

1 **Title Page**

2 **Article Title:** An old worm with new tricks? The applications for *Lumbriculus variegatus* as an
3 invertebrate model for education.

4 **Short title:** *L. variegatus* as an animal model in teaching

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11 **Abstract:**

12 *Lumbriculus variegatus* is an aquatic worm which has gained recent renewed interest as a
13 practical model for biomedical education, enabling *in vivo* behavioural, physiological,
14 toxicological, and biochemical responses to biologically active compounds to be assessed in
15 educational settings. As invertebrates, *L. variegatus* offers easy integration into educational
16 settings without regulatory constraints, and this positions *L. variegatus* as a valuable tool for
17 practical *in vivo* learning. This review provides an overview of methodologies utilised within
18 studies of *L. variegatus*, including toxicity, locomotive behavioural assays, measurement of
19 dorsal blood vessel pulsation, metabolic profiling, and molecular endpoints, and describes
20 their current and potential applications within educational settings.

21

22 **Keywords:** *L. variegatus*, *Lumbriculus variegatus*, invertebrate, animal models, education.

23 Main Text

24 Introduction

25 *Lumbriculus variegatus* (Annelida: Clitellata: Lumbriculidae), commonly known as blackworms
26 or California blackworms, are aquatic, asexual, regenerative worms, taxonomically related to
27 leeches and other mud-dwelling clitellates (1). These freshwater worms are detritivore species
28 that play important roles in soil formation and nutrient cycling and inhabit shallow ponds,
29 lakes and marshes across Europe, North America, and Asia (2).

30

31 *L. variegatus* range between 50 – 80 mm in size (3) and contain ~150 body segments (4) with
32 a single smooth muscle tube, the dorsal blood vessel (DBV), being easily visible through its
33 transparent body wall (Figure 1A), and peristaltically moves blood from the posterior to the
34 anterior of the worm through a closed circulatory system (5). These worms display two
35 stereotyped escape reflex behaviours (6) whereby stimulation of the anterior 1/3 of segments
36 results in bending movements, which reverse head and tail positions (Figure 1B), and
37 stimulation of the posterior 2/3 of segments evokes a “cork-screw”, or helical swimming,
38 movement characterised by rapid helical body bends (6) (Figure 1C). These reflexes are
39 mediated by three giant fibres: one medial giant fibre (MGF), which is activated following
40 anterior stimuli, and two paired lateral giant fibres (LGF), which are activated following
41 posterior stimuli (7). These stereotypical behaviours are analogous to the body bends and
42 locomotor activity observed in *Caenorhabditis elegans* (8) or the “gliding” and “scrunching”
43 behaviour of planarian flatworms (9), with *L. variegatus* behaviours capable of being viewed
44 with ease owing to their larger size and thereby negating microscopy techniques.

45

46 Most research conducted using *L. variegatus* to date pertains to its relevance as an ecological
47 indicator species, with *L. variegatus* being a recommended test organism in water and
48 sediment quality evaluation (10) and used extensively in ecological pollutant studies (11–16).
49 However, the applications of *L. variegatus* are not limited to these fields.

50

51 The use of *L. variegatus* as an accessible model within education was first proposed by Charles
52 Drewes almost three decades ago (17). Given that *L. variegatus* can be established and
53 maintained without specialist equipment and low maintenance requirements, as outlined by
54 Seeley *et al.* (18), this organism is scalable and financially accessible, even under constrained
55 budgets. Use of *L. variegatus* has seen renewed interest within educational settings, with
56 recent studies describing *in vivo* practical classes where students observe and quantify
57 stereotypical behaviours in response to pharmacological compounds (18,19). These classes
58 integrate learning outcomes on hypothesis formulation, drug dilutions, and behavioural
59 observation, which have been positively received by students (3,19). As *L. variegatus* are not
60 regulated by the Animal Welfare Act Regulations or the Public Health Service Policy on
61 Humane Care and Use of Laboratory Animals in the United States, nor the Animal (Scientific
62 Procedures) Act 1986 in the United Kingdom, with similar legislation existing in many
63 countries, *L. variegatus* offers the opportunity for utilisation within educational settings.

64

65 This review provides a comprehensive overview of the utilisation of *L. variegatus* and the
66 variety of methodologies, from both pedagogical studies and primary scientific research, for
67 use in this organism within biomedical education.

68

69 **Toxicological measurements in *Lumbriculus variegatus***

70 A significant proportion of studies utilising *L. variegatus* examine the toxicity of a diverse range
71 of compounds, commonly determined by survival (11,20) or mortality rates (21). Those
72 characterised include pesticides (20), synthetic musk (11), histamine (19), nicotine (21), and
73 cannabidiol (CBD) (22), within timeframes from 24 hours (19,21,22), ten days (20) and twenty-
74 eight days (11). In his study on CBD, Williams *et al.* added additional nuance by defining
75 toxicity in *L. variegatus* as “decomposition, determined by partial or complete degeneration,
76 and tissue pallor” (22), extending toxicity measurements from just alive or dead *L. variegatus*.
77 Furthermore, Aikins *et al.* raise an interesting point regarding long-term assays, whereby
78 asexual reproduction of *L. variegatus* can result in control survivorship >100%, and so long-
79 term studies of toxicity in *L. variegatus* could be interpreted as the effect on reproduction, not
80 just survival (11). Commonly, *L. variegatus* reproduction, alongside dry biomass, are also used
81 as an indicator of toxicity in longer-term studies, ranging from 28 to 98 days (15,23,24). The
82 relative simplicity of these endpoints emphasises *L. variegatus* as an accessible model for
83 introducing core toxicological principles in educational settings.

84

85 **Whole-organism behavioural responses of *Lumbriculus variegatus***

86 Since Drewes first described *L. variegatus*' stereotypical movements (6), several studies have
87 examined the impact of biologically active compounds on *L. variegatus* locomotor behaviours.
88 Recently, two behavioural assays using three different behavioural endpoints were described
89 (3): the first was a semi-quantitative assay for evaluating tactile stimulation to elicit both body
90 reversal and helical swimming behaviours (Figure 1B-C) using a three-point scale following
91 anterior or posterior stimulation of *L. variegatus*, respectively. The second was a quantitative
92 image analysis of *L. variegatus*' unstimulated locomotor activity, with the effects of
93 dantrolene, lidocaine and quinine, were described using these assays (3).

94

95 Subsequent studies used these assays to evaluate the effects of diverse compounds, including
96 histamine, loratadine, mepyramine (19), ethanol (25), nicotine (21), CBD (22) and gamma-
97 aminobutyric acid (GABA) (18) within ≤ 24 hours. Similar methods for measuring *L. variegatus*
98 responses to physical stimuli have been described by Wang *et al.*, who examined the effect of
99 per- and polyfluoroalkyl substances after an exposure duration of 12 days (26). Analysis of *L.*
100 *variegatus*' unstimulated locomotor activity has also been used to evaluate the effects of
101 diverse compounds, with exposure times from 10 minutes up to 24 hours (3,21,22,25). Using
102 this technique, analysis of *L. variegatus*' locomotor activity when exposed to ethanol or
103 nicotine was shown to induce rapid reductions in locomotor activity within two minutes
104 (21,25), with nicotine having readily reversible effects at 0.1 – 0.25 mM within 10 minutes
105 (21). Furthermore, Ji *et al.* developed an automatic line-tracking system for measuring *L.*
106 *variegatus* locomotor activity when exposed to copper sulphate for six hours (27). The short
107 exposure times for these behavioural measurements enable educators to implement these
108 assays within standard laboratory practical teaching timeframes, normally of a few hours, or
109 extend these over a period of days.

110

111 An emerging behaviour of interest is the ability of *L. variegatus* to entangle their bodies into
112 dense, intertwined worm "blobs" (28,29) (Figure 1D). ≥ 10 *L. variegatus* spontaneously
113 aggregate within minutes to shield themselves against external stressors (29) (Figure 1E) but
114 are capable of disentanglement within milliseconds (30). The collective responses of these
115 blobs to environmental stimuli highlight an additional dimension of *L. variegatus* behaviour,
116 reinforcing their utility as a model for studying both individual and group behavioural

117 dynamics in educational settings. For example, when exposed to thermal gradients, these
118 blobs displayed both thermotactic (28,29), and phototactic behaviours (29).

119

120 The increasing repertoire of methods for studying *L. variegatus* behaviours highlights the
121 growing utilisation of *L. variegatus* as a behavioural model. These rapid responses to a range
122 of both chemical and physical stimuli, alongside the simplicity of the methodology, make these
123 behaviours well-suited to educational evaluations.

124

125 **Physiological responses in *Lumbriculus variegatus***

126 Several studies have examined the impact of biologically active substances on *L. variegatus*
127 physiology. Various approaches have been used to study *L. variegatus* respiration, such as
128 contactless oxygen sensors (31) or dissolved oxygen meters (22), with both approaches
129 allowing non-invasive, non-destructive measurements which can be repeated over time.
130 Conversely, Silva *et al.* used p-iodonitrotetrazolium to measure aerobic energy production in
131 *L. variegatus* homogenates at experiment endpoints (15).

132

133 Lesiuk & Drewes described the first classroom activity for measuring DBV pulsations in *L.*
134 *variegatus* (32) in which a 15-minute exposure to 0.1 and 0.25 mM nicotine increased DBV
135 pulsations, with pulsations inhibited entirely at 1 mM nicotine, and 1 - 10 mM caffeine caused
136 increased pulsation rates (32). Investigative laboratory classes have also examined the effects
137 of herbal supplements and ethanol on DBV pulsations (4,33). Regulation of the DBV has been
138 shown to be regulated by estrogenic mechanisms (34) and biogenic amines, which were
139 modulated by using the selective serotonin reuptake inhibitor, fluoxetine, or various tricyclic
140 antidepressants (5).

141

142 The transparent body wall of *L. variegatus* (Figure 1A) enables observation of the DBV without
143 dissection or invasive techniques (Figure 1F), making it especially suitable for educational
144 contexts. DBV pulsations have been quantified using various methodologies which are low-
145 cost allowing translation to the classroom; either using a Parafilm or paraffin wax viewing
146 chamber (4,32), a 3D printed viewing chamber, glass capillary tubes (33), placing between two
147 layers of tape (35), or transferring to microscopy slides and gentle compression beneath a
148 coverslip (5,22,34) before viewing under a light microscope.

149

150 Recently, Lybrand *et al.* provided compelling evidence for the use of *L. variegatus* in
151 neurophysiology. In their study, electrophysiological techniques were used to investigate the
152 escape circuitry of *L. variegatus* (36). Here, a five-minute treatment with 25 μ M of the
153 AMPA/kainate receptor antagonist, CNQX, but not 100 μ M of the N-methyl-D-aspartate
154 (NMDA) receptor antagonist AP5, abolished MGF activation (36). These observations offer
155 wider applications of *L. variegatus* for use in electrophysiology and neuropharmacology
156 education.

157

158 **Regeneration studies using *Lumbriculus variegatus***

159 While planaria have remarkable regenerative capacities (37), *L. variegatus* are also an
160 established regeneration model (1), owing to its robust segmental regrowth and neural
161 plasticity (38). Regenerative studies are readily adaptable to teaching, ranging from qualitative
162 observation (39) to quantitative endpoints such as regenerated segment counts (40) or tissue
163 area measurements (22,41), as well as more advanced approaches including BrdU
164 incorporation to assess cell proliferation (39). Several studies have also examined the effects

165 of pharmacologically active compounds on regeneration (22,39–41), supporting hypothesis-
166 driven learning. An extensive review of *L. variegatus* regeneration is provided by Martinez
167 Acosta et al. (1).

168

169 **Molecular and biochemical markers in *Lumbriculus variegatus***

170 While behavioural and physiological assays reveal whole-organism responses, these often lack
171 mechanistic detail, which biochemical measurements can provide by quantifying the
172 underlying molecular and cellular processes.

173

174 At the whole-organism level, studies have utilised absorbance or colorimetric assays to
175 quantify protein, carbohydrate and lipid levels to measure total energy budget in *L. variegatus*
176 homogenates (15,22). Furthermore, reactive oxygen species (ROS) analysis in *L. variegatus* is
177 reported in ecotoxicological studies, whereby glutathione quantification, glutathione-S-
178 transferase, catalase, and superoxide dismutase activity, and oxidation damage by lipid
179 peroxidation, are utilised as experimental endpoints (15,26,42). More recently, Beinart &
180 Gillen demonstrated that H₂DCFDA, which undergoes rapid oxidation in the presence of ROS
181 to fluorescent 2',7'-dichlorofluorescein, can be used to visualise ROS in regenerating *L.*
182 *variegatus* (41).

183

184 Furthermore, confirmation of absorption of pharmacologically active compounds, namely
185 ethanol, has also been achieved using *L. variegatus* homogenate using colorimetric assays,
186 following short 10-minute exposure periods (25). The neurotransmitter acetylcholine has also
187 been measured in *L. variegatus* homogenates (21), and several studies have examined
188 cholinesterase activity in *L. variegatus* homogenates using Ellman's method (15,21,42,43).

189 Collectively, these methods broaden the potential of *L. variegatus* as a model for teaching by
190 aligning observed phenotypes with biochemical changes.

191

192 **Genomic studies of *Lumbriculus variegatus***

193 Compared to *C. elegans*, *Drosophila melanogaster* and planaria, there are few genomic
194 studies of *L. variegatus* (11,44–47). However, transcriptome analysis of *L. variegatus*, either in
195 response to chemical contaminants or during regeneration, has identified several differentially
196 expressed genes, including ATP-binding cassette transporters, heat shock proteins, and
197 glutathione-S-transferases (44,45). Despite seeing no significant difference, Aikins *et al.*
198 quantified mRNA expression of heat shock protein 70 when exposed to Galaxolide (11),
199 representing an exciting new avenue for the use of *L. variegatus* in research and education.

200

201 Additionally, the Aquatic Symbiosis Genomics Project are currently sequencing data to
202 facilitate *L. variegatus* genome assembly and annotation (48) with advances in genomic tools
203 offering a clear pathway for *L. variegatus* to transition from descriptive studies to
204 mechanistically grounded investigations.

205

206 **Concluding remarks**

207 *Lumbriculus variegatus* presents an inexpensive and accessible invertebrate model for *in vivo*
208 education, with the growing interest in molecular and genomic tools further expanding *L.*
209 *variegatus'* utility beyond introductory education to research-led teaching. There are several
210 studies which provide clear descriptions for the implementation of *L. variegatus* within
211 educational settings, covering procurement, maintenance, experimental design, and potential
212 assessment methods (1,3,18,19,33), with a range of methodologies suitable for adaptation to

213 individual curricula. Collectively, these support the wider adoption of *L. variegatus* for
214 practical *in vivo* teaching and highlight *L. variegatus* as an emerging model organism for use
215 in biomedical sciences education.

216

217 **Acknowledgements**

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219 shown in Figure 1A and Figure 1F, acquired within the Swansea Worm Integrative Research
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221

222 **References and recommended reading**

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283 This sourcebook provides a step-by-step protocol for educators on culturing and maintaining
284 *L. variegatus* and how to conduct stereotypical movement assays within practical classes,
285 including video examples of *L. variegatus* stereotypical movements. Further, it provides a
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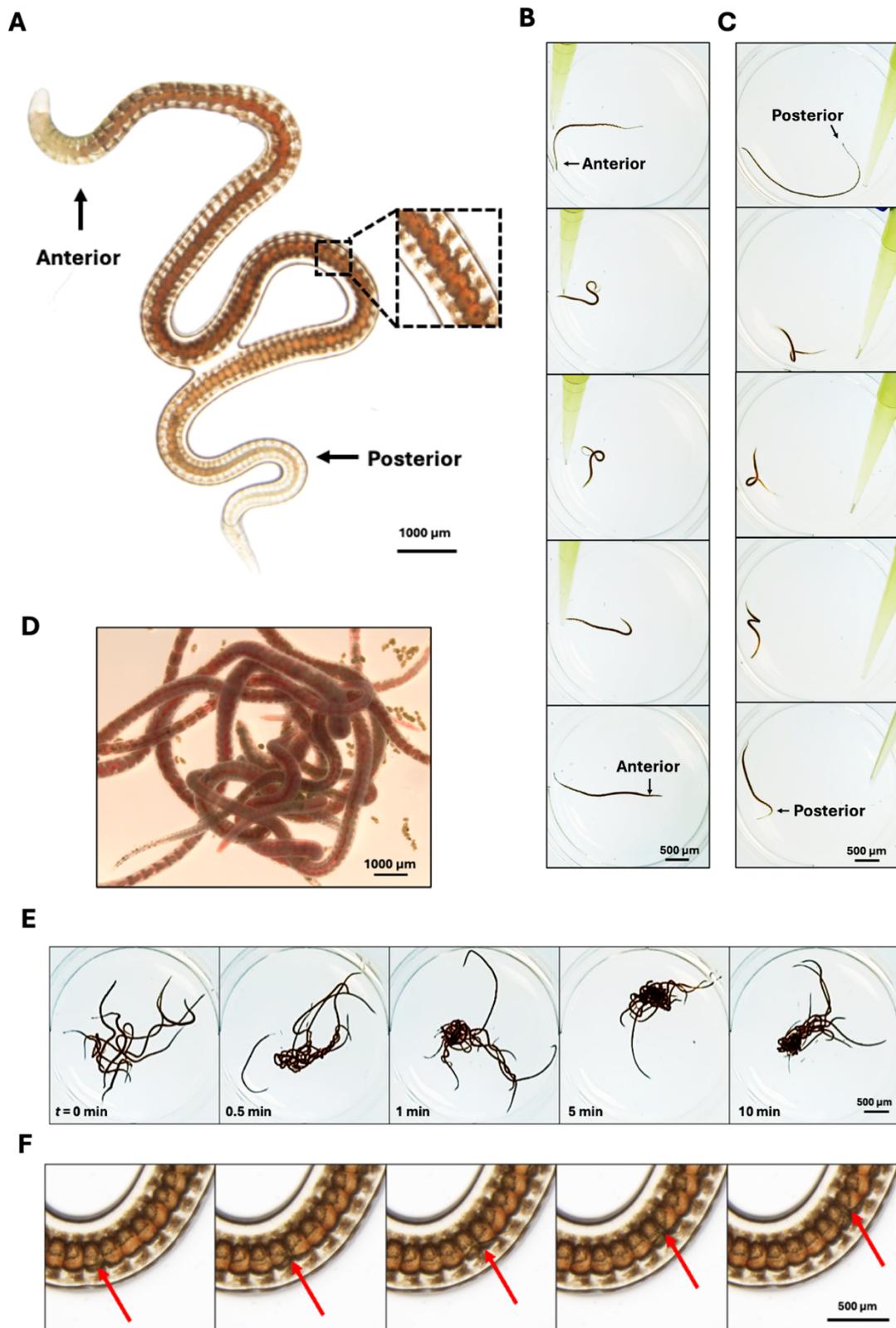
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401 **Figure 1. *Lumbriculus variegatus* behaviours and physiology for use as a model organism in**
402 **education. (A)** Representative image of *L. variegatus*, taken on a Nikon SMZ1270i
403 stereomicroscope. The highlighted region shows the dorsal blood vessel (DBV) through the
404 transparent body wall of *L. variegatus*. Arrows identify the anterior and posterior regions of
405 *L. variegatus*, with the anterior region characterised by the darker pigmentation, while the
406 posterior region of *L. variegatus* is characterised by the lighter pigmentation. **(B)** When the
407 anterior or posterior of *L. variegatus* is stimulated, this results in **(B)** bending movements that
408 reverse the head and tail positions and **(C)** helical swimming movements characterised by
409 rapid helical body bends, respectively, as described in ref 3. Images shown demonstrate the
410 stereotypical movements occurring over a 2 – 3 second period, based on ref 18. **(D)** A close
411 view of the entangled formation of a worm blob of ten *L. variegatus* taken on a Nikon
412 SMZ1270i stereomicroscope. **(E)** The formation of entangled worm blobs in *L. variegatus*
413 occurs within minutes, as described in refs 28,29. Representative frames taken using an eight
414 megapixel camera, recorded at 30 fps, over ten minutes, showing formation of worm blobs.
415 **(F)** Representative images of the peristaltic pulsation of the DBV in *L. variegatus* taken at one-
416 second intervals on a Nikon SMZ1270i stereomicroscope. *L. variegatus* was transferred to a
417 glass microscopy slide and gently compressed beneath a coverslip, as per refs 5,22,33. Red
418 arrows highlight the peristaltic pulsations.
419