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ADVANCES IN PHYSIOLOGY EDUCATION

SOURCEBOOK OF LABORATORY ACTIVITIES IN PHYSIOLOGY

How to get physiologically relevant data with students using *Lumbriculus variegatus*

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Abstract

The decline of in vivo teaching in higher education has resulted in graduates lacking essential experimental skills. To address this gap, we present an easy and cost-effective practical class using the emerging invertebrate model organism *Lumbriculus variegatus* as an additional in vivo model for education. This practical class enables students to observe the effects of pharmacologically active compounds on the stereotypical behaviors of body reversal and helical swimming in *L. variegatus* through tactile stimulation. During this class, students will conduct drug dilution calculations, administer test compounds, and conduct an in vivo behavioral experiment. Results from this class demonstrate drug effects in vivo and enable students to observe reversible or irreversible behavioral effects, depending on the compound tested. This class demonstrates *L. variegatus* as a model for hands-on in vivo teaching, providing students with critical laboratory experience without the need for vertebrate or higher-order mammal models. Furthermore, the approach outlined here is scalable and an adaptable teaching methodology that enhances student engagement with in vivo teaching without costly equipment or complex animal husbandry.

NEW & NOTEWORTHY There are increasing societal expectations that the extent of experimentation using animals is reduced in both research and educational fields. This leaves educators and researchers with a problem: how to foster the development of skills and advances in knowledge in future generations? This article seeks to address this problem by providing clear examples of an experimental model that can be used to study fundamental physiological and pharmacological processes using the invertebrate organism *Lumbriculus variegatus*.

Lumbriculus variegatus; partial replacement; 3Rs; undergraduate experiments

INTRODUCTION

Animal use in education has seen a decline, with an 88% decrease in animals used for education and training since 2001 in the United Kingdom alone (1, 2), which has resulted in students completing their degree without being taught in vivo skills (3). Learned societies have highlighted the importance of animal use in education, such as the British Pharmacological Society (4), the Human Anatomy & Physiology Society (5), and the American Physiological Society (6), and experiences of using living systems are impactful learning experiences for students (7, 8). Furthermore, this decline in in vivo teaching has produced an in vivo skills gap in graduates (9, 10), and so additional teaching methodologies for delivering in vivo teaching are required to address this.

Invertebrate models, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, while extensively used in research, have extensive applications in education (11). The ease of rearing invertebrates, the low costs of acquisition and maintenance, and their similarities, or differences, to vertebrate counterparts all contribute to their value as experimental subjects for use in education (12).

The freshwater annelid *Lumbriculus variegatus* was first proposed as an inexpensive and accessible organism for education by Charles Drewes in 1996 (13). As invertebrates, *L. variegatus* are not covered under the Animal (Scientific Procedures) Act 1986 in the United Kingdom or the Animal Welfare Act Regulations or the Public Health Service Policy on Humane Care and Use of Laboratory Animals in the United States, with similar legislation in many other countries allowing widespread adoption of the techniques outlined in this article. This organism, therefore, offers the opportunity for utilization within education settings. The use of invertebrate models for practical teaching, which traditionally utilizes mammalian species, can serve as a partial replacement in line with the principles of replacement, reduction, and refinement (3Rs), while still offering students valuable hands-on experience for behavioral studies.

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Objectives and Overview

The use of *L. variegatus* within biomedical education has recently had a small resurgence owing to their low cost and ease of use, the lack of regulatory restrictions, and utilization of commonly used equipment and resources (14, 15).

The practical class described here provides the opportunity to observe the effects of drug compounds in the invertebrate model *L. variegatus*, using methodology initially described by Seeley et al. (14). The selection of test compounds can easily be aligned to a range of physiological applications, and students performing the outlined practical class will observe the effects of diverse compounds on *L. variegatus*' ability to perform stereotyped behaviors before, during, and after exposure to compounds.

The objectives of the activities described in this exercise are to *1*) conduct drug dilution calculations and prepare drug solutions, *2*) complete a behavioral study examining the effects of drug administration on stereotypical movements of body reversal and helical swimming by *L. variegatus* through collection of quantitative data, and *3*) evaluate and analyze data relating to changes in stereotypical movements of *L. variegatus* following the addition and removal of drug solutions with basic statistical techniques.

Background

Lumbriculus variegatus, more commonly known as the blackworm or Californian blackworm, is an asexually reproducing, regenerative detritivore species that inhabits shallow freshwater ponds, lakes, and marshes (14, 16). Initially described by Drewes in 1999, *L. variegatus* display easily quantifiable stereotyped locomotor behaviors of body reversal and helical swimming following tactile stimulation (16), whereby tactile stimulation of the anterior segments of *L. variegatus* evokes body reversal through bending movements that reverse the head and tail positions (Fig. 1*A* and Supplemental Video S1) whereas stimulation of the posterior region evokes helical swimming movements characterized by rapid helical body bends (Fig. 1*B* and Supplemental Video S2). These behaviors are analogous to the behaviors observed in the extensively characterized *C. elegans* model, which displays quantifiable body bending and locomotor activity (17), but the larger size of *L. variegatus* (50–80 mm) compared with *C. elegans* (≈1 mm) makes them easier to view as individuals without microscopy techniques.

A 2021 study by Seeley et al. (14) described methods for quantifying the effects of exposure to compounds on inhibition of *L. variegatus* stereotypical movements in response to tactile stimulation. These stereotypical movement assays can be utilized to give students hands-on in vivo training, using a simple three-point scale (14), enabling students to measure the effects of compounds on reducing tactile stimulation to elicit two different behaviors (body reversal and helical swimming). It should be noted that this assay can only measure a decreased response to tactile stimulation, so it allows students to distinguish *L. variegatus* that perform body reversal or helical swimming movements in response to tactile stimulation (Fig. 1, Supplemental Videos S1 and S2) from *L. variegatus* that do not (Supplemental Video S3). The relative ease of the tactile stimulation application and the simplicity of the three-point scale minimize the risk of misinterpretation of the movements and limit variation between students conducting the assay.

L. variegatus have been studied extensively for the impact of pollutants (18–23) and are increasingly being used to study diverse compounds including the selective serotonin reuptake inhibitor fluoxetine (24), nonsteroid anti-inflammatory drugs such as diclofenac (25), the channel blockers dantrolene, quinine, and lidocaine (14), antihistamines such as mepyramine and loratadine (15), alcohol (26), nicotine (27), and cannabidiol (CBD) (28). These studies have examined behavioral effects such as reproduction, feeding, tactile stimulation to elicit stereotyped behaviors of body reversal and helical swimming, and unstimulated locomotor activity as experimental end points. Additional studies have described the effects of biogenic amines in *L. variegatus* (29), demonstrated the presence of glutaminergic neurons (30), and proposed the presence of a cholinergic system within this organism through quantification of acetylcholine levels and cholinesterase activity (27). These findings demonstrate the

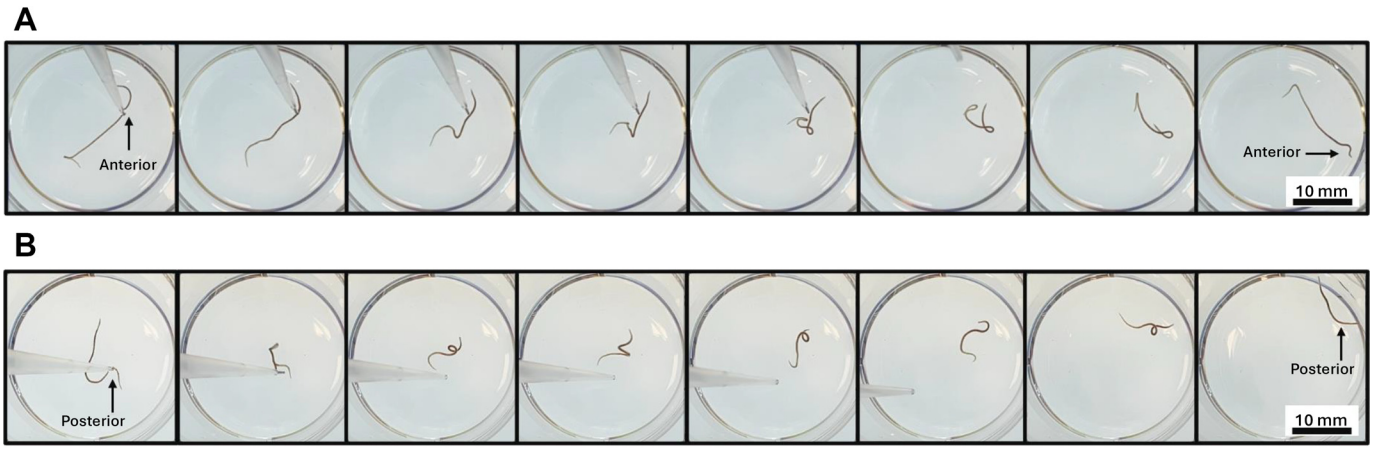

Figure 1. Stereotypical movements of *Lumbriculus variegatus* following tactile stimulation. Images show the stereotypical movement body reversal (*A*), whereby bending movements reverse the head and tail positions, and helical swimming (*B*), characterized by rapid helical body bends, of *L. variegatus* after tactile stimulation with a pipette tip on the anterior and posterior regions, respectively. Images show the stereotypical movements over a 2- to 3-s period after stimulation.

applicability of *L. variegatus* for use in physiology education while avoiding the costly and complex issues associated with vertebrate teaching models.

Here, we provide a comprehensive guide for administering the stereotypical movement assay (14) as an in vivo behavioral practical class using *L. variegatus* within a teaching environment.

Learning Objectives

After completing this practical class, students should be able to do the following:

1) Prepare, dilute, and administer drug compounds to *L. variegatus*.
2) Observe the effects of compounds on the body reversal and helical swimming stereotypical behaviors of *L. variegatus*, using the three-point scale from Seeley et al. (14).
3) Measure and plot relationships between drug concentrations and stereotypical behavior responses following tactile stimulation.
4) Apply basic statistical analyses to determine the effects of drugs of interest on the ability of *L. variegatus* to perform stereotypical behaviors during and after drug exposure.

Activity Level

The practical class presented here has been used with all levels of undergraduate students and Masters students in the general biomedical science disciplines. This method provides an early opportunity for students to engage with in vivo techniques, learn new skills, and gain knowledge of a research environment while generating potentially meaningful data. Furthermore, these techniques can, and have been, easily adapted for use in outreach activities with the general public.

Prerequisite Student Knowledge or Skills

Before students perform this practical class, they should have a good understanding of animal models in research and should be able to use a micropipette and calculate drug dilutions before the class commences, or additional instruction should be provided before conducting the class. Students would benefit from an understanding of the principles of replacement, reduction, and refinement (the 3Rs), and students may also benefit from prereading materials on the use of *L. variegatus,* such as the previous studies on using *L. variegatus* in education settings (14, 15).

Time Required

The class described here can be completed within a 3-h period, which includes a brief lecture covering the use of animals in research, the 3Rs, and the *L. variegatus* model and its stereotyped behaviors.

MATERIALS AND METHODS

Equipment and Supplies

Where suppliers have been cited below, alternative supplies are routinely available in many countries. Where a supplier has not been listed, these are available from standard scientific supply companies or directly from commercial sources.

- *L. variegatus* can be purchased from Carolina Biological Supply (United States, Catalog No. 141720) or Blades Biological Ltd (United Kingdom) or purchased from local aquarium stores before the class.
- Artificial pond water constitutions: sodium chloride (Melford Laboratories Ltd, Ipswich, United Kingdom, Catalog No. S23020), potassium chloride (Melford Laboratories Ltd, Ipswich, United Kingdom, Catalog No. P41000), calcium nitrate tetrahydrate (Duchefa Biochemie, Haarlem, The Netherlands, Catalog No. C0505), magnesium sulfate heptahydrate (Duchefa Biochemie, Haarlem, The Netherlands, Catalog No. M0513), HEPES (Sigma-Aldrich, United Kingdom, Catalog No. 54457), deionized water
- Equipment for short-term culturing (up to 4 wk) of *L. variegatus*: small commercial aquaria or buckets and brown paper towels
- (Optional) Equipment for longer-term culturing (longer than 4 wk) of *L. variegatus*: commercial aquaria, water filters, air stones, a timed light source, sandy or fine sediment, and spirulina powder
- TetraMin flakes
- Micropipettes (10, 20, 200, 1,000 μL volumes)
- Standard laboratory plasticware: six-well plates, Pasteur pipettes, pipette tips, 15-mL Falcon centrifuge tubes
- Marker pens
- Liquid waste containers
- Timers
- Drug compounds for testing; exemplary compounds are shown in Table 1.
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, United Kingdom, Catalog No. 34869) if a drug vehicle is required, as indicated in Table 1
- 70% ethanol solution

Animal Subjects

For short-term cultures, which are sustainable for up to 4 wk before the practical class, *L. variegatus* cultures should be maintained in artificial pond water composed of 1 mM sodium chloride, 13 μM potassium chloride, 4 μM calcium nitrate tetrahydrate, 17 μM magnesium sulfate heptahydrate, and 71 μM HEPES buffer in UV-treated deionized water (14, 20) at room temperature (18–21°C) in small aquaria or buckets with strips of brown paper towels (31), which mimic leaf litter of natural habitats (32), and fed TetraMin flakes weekly.

Instructors may, optionally, establish longer-term cultures, which can be maintained for periods of years. Instructors may wish to establish longer-term cultures if there are multiple iterations of the practical class throughout the academic year or to reduce the reordering of cultures ahead of the class. If electing to culture *L. variegatus* in the longer term, cultures can be laboratory reared in artificial pond water, as above, and should be maintained at room temperature and subjected to a 16:8-h light-dark cycle in aquaria with sandy or other fine sediment added to the aquaria. *L. variegatus* cultures should be fed TetraMin flakes and 10 mg/L spirulina weekly. Continuous water aeration and filtration can be achieved by the use of commercial air stones

Table 1. *Test compounds known to affect L. variegatus' response to tactile stimulation*

Test Compound	Known Target in Mammals	Stock Concentration	Working Concentration	Concentration That Decreases Stereotypical Movements after 10 min
Acetylcholine	Nonselective acetylcholine receptor agonist	100 mM	100 mM	≥25 mM
Baclofen	$GABA_B$ agonist	20 mM	20 mM	No effect
Bicuculline#	$GABA_A$ antagonist	50 mM	250 μM	≥50 μM
Cannabidiol (CBD)#	Various	4 mM	20 μM	≥5 μM*****
Dantrolene#	Ryanodine receptor (RyR1) antagonist	10 mM	50 μM	≥25 μM*
Donepezil	Acetylcholinesterase inhibitor	100 μM	100 μM	≥50 μM
Dopamine	Nonselective dopamine receptor agonist	100 mM	100 mM	≥10 mM
Ethanol	Various	500 mM	500 mM	≥250 mM***
GABA +	Nonselective GABA receptor agonist	100 mM	100 mM	≥10 mM
Glycine	Nonselective glycine receptor agonist	500 mM	500 mM	500 mM
Histamine	Nonselective histamine receptor agonist	10 mM	10 mM	≥1 mM**
Haloperidol#	Dopamine receptor (D_2) antagonist	20 mM	100 μM	≥50 μM
Lidocaine	Voltage-gated sodium channel inhibitor	1 mM	1 mM	≥0.5 mM*
Loratadine#	Histamine receptor 1 (H1) antagonist	12 mM	60 μM	≥6 μM**
Mecamylamine	Nonselective antagonist of the nicotinic acetylcholine receptor	100 μM	100 μM	≥50 μM
Mepyramine	Histamine receptor 1 (H1) antagonist	10 mM	10 mM	≥5 mM**
Naproxen	Cyclooxygenase (COX) inhibitor	25 mM	125 μM	≥50 μM
Nicotine	Nicotinic acetylcholine receptor agonist	1 mM	1 mM	≥0.1 mM****
Quinine#	A nonselective sodium and potassium channel blocker	200 mM	1 mM	≥0.1 mM*
Serotonin	Nonselective 5-HT receptor agonist	2 mM	2 mM	≥0.25 mM
Tubocurarine	Nonselective antagonist of the nicotinic acetylcholine receptor	100 μM	100 μM	≥25 μM

Suggested stock and working concentrations are provided, along with the known mammalian molecular target and known effect in *Lumbriculus variegatus*. Test compounds are soluble in artificial pond water up to the stock concentration, unless denoted by #, which indicates that the compound requires to be initially dissolved in 100% DMSO and then diluted to 0.5% (vol/vol) DMSO in artificial pond water before use. +Shown in Fig. 2, *see Ref. 14, **see Ref. 15, ***see Ref. 26, ****see Ref. 27, *****see Ref. 28.

and aquarium filters, respectively. As per previous studies, it is recommended that populations be maintained for a minimum of 3 mo before use to allow for population expansion by asexual reproduction (14, 15, 26–28).

Instructors of this class are responsible for following the regulations for animal research within their home institution.

Instructions

The following are specific instructions for setting up the practical class.

Preparations before student arrival.

1) Prepare artificial pond water, as outlined above. This can be prepared as a 100× solution and diluted to 1× ahead of the practical class. Artificial pond water can be maintained at room temperature for up to 6 mo.
2) The instructor should separate *L. variegatus* into individual wells of a six-well plate 18–24 h before the practical class begins, to allow for *L. variegatus* acclimatization, as per previous studies (14, 15, 26–28). Each well should contain ~4 mL of artificial pond water. Individual *L. variegatus* should be randomly selected, lacking any obvious morphological defects and ranging from 2 to 8 cm in length as per previous studies (14, 15, 26–28). The number of plates required will depend on class size, but we recommend one six-well plate for each pair of students. This can easily be modified depending on class size and student level.
3) Prepare drug solutions to be used during the practical class by dissolving the appropriate mass in artificial pond water. Example drug compounds and their tested concentration ranges, as well as effective concentrations, are shown in Table 1. Drug compounds may require a vehicle, and we have observed that 0.5% (vol/vol) DMSO does not affect *L. variegatus* stereotypical behaviors (14, 15, 28). Furthermore, 0.58% (vol/vol) ethanol has been shown to have no significant effect on *L. variegatus* (26), and so, depending on the drug selected, ethanol may be used as a vehicle control at ≤0.5% (vol/vol). If a vehicle is required, then instructors should utilize a suitable vehicle control, such as 0.5% (vol/vol) DMSO or ethanol in artificial pond water. If the drug compound is water soluble and does not require a vehicle, then artificial pond water only should be used as an experimental control.

Instructions for students.

1) After the 18- to 24-h acclimatization period, students should remove all artificial pond water from each well of the six-well plate, using a Pasteur pipette, to remove any debris. This should be immediately replaced with 4 mL of fresh artificial pond water.
2) Before administering drug compounds, the baseline ability of the worm to perform stereotypical behaviors should be tested and recorded. This is done by alternately stimulating the anterior or posterior end of the body with a clean plastic pipette tip or Pasteur pipette to stimulate body reversal (Fig. 1*A* and Supplemental Video S1) and helical swimming (Fig. 1*B* and Supplemental Video S2). Tactile stimulation should be done by gently swiping the tip across the plate to touch the worm. Tactile stimulation should not be done in a stabbing motion, as this can induce autotomy: the sudden separation of *L. variegatus* into two or more segments due to compression (33).
3) Students should administer tactile stimulation to each *L. variegatus* five times on the anterior and posterior ends, with a 5- to 10-s time interval between tactile

stimuli. Students should score these movements with the three-point scale (14) whereby:

A score of 3 corresponds to full stereotypical movements, as demonstrated in Fig. 1 and Supplemental Videos S1 and S2.

A score of 2 corresponds to partial stereotypical movement, whereby movement is less than Fig. 1 and Supplemental Videos S1 and S2 but more than Supplemental Video S3.

A score of 1 corresponds to no stereotypical movement following tactile stimulation, as shown in Supplemental Video S3.

Scores should be recorded for each stimulation at both the anterior and posterior tactile stimulation of *L. variegatus* and recorded in Supplemental Table S1.

4) Once students have completed their baseline scores, the artificial pond water should then be removed and immediately replaced with 4 mL of a vehicle or experimental control or test drug solution.
5) After a 10-min incubation with the drug solution and vehicle or experimental control, *L. variegatus* can be tested again, using the same procedure to evaluate the effects of drug exposure on stereotypical movements.

Students should administer tactile stimuli beginning with the vehicle or experimental control and then progressing from the lowest to the highest concentrations, after which students should discard the pipette tip before starting the next round of tactile stimuli to prevent cross-contamination with higher concentrations of the test compound.

The class can be terminated after evaluating the effects of drug exposure on *L. variegatus*' response to tactile stimuli should instructors wish to reduce the overall time of the practical class, or students can evaluate the effects following the removal of the drug.

6) To evaluate the effects following the removal of the drug compounds, the drug solutions and vehicle or experimental control are aspirated from the well and disposed of in suitable liquid waste containers.
7) To remove any latent drug and vehicle or experimental control residue, 4 mL of fresh pond water should be added and immediately aspirated and then replaced with 4 mL of fresh artificial pond water.
8) The ability of *L. variegatus* to perform stereotypical movements can then be retested after 10 min to measure reversibility of any observed effects.
9) Depending on time constraints, *L. variegatus* can be retested 24 h after exposure to examine the effects on stereotypical movements and to measure toxicity or lethality. Toxicity in *L. variegatus* is defined as tissue pallor or partial tissue degeneration, with lethality resulting in complete tissue degeneration.
10) Upon completion of the practical class, *L. variegatus* should not be returned to cultures because of the unknown long-term effects of exposure to test compounds. At experimental end points, *L. variegatus* should be euthanized by rapid submersion in 70% ethanol.

Quantification and Statistical Analyses

Students should be encouraged to plot the effects of the drug compound on the ability of tactile stimulation to elicit stereotypical movements. Students should calculate the average score for each *L. variegatus* at the different drug concentrations and time points tested for both movements, and data can be normalized to baseline ability by using the calculation:

$$\text{Ability to complete movement (relative to baseline)} = \frac{\text{Average movement score of } L.variegatus \text{ at the tested concentration or time point}}{\text{Average movement score } of\ L.\ variegatus \text{ at baseline}}$$

Using this calculation, students should have a baseline score equal to 1, and if the drug has affected the ability of tactile stimulation to elicit stereotypical movements in *L. variegatus* then a score of <1 will be calculated. Student data can be visualized for each student or collated to show the impact of repeated experiments. Plotting the collated data will emphasize to students the requirement for technical and experimental replicates.

The type of statistical analyses applied will vary depending on student level and access to statistical software. Students could compare stereotypical movements of *L. variegatus* during drug exposure conditions with stereotypical movements taken at baseline by paired nonparametric two-tailed *t* tests, in line with previous publications (14, 15, 26–28). For analysis of stereotypical movements after the removal of the drug compound, students may analyze these using a two-way ANOVA with Dunnett's posttest to compare baseline stereotypical movements with stereotypical movements at both the 10-min and 24-h recovery time points, as in previous studies utilizing this method (14, 15, 26–28).

Troubleshooting

As *L. variegatus* undergo asexual reproduction they may separate during the acclimatization period, with the newly separated *L. variegatus* having reduced tactile responses. Additionally, a small number of *L. variegatus* may expire during the acclimatization process. It is recommended that instructors have a small number of additional *L. variegatus* for the class that have been acclimatized to replace any *L. variegatus* that have undergone asexual separation into two or more *L. variegatus* or those that may have expired during the acclimatization stage.

L. variegatus will show reduced stereotypical movements if continuously stimulated and may stop responding if repeatedly stimulated without an interval. As outlined above, each stimulus should be administered after a 5- to 10-s time lapse

between tactile stimuli. Instructors should suggest that students stimulate each worm individually at the anterior, then stimulate each worm individually at the posterior, and repeat for five stimulation events at the anterior and posterior to prevent a diminished response of *L. variegatus*.

Additionally, students may report that their worm has suddenly split into two worms. Although mechanical pressure has been shown to induce autotomy of *L. variegatus* (33), the sudden separation of the worm in these classes is normally due to excessive force during stimulation. The method for tactile stimulation of the stereotypical behaviors is shown in Supplemental Videos S1–S3.

New test compounds can be selected to align with course curricula, but some compounds may not be soluble in artificial pond water or produce an effect in *L. variegatus*. Instructors are recommended to check the solubility of test compounds before the practical class, using either DMSO or ethanol as a vehicle, not exceeding 0.5% (vol/vol). Furthermore, the effects of some compounds, such as mecamylamine, may not appear after a 10-min exposure to the compound but effects may be observed at recovery time points (10 min or 24 h after exposure and incubation in artificial pond water only), and so it is recommended that recovery time points be completed to observe any delayed effects of drug exposure. Instructors may wish to include a compound that has been shown to inhibit stereotypical movement in response to tactile stimulation of *L. variegatus* as a positive control. Compounds that produce a known effect are shown in Table 1.

Safety Considerations

When preparing the artificial pond water, instructors should wear appropriate personal protection equipment, including gloves, goggles, and a mask, as calcium nitrate tetrahydrate is harmful if swallowed and causes serious eye damage.

Instructors should check with their institutional review boards regarding the use of proposed test compounds with students to ensure compliance with regional and institutional regulations. Each test compound will have its specific safety considerations, but, at a minimum, standard laboratory personal protective equipment should be used throughout the practical class. Instructors are advised to consult the manufacturer's safety data sheet on the safe disposal of test compounds. Furthermore, instructors are responsible for ensuring that appropriate health and safety documentation is completed for any drug compound selected for investigation and verifying whether their institute has any ethical requirements for the use of *L. variegatus*.

L. variegatus should not be released into the environment, to prevent environmental and ecological contamination, and should be euthanized in 70% ethanol and disposed of by appropriate disposal routes.

RESULTS AND DISCUSSION

Expected Results

Depending on the test compound selected, students will observe a dose-dependent inhibition of body reversal and/or helical swimming stereotypical movements. Tested compounds with known effects on the response to tactile stimulation to elicit stereotypical movements in *L. variegatus* are shown in Table 1. Exemplary data from the use of this methodology are shown in Fig. 2, which demonstrates the effects of acute exposure on the ability of tactile stimulation to elicit body reversal (Fig. 2*A*) and helical swimming (Fig. 2*B*). The ability of *L. variegatus* to perform these movements 10 min and 24 h after exposure is also shown (Fig. 2, *C* and *D*), demonstrating the irreversible effects of this test compound. Compounds that affect stereotypical movements reversibly include lidocaine, mepyramine, loratadine, and ethanol (14, 15, 26), whereas irreversible inhibition occurs with exposure to quinine, histamine, or nicotine (14, 15, 27).

If instructors are selecting a novel drug compound to test in *L. variegatus*, exposure may result in *1*) no observable effect on *L. variegatus*' ability to perform stereotypical movements after tactile stimulation, *2*) partial inhibition of stereotypical movements whereby *L. variegatus* responds to tactile stimulation but movements may be slowed or incomplete, *3*) complete inhibition of stereotypical movements whereby *L. variegatus* has no response to tactile stimulation, or *4*) rapid toxicity characterized by rapid writhing of *L. variegatus* followed by a period of stasis and potential decomposition observed up to 24 h after exposure.

Misconceptions

A common point of confusion is that the inhibition of stereotypical movements in *L. variegatus* following tactile stimulation is interpreted as lethality following drug exposure or recovery time points. However, the toxicity of drug compounds is confirmed by visible partial tissue degeneration and/or pallor and lethality as complete tissue degeneration or whole organism tissue pallor, which is observable 24 h post drug exposure. The inability of *L. variegatus* to respond to tactile stimulation at any time point is not indicative of organism expiry, as this may be a long-term inhibitory effect of drug exposure on stereotypical movement.

Evaluation of Student Work

Experiments described here using the *L. variegatus* model have been used from the first year of undergraduate studies through to more advanced levels of study. Students may present their results in several different ways, such as a laboratory report, or we have had students use their findings to produce a scientific poster and present their poster with a 2-min summary. In this format, students gain valuable experience in presenting their findings clearly and concisely, while gaining skills in science communication. Anecdotally, students have reported that presenting their own data provides them with increased confidence in their presentation skills. For students who prefer not to work with living animals, datasets from the class can be shared, or exemplary data can be obtained from the corresponding author.

Inquiry applications.

Commonly, students will enquire what it is they should see rather than reporting what they see. To conduct these experiments ethically and ensure, we are continually adding to the knowledge base on *L. variegatus* responses: we often test new compounds rather than ones with known effects. In this manner, there is no desired result, and students are encouraged to report on what they see, not what they think they should see. To remove this confusion, students are

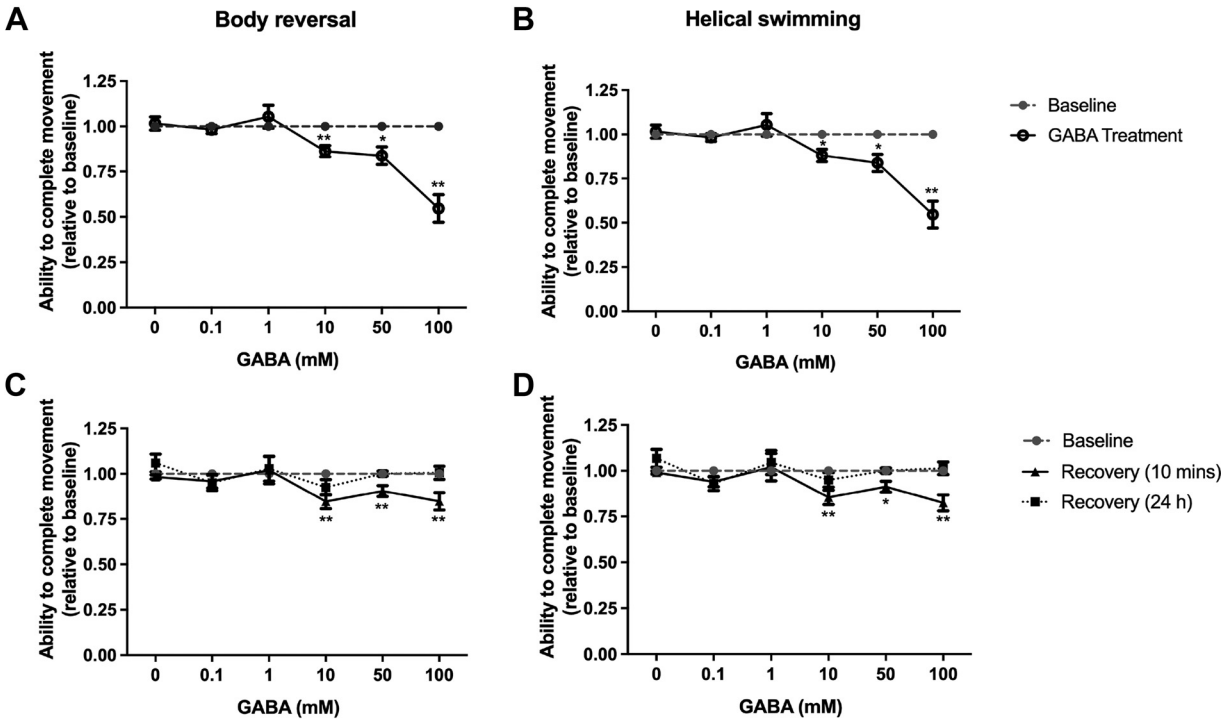

Figure 2. The effects of gamma-aminobutyric acid (GABA) on *Lumbriculus variegatus* behavior. *L. variegatus* were exposed to GABA (0–100 mM) and tested for the ability of tactile stimulation to elicit body reversal (*A*) or helical swimming (*B*). GABA was then removed, and the ability of *L. variegatus* to perform body reversal (*C*) or helical swimming (*D*) was tested 10 min after removal [Recovery (10 min)] and 24 h after removal [Recovery (24 h)]. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. Error bars represent SE. $n = 8$, with a single *L. variegatus* exposed to each concentration per experimental repeat. Statistical significance between baseline and GABA exposure or statistical significance between baseline and Recovery (10 min): $*P < 0.05$, $**P < 0.01$; no statistical significance between baseline and Recovery (24 h) was observed.

blinded to compounds to prevent any bias and relieve student pressure on aiming to get the "required" result.

Although the methods described above most obviously lend themselves to a facilitated inquiry approach, they have also more recently been used as a vehicle for cocreation of the curriculum (15) whereby students at final year undergraduate level, as well as Master's level, are involved in the selection of compounds, the laboratory setup, class delivery, and analysis of collated data, with these data directly feeding into their projects. This aligns with an open inquiry approach where students themselves generate hypotheses and then design the experiments to test the hypotheses. This ensures that the data generated from these organisms have research applications and are a more ethical use of animals for education and training purposes. Moreover, the methodologies outlined here are well suited for independent student projects and can easily be tailored to systems/drugs of interest, highlighting their application in physiology education.

Wider applications.

This practical class can be used to explore the behavioral effects of diverse pharmacologically active compounds after short-term (10 min) exposure, as outlined here, the effects of repeated exposures, or longer-term exposures, up to 24 h, as found within the literature (27, 28).

As a problem-based learning exercise, students could be provided with compounds known to produce effects, such as GABA (Fig. 2), and drugs that do not produce an effect, such as the GABA agonist baclofen (Table 1), and asked to identify the compounds. Alternatively, students could be tasked with identifying a parental compound and inactive metabolites, which have diminished effects on behavior (28). Furthermore, students could investigate agonist and antagonist pairings and compare the effects in isolation, and then in combination, which has previously been utilized for cocreated practical classes using *L. variegatus*, examining the effects of histamine and the antihistamine drugs loratadine and mepyramine (15).

Additional experiments using *L. variegatus* could run across multiple practical classes, or as student projects, to enable students to investigate single compounds in depth and produce a more comprehensive report of their findings. Indeed, multiple studies have quantified unstimulated locomotor activity of *L. variegatus* with low-cost equipment and free-to-use software (14, 26–28) or investigated effects of compounds on dorsal blood vessel pulse rates with a variety of techniques (23, 28, 29, 31, 34). These manipulations or additions open up a range of new experimental possibilities for the utilization of *L. variegatus* within practical classes.

ADDITIONAL RESOURCES

For additional information demonstrating the effect of pharmacologically active compounds on *L. variegatus*, please see Refs 14, 15, 26–28. Additional information and support for implementing this resource can be found at www.SWIRLswansea.com.

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL MATERIAL

Supplemental Video S1: https://doi.org/10.6084/m9.figshare.28727045.v3.
Supplemental Video S2: https://doi.org/10.6084/m9.figshare.28727051.v2.
Supplemental Video S3: https://doi.org/10.6084/m9.figshare.28727042.v3.
Supplemental Table S1: https://doi.org/10.6084/m9.figshare.28727048.v2.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.S., L.F.C., J.L.R., and N.S.F. conceived and designed research; A.S. performed experiments; A.S. analyzed data; A.S. interpreted results of experiments; A.S. prepared figures; A.S., L.F.C., J.L.R., and N.S.F. drafted manuscript; L.F.C., J.L.R., and N.S.F. edited and revised manuscript; A.S. approved final version of manuscript.

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