of locomotive activity in *Drosophila* exposed to ~0.5 mM of caffeine for 24 hours demonstrated increased locomotor activity, with similar effects observed at ~2.6 mM and ~5.1 mM (Lin et al., 2010). Moreover, 10 mM caffeine exposure has been shown to increase *C. elegans* locomotion after 72 hours but did not significantly change locomotive behaviours after 24 hours of exposure (Manalo and Medina, 2020), as used within this study, suggesting increased sensitivity of *L. variegatus* compared to *C. elegans* and *Drosophila*. Of note, the route of exposure to caffeine in these studies using *C. elegans* and *Drosophila* was through supplementation of solid media. Exposure of given concentrations in solid media are not directly experimentally comparable to the same concentrations administered in liquid media (Matta et al., 2007), and so the differences in caffeine toxicity and locomotive effects observed herein may be due to differences in total surface area contact, osmoregulation or cuticular permeability (Davies et al., 2025; Matta et al., 2007).

As caffeine has been shown to affect energy metabolism and stores in invertebrates (Du et al., 2018; Naing et al., 2025), we next sought to investigate the effects of caffeine exposure on energy stores, the total E_a, in *L. variegatus*. Here, we observed that exposure to caffeine for 24 hours had no significant effect on protein, carbohydrate, or lipid levels, nor total E_a, indicating that caffeine exposure does not affect energy stores of *L. variegatus*. These findings conflict with previous invertebrate studies in *Lucilia sericata* and *C. elegans*, which showed that caffeine affected lipids (Du

et al., 2018; Naing et al., 2025). Our observations also contrast with previous studies using *L. variegatus* that observed exposure to polyethylene microplastics decreased lipid and carbohydrate levels, with minimal effects on total E_a (Silva et al., 2021), and exposure to CBD decreased carbohydrate and increased lipid levels, with no significant difference in total E_a (Williams et al., 2025). Here, our observations on total E_a do not elucidate the mechanism underlying caffeine-induced lethality or behavioural changes, but they indicate that such effects are unlikely to be associated with alterations in metabolic energy stores.

Within the scientific community more broadly, there is a consensus of a reproducibility crisis in science (Baker, 2016), with concerns that replication of published results may only be possible when using homogeneous animal strains and strict experimental conditions, thereby limiting the generalisation of study findings (França and Monserrat, 2018; Voelkl et al., 2018).

However, we observed that using caffeine as a test compound and established behavioural techniques for using *L. variegatus* within practical classes (Carriere et al., 2023; Seeley et al., 2025, 2021) led to data akin to those seen under laboratory conditions, with broadly consistent results observed across the undergraduate practical classes from three institutions. While ≥5.0 mM caffeine was shown to inhibit body reversal movements across the three institutions, the effects of 5.0 mM caffeine on helical swimming were not observed within the University College Cork data set. Notably, this data set had the lowest replicate number and, consequently, exhibited greater variability, with six replicates compared to eight replicates employed in previous studies utilising this methodology (Carriere et al., 2023; Davies et al., 2025; Seeley et al., 2024, 2021; Williams et al., 2025). Variation in responses was expected to be seen between *L. variegatus* cultures, as it has been suggested that *L. variegatus* exists within two clades, with clade I specimens being highly polyploid and clade II specimens being diploid (Gustafsson et al., 2009), without morphological differences between the

clades (Zhou et al., 2023). Despite this, the observations of caffeine's effects within the practical classes were broadly consistent across the institutions, despite the ploidy level of *L. variegatus* being unknown and the cultures coming from different commercial sources.

While these data demonstrate clear dose-dependent behavioural and toxicological effects, the molecular targets remain to be elucidated. A key limitation of the work presented here, and the use of *L. variegatus* more broadly, is the lack of a full genomic sequence and the limited genomic information available (Agbo et al., 2013; Tellez-Garcia et al., 2021). As such, it is not known if *L. variegatus* express the sites of action for caffeine. That said, given the previously described physiological responses to adenosine (Crisp et al., 2010) and adenosine receptor antagonists (Crisp et al., 2010; Lesiuk and Drewes, 1999) alongside the findings of this study, these collectively suggest an 'adenosinergic-like' system within *L. variegatus*.

Herein, we show that caffeine produces concentration-dependent reductions in responses to tactile stimulation and locomotion in *L. variegatus* without affecting energy stores. Our findings were broadly consistent across independently maintained cultures and replicated in undergraduate teaching environments, underscoring the robustness of *L. variegatus* as a model while also emphasising the application of *L. variegatus* as a model for pharmacological research and education. While environmental monitoring finds caffeine at µg/L levels in environmental samples (Li et al., 2020; Wilkinson et al., 2022), far below the concentrations used here, our results at these higher doses establish a hazard profile for future studies examining the effects of caffeine in *L. variegatus*.

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6. Author Contributions

GEL: Investigation, Formal analysis, Visualization, Writing – original draft, RKL: Investigation, Resources, Writing – original draft, MRC: Investigation, Resources, Writing – original draft, NAD: Supervision, Funding acquisition, SGR: Investigation, Supervision, Writing – original draft, AS: Conceptualization, Methodology, Investigation, Formal analysis, Supervision, Funding acquisition, Visualization, Project administration, Resources, Writing – original draft, Writing – review and editing.

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9. Figure Legends

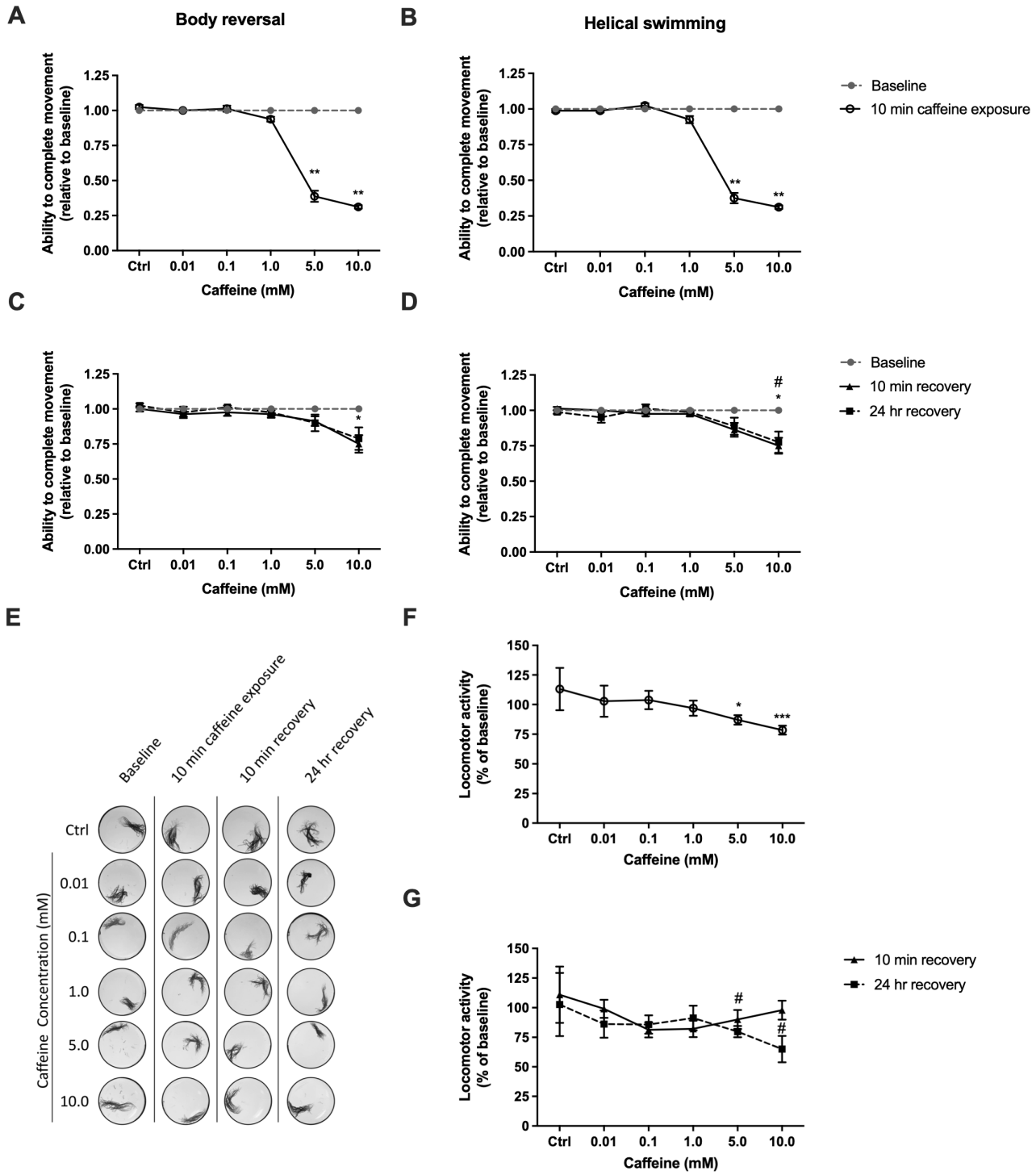

Figure 1. The effect of 10-minute exposure to 0.01 - 10 mM caffeine on *Lumbriculus variegatus* behaviours. *L. variegatus* were exposed to caffeine (0.01 - 10 mM) for 10 minutes and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. Following removal of caffeine, the

ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 minutes and 24 hr. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** Representative superimposed images analysed in ImageJ showing the effects of 10 minutes of exposure to caffeine on locomotor activity measured before caffeine exposure (baseline), after 10 minutes exposure to 0.01 - 10 mM caffeine (10 min caffeine exposure), 10 minutes after caffeine removal (10 min recovery) and 24 hours after caffeine removal (24 hr recovery). Quantification of the area covered by *L. variegatus* following **(F)** 10 minutes exposure to 0.01 - 10 mM caffeine and **(G)** removal of caffeine for 10 minutes (10 min recovery) and 24 hours (24 hr recovery), expressed as a percentage of the locomotor activity at baseline. Analyses were conducted by comparing caffeine exposure conditions with baseline conditions by paired nonparametric two-tailed *t*-test 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-minute and 24-hour recovery time points compared with baseline conditions for *L. variegatus*. */# $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; where * refers to statistical significance between Baseline and 10 minute caffeine exposure or statistical significance between Baseline and 10 minute recovery, # refers to statistical significance between Baseline and 24 hour recovery. Data are reported as the mean ± SEM, $n = 8$. Ctrl = artificial pond water only.

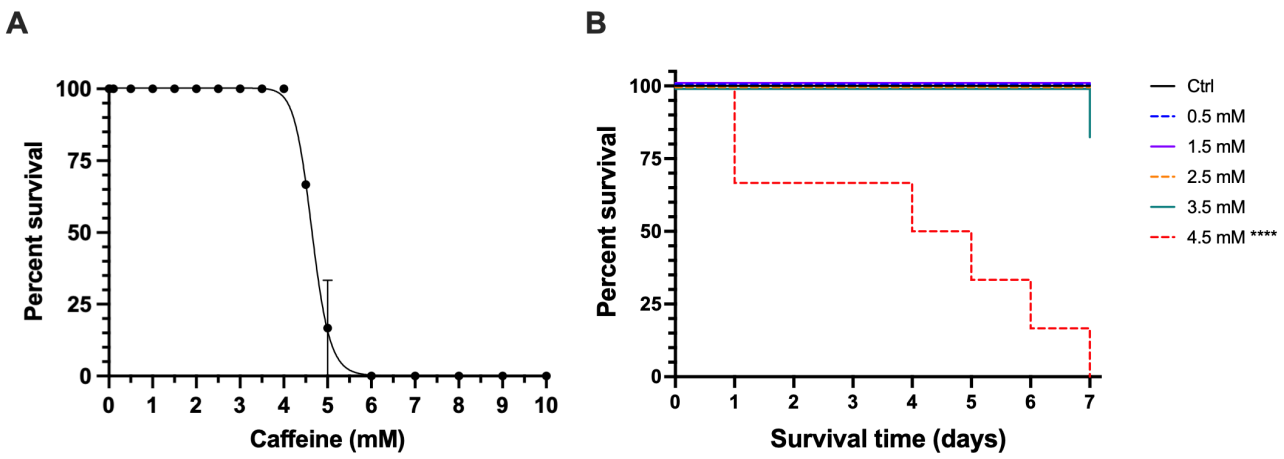

Figure 2. Lethality of caffeine in *Lumbriculus variegatus*. (A) *L. variegatus* were exposed to caffeine (0 – 10 mM) for 24 hours. After 24 hours of exposure, surviving *L. variegatus* were counted and expressed as a percentage. Data are reported as the mean ± SEM. $n \geq 6$ per concentration. **(B)** Survival assays of *L. variegatus* exposed to 0 – 4.5 mM caffeine for 7 days. The log-rank (Mantel-Cox) test was used to analyse survival compared to Ctrl, **** $p < .0001$. $n \geq 6$ per concentration. Ctrl = artificial pond water only.

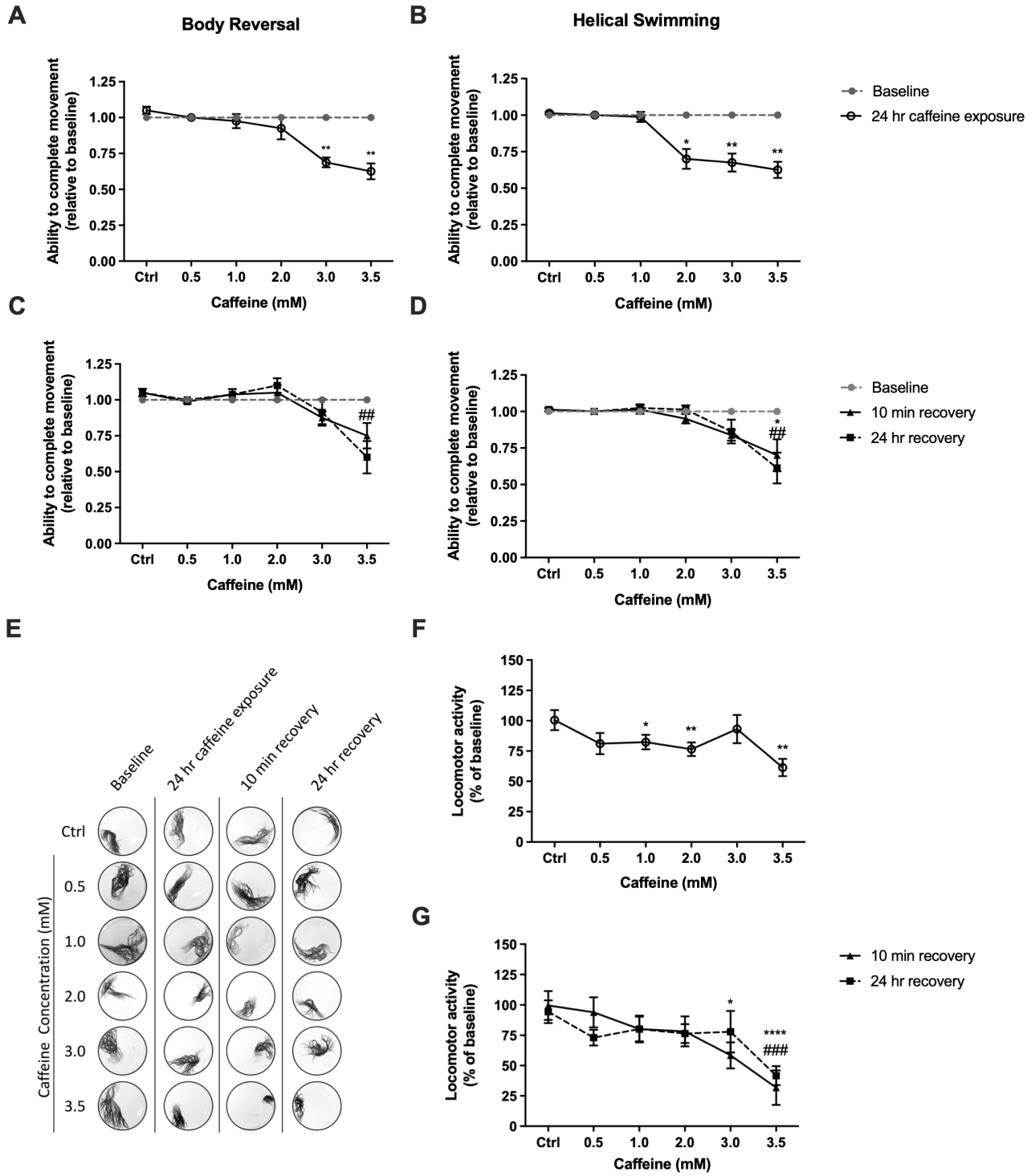

Figure 3. The effect of 24-hour exposure to 0.5 - 3.5 mM caffeine on *Lumbriculus variegatus* behaviours. *L. variegatus* were exposed to caffeine (0.5 - 3.5 mM) for 24 hours and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. Following the removal of caffeine, the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 minutes and 24

hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** Representative superimposed images analysed in ImageJ showing the effects of 24 hours of exposure to caffeine on locomotor activity measured before caffeine exposure (baseline), after 24 hours of exposure to 0.5 -3.5 mM caffeine (24 hr caffeine exposure), 10 minutes after caffeine removal (10 min recovery) and 24 hours after caffeine removal (24 hr recovery). Quantification of the area covered by *L. variegatus* following **(F)** 24-hour exposure to 0.5 - 3.5 mM caffeine and **(G)** removal of caffeine for 10 minutes (10 min recovery) and 24 hours (24 hr recovery), expressed as a percentage of the locomotor activity at baseline. Analyses were conducted by comparing caffeine exposure conditions with baseline conditions by paired nonparametric two-tailed *t*-test 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-minute and 24-hour recovery time points compared with baseline conditions for *L. variegatus*. * $p < 0.05$, **/## $p < 0.01$, ###, $p < 0.001$, **** $p < 0.0001$; where * refers to statistical significance between Baseline and 24 hour caffeine exposure or statistical significance between Baseline and 10 minute recovery, # refers to statistical significance between Baseline and 24 hour recovery. Data are reported as the mean ± SEM, $n = 8$. Ctrl = artificial pond water only.

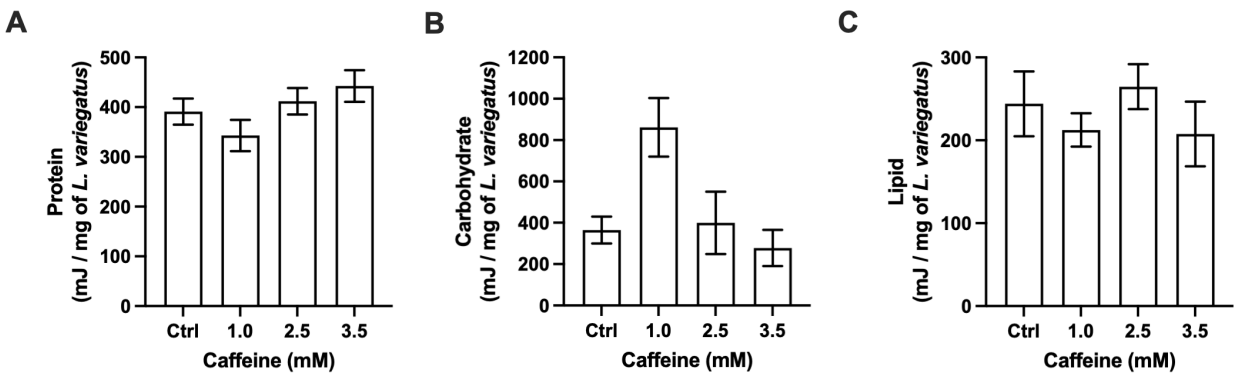

Figure 4. Quantification of energy stores (protein, carbohydrate and lipid contents) of *Lumbriculus variegatus* after exposure to caffeine. Levels of energy stores for **(A)** protein, **(B)** carbohydrates, and **(C)** lipids when *L. variegatus* were exposed to 1.0 - 3.5 mM caffeine for 24 hours; *n* = 6 with 10 *L. variegatus* per replicate measured in triplicate for each concentration. A one-way ANOVA with Dunnett's post-test was used to analyse the data comparing caffeine exposure with Ctrl. Data are reported as the mean ± SEM. Ctrl = artificial pond water only.

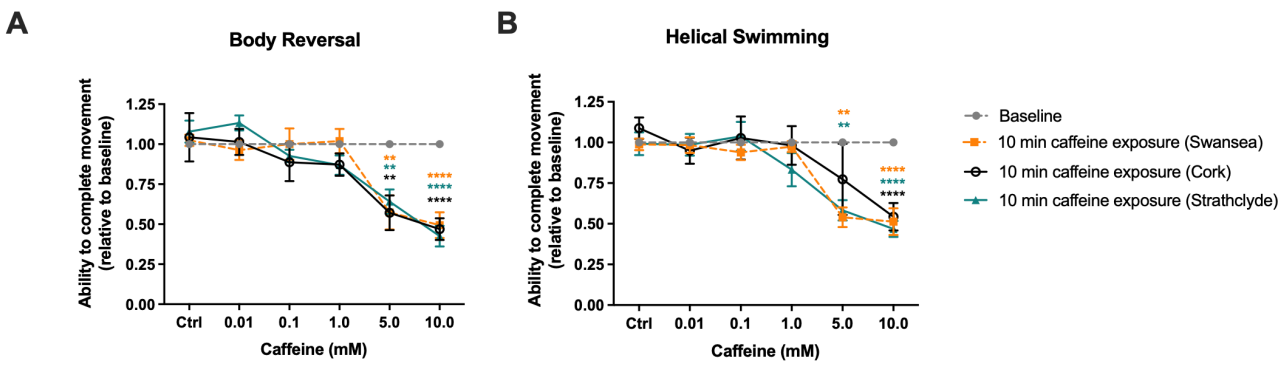

Figure 5. Comparison of the effects measured in practical classes across three higher education institutions of 10-minute exposure to 0.01 - 10 mM caffeine on *Lumbriculus variegatus* behaviours. *L. variegatus* were exposed to caffeine (0.01 - 10 mM) for 10 minutes and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming during a practical class conducted at Swansea University, United Kingdom (10 min caffeine exposure [Swansea]), University College Cork, Ireland (10 min caffeine exposure [Cork]) and University of Strathclyde, United Kingdom (10 min caffeine exposure [Strathclyde]). Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. A one-way ANOVA with Tukey's post-test was used to analyse the data. ** $p < 0.01$, **** $p < 0.0001$; where * refers to statistical significance between Baseline and 10-minute caffeine exposure. No significant difference was observed between the data obtained from University College Cork, University of Strathclyde and Swansea University. Data are reported as the mean ± SEM, $n = 8$ for 10-minute caffeine exposure (Swansea), $n = 6$ for 10-minute caffeine exposure (Cork), and $n = 10$ for 10-minute caffeine exposure (Strathclyde). Ctrl = artificial pond water only.

10. Table Legends

Table 1. Total energy stores of *Lumbriculus variegatus* after 24-hour exposure to caffeine. The total energy available, E_a, is the sum of $E_{Protein}$ + $E_{Carbohydrate}$ + E_{Lipid} (expressed as mJ / mg of *L. variegatus*). Data are reported as the mean ± SEM. No significant difference in E_a was observed in *L. variegatus* after caffeine exposure when analysed by one-way ANOVA with Dunnett's post-test compared to Ctrl. Ctrl = artificial pond water only.

Caffeine concentration	**E_a ($E_{Protein}$ + $E_{Carbohydrate}$ + E_{Lipid})**
Ctrl	999.7 ± 117.1
1.0 mM	1417.0 ± 105.4
2.5 mM	1076 ± 124.4
3.5 mM	927.7 ± 108.8

Note. E_a = total energy available, $E_{Protein}$ = energy from protein; $E_{Carbohydrate}$ = energy from carbohydrates; E_{Lipid} = energy from lipids.