Environmental Conditions and Husbandry Approach Affect the Survival and Physiology of the California Blackworm (*Lumbriculus variegatus*)

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Abstract: The California Blackworm (Lumbriculus variegatus) is a freshwater segmented worm species that has been used by biology instructors as a model system for inquiry-based student investigations. The blackworm dorsal blood vessel pulsation rate is easily quantified. Moreover, this species can facilitate the study of neuromuscular functioning via its photosensitive escape behavior which can be quantified as a segmental reflex rate. Both of these variables can be used to examine the physiological response of the *L. variegatus* circulatory and neuromuscular systems to environmental changes. Because knowledge about this species and its optimal environmental conditions is limited, we studied dorsal vessel pulsation and segmental reflexes of *L. variegatus* maintained at differing lighting, temperature and water cleaning frequency conditions. Our data strongly indicate that *L. variegatus* circulatory and motor functions are significantly affected by environmental conditions. We provide evidence -based recommendations for the careful control of environmental conditions that will allow instructors, students and researchers to collect robust data on *L. variegatus* and better utilize this model organism in their investigations.

Key Words: Anatomy and/or Physiology, Animal Behavior (Ethology) and Environmental Biology

Introduction

Lumbriculus variegatus, commonly known as California Blackworm, are freshwater segmented worms in the Annelida phylum (Jamieson, 1981). They tend to feed on decaying vegetation and microorganisms (Drewes, 2004). Therefore, they are often found at the shallow edges of ponds and lakes, the temperatures of which often range between 4°C and 15°C.

In the past decade and a half, there has been an increasing interest in this species as a model organism and tool to aid in the understanding of biological systems, such as the circulatory system. *L. variegatus* worms are suited to this task as the body wall is transparent which allows for the macroscopic inspection of the dorsal blood vessel, the larger of two major blood vessels, which runs along the posterior side of the worm (Lesiuk & Drewes, 1999). As *L. variegatus* worms lack true cardiac tissue, they utilize rhythmic contractions of smooth tissue surrounding the dorsal blood vessel to facilitate the flow of blood throughout the body within a closed circulatory system. Therefore, inspecting the dorsal blood vessel can be a convenient and informative approach to examine the *L. variegatus* circulatory system. However, as research studying *L. variegatus* physiology is relatively new, the extent of what we know about this species is limited. Gaining a clearer understanding about this physiology would help researchers, science instructors, and students to better utilize this model organism in their investigations. A detailed understanding of its husbandry is fundamental to such investigation.

L. variegatus are a useful model organism for the study of other physiological systems such as the neuromuscular system (Jamieson, 1981; Drewes & Fortner, 1989). *L. variegatus* are sensitive to a variety of environmental conditions, such as light and slight pressure, that elicit discrete behavioral phenomena such as segmental shortening. Also, we have observed in an oxygen deficiency study (personal observations) that *L. variegatus* movement and dorsal blood vessel pulsation rates change when their environmental water is not frequently replenished. Additionally, vascular performance is highly dependent on temperature. For example, Fillafer and Schneider (2013) showed that cardiac arrest could occur in *L. variegatus* after heat shock treatment reaches a critical point of 37.2 °C. Although educational settings may most easily use environmental conditions that are similar to room temperature for humans, *L. variegatus* typically inhabit environments with temperatures that deviate from room temperature (Drewes, 2004). Therefore, it is important to establish optimal laboratory husbandry approaches that help maintain these organisms at maximal metabolic function to help the study of their systems, since blood circulation is directly related to metabolic function (Biga et al, n.d.).

To establish these optimal environmental conditions, we studied dorsal vessel pulsation and segmental reflexes of *L. variegatus* maintained at differing lighting, temperature and water cleaning frequency conditions. We hypothesized that dorsal vessel pulsation and segmental reflex rates in *L. variegatus* would be significantly related to these environmental conditions. The range of environmental conditions studied included those typical of the natural habitat of *L. variegatus*. Dorsal vessel pulsation and segmental reflex rates, as well as a qualitative assessment scale of *L. variegatus* cardiovascular health, were utilized to test this hypothesis.

Due to COVID-19 restrictions, the experiments detailed in this manuscript were conducted in the premises of a conventional dormitory room. While a simple light microscope and disposable pipettes were lent by the UW-Madison Biocore department, most of the materials described in this manuscript are common household items. Furthermore, the methodology was designed to be easily executable in a typical dormitory room with little required previous laboratory training. Therefore, while the primary goal of this investigation was to inspect how environmental conditions affect the physiology and survival of *L. variegatus*, the methodology detailed in this manuscript could be utilized as a guideline for biology teachers who instruct students interested in investigating *L. variegatus* physiology and survival in a variety of settings, from traditional science lab classrooms to student dorm rooms, at either the high school or a college level.

Materials and Methods

Timeline

Prior to being used in the study, all worms were refrigerated at 4℃. For all experiments, we housed worms in 10 cm x 10 cm x 8 cm plastic containers, each containing 5 individual *L. variegatus* specimens. Each container was filled with 560 mL of tap water to normalize for the effects of water type and quantity. The effects of air availability were normalized by the use of an air pump in all treatments. An environmental temperature of 10 °C, which was maintained by using a replenishable ice bath, a water cleaning schedule of twice/week and a 24-hour light treatment that utilized commercial 150-watts neon lighting with a distance of 1.8 meters between the container and the light source were considered as control conditions and were used when not otherwise stated.

There were three experimental conditions - elevated temperature, reduced cleaning frequency, and reduced lighting - considered in this study. We conducted studies with these conditions twice: first for quantitative data collection and then for qualitative (body color) data collection. New worms were selected for each study. Individual worms were used only once. All worms were exposed concurrently to their respective environmental conditions for two weeks. At that time, for the quantitative analysis, circulatory and reflexive data (details below) were collected; for the qualitative analysis, color data were collected. Subsequently, all worms were placed in a separate container for disposal. Within each of the two sets of studies - quantitative data and qualitative data - all containers were under study at the same time.

For quantitative data collection, 9 containers were utilized as experimental treatment groups with 3 containers for each treatment group. Due to time restriction and local *L. variegatus* shortage at the time, only 3 containers were utilized as control for all experimental conditions. For qualitative data collection, 9 containers with 3 containers per experimental treatment group were utilized. Because qualitative data collection occurred at a later time when there was less of a local *L. variegatus* shortage, 3 distinct sets of containers were intended to be utilized as control for each environmental condition, for a total of 9 control containers. However, the 3 control containers dedicated to temperature comparison contained old and new worms (which at the time of the study were assumed to be physiologically equivalent). This assumption was called into question when these 3 control containers yielded data that conflicted substantially from the other controls (see appendix A). Hence, these data were discarded, and the other 6 control containers were pooled together to serve as controls for all three experimental treatments. This decision was supported by the fact that the old worms had been kept refrigerated for 3 weeks longer than the new worms. This may have influenced their vascular performance (Fillafer & Schneider, 2013).

Environmental Conditions

For the assessment of environmental temperature, worms in the 3 increased temperature treatment containers were reared at a temperature of 22 °C which was maintained by regulating the room thermostat. For reduced cleaning frequency, 3 containers were maintained using a once/week water cleaning schedule. For the reduced lighting condition, 3 containers were exposed to a 24-hour dark treatment enforced via an inverted cardboard box. For each of these three experimental treatment situations, the two factors not under direct assessment were kept at control conditions.

The protocol described in the previous paragraph was used for both the quantitative and qualitative data studies. As noted, the same 3 containers were used as the control for each of the environmental conditions for the quantitative data studies. The quantitative data studies utilized 6 containers as control for the three experimental conditions (as explained above).

Quantitative Data Collection

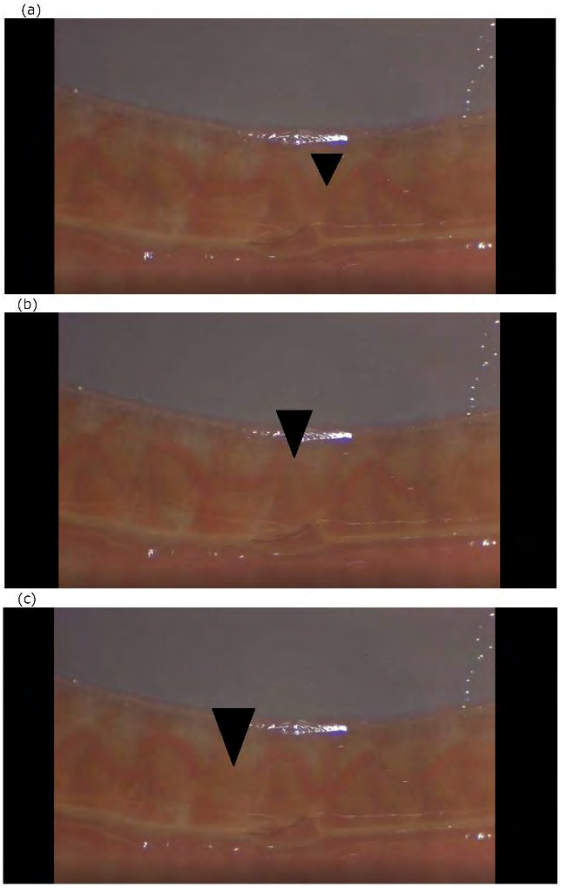
A. Dorsal Vessel Pulsation Rate Calculation

Each *L. variegatus* was placed between two layers of scotch tape. After that, the tape was placed under a light microscope while dorsal vessel pulsations were counted for 30 seconds. Each macroscopic contraction of the dorsal vessel was counted as one pulsation (Figure 1). The number of the pulsations of the dorsal vein in 30 seconds were counted and multiplied by 2 to obtain a dorsal blood vessel pulsation rate with units of pulses/minute (Eq. 1).

$$\text{(Eq.1) } Dorsal\ Vessel\ Pulsation\ Rate\ \left(\frac{pulse}{minute}\right) = \#\ of\ pulsations\ per\ 30\ seconds\ \cdot 2$$

Figure 1. Microscopic view of dorsal vessel pulsation in *L. variegatus*

The black arrowhead shows how the diameter of one location on the vessel decreased as the smooth muscle surrounding the dorsal blood vessel contracted along the length of the dorsal blood vessel, for one pulse, at different timepoints: (a) 0 seconds. (b) 1 second. (c) 2 seconds. Images taken at a (x35) magnification level.

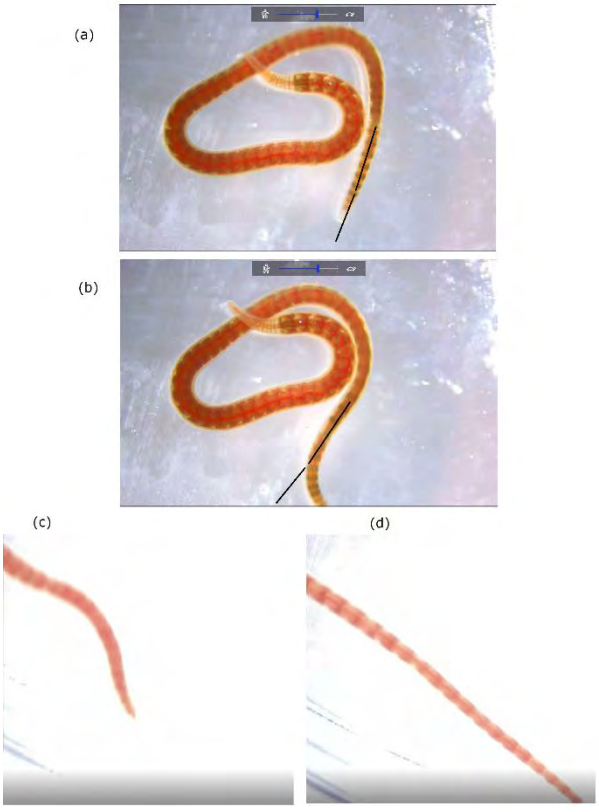


B. Segmental Reflex Rate Calculation

To fully gauge segmental reflex to photic stimulation, one *L. variegatus* worm was placed in a petri dish containing 3 mL of tap water under a light source, such as the lamp of a light microscope. Each head or tail thrashing across the body axis and each attempt to lengthen or shorten segment length were counted as one segmental reflex response (Figure 2). After the first response was recorded, any additional segmental reflex response was recorded for 30 seconds and multiplied by 2 to obtain a segmental reflex rate with units of segmental reflexes/minute (Eq.2).

$$\text{(Eq.2)}\ \ Segmental\ Reflex\ Rate\ \left(\frac{pulse}{minute}\right) = \#\ of\ segmental\ reflex\ responses\ per\ 30\ seconds\ \cdot 2$$

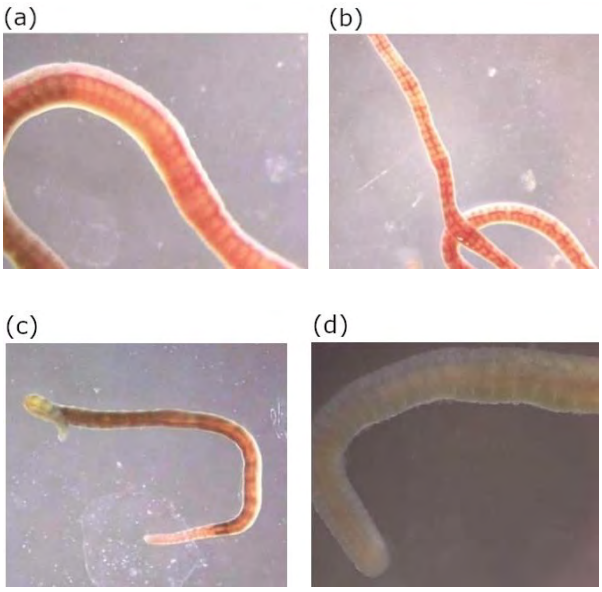
Figure 2. Neuromuscular response of *L. variegatus* to light stimulus
(a) *L. variegatus* at resting position. Note that the segment is aligned along the body axis which is represented with a dashed black line. (b) *L. variegatus* thrashing away from the body axis which is represented with a dashed black line. (c) Segmental shortening of *L. variegatus*. (d) Segmental lengthening of *L. variegatus*. Images taken at a (x28) magnification level.



Circulation Assay (for qualitative analysis)

The circulatory performance of *L. variegatus* was assessed using a qualitative color assay where each worm was categorized into one of four ordered groups based on the degree to which the intensity of the red color of the blood vessels as inspected by light microscopy resembled one of 4 discrete patterns (see figure 3). Worms with intensities that were in between two consecutive patterns were classified into one of three intermediate categories (see figure 3). Thus, each worm was classified to one of 7 ordered categories. To increase consistency, a scale (figure 3) was used as a reference for value allocation. An assay score below 2 indicated poor circulatory health.

Figure 3. Circulation assay reference:
(a) The highest level of circulation score of 3 was indicated by diffused and bright redness along the worm length. (b) An intermediate level of circulation which was indicated by diffused and bright redness intermittent along the worm length was granted an index of 2. (c) A low level of circulation which was indicated by a dim redness and the presence of pale-yellow regions was granted an index of 1. (d) The lowest level of circulation which was indicated by greyness along the worm length was granted an index of 0. Worms that appeared as an intermediate between two of these classifications were assigned an average of the value associated with the two classifications under inspection. Total magnification levels used to obtain images for (a), (b), (c) and (d) were (x28), (x16), (x8) and (x28), respectively.



Statistical Analysis

We used RStudio software for all statistical analyses (RStudio Team, 2019). For quantitative data analyses, we used a nested model with container as the error term for testing environmental condition (and worm as the sub-plot error) to determine if there was a significant difference in the mean blood vessel pulse rates and mean segmental reflex rates of *L. variegatus* worms for the different environmental conditions. A two-sided Fisher's Exact Test was used to determine if there was a significant difference between the qualitatively assigned circulation categories.

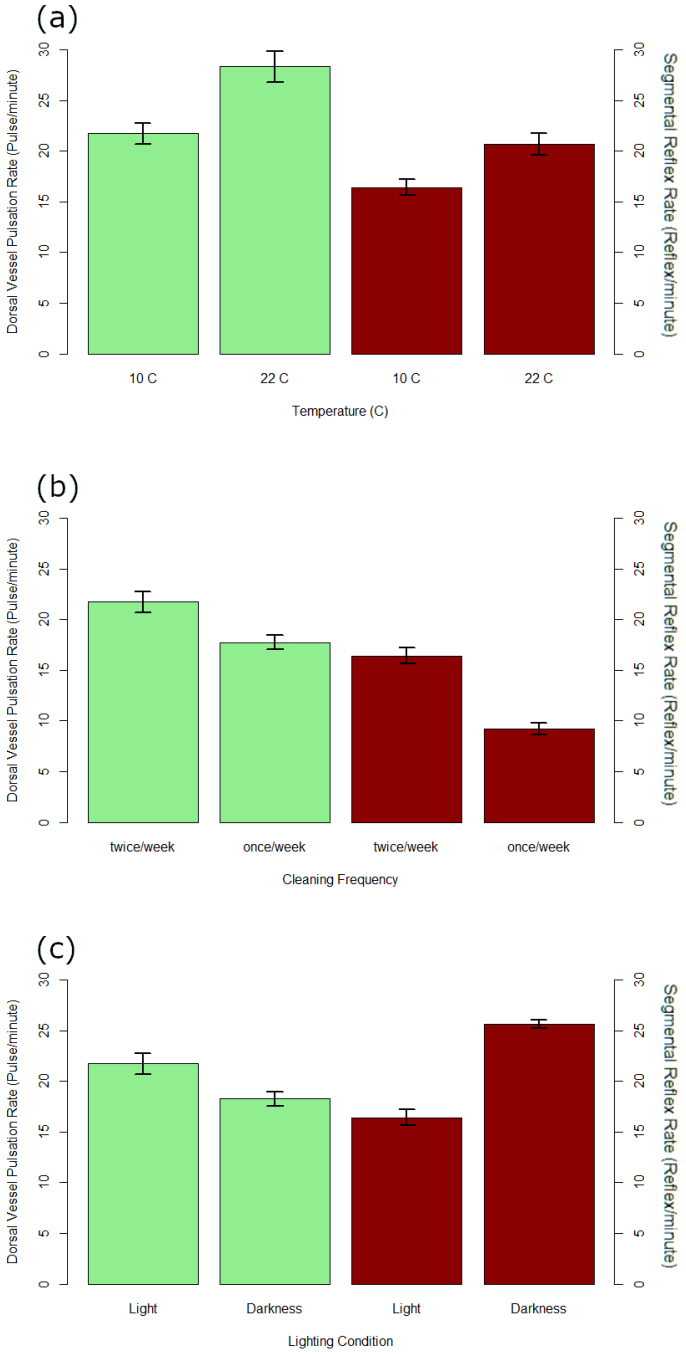
Results

Temperature

As shown in Figure 4a, the mean dorsal vessel pulsation rate of *L. variegatus* worms housed at 10°C was 21.7±4.0 pulsations per minute while *L. variegatus* worms kept at 22°C had an average pulsation rate of 28.3±5.9 pulsations per minute. There was a statistically significant effect at 5% of temperature on dorsal vessel pulsation rate

Figure 4. Effects of Environmental Conditions on Dorsal Vessel Pulsation and Segmental Reflex Rate

(a) Bar plots of mean dorsal vessel pulsation and segmental reflex rate of *L. variegatus* reared in a 10 °C water (control) temperature vs a 22 °C water temperature. Error bars represent +/- 1 SE (n=15). (b) Bar plots of mean dorsal vessel pulsation and segmental reflex rate of *L. variegatus* reared in a water container cleaned twice/week (control) vs *L. variegatus* reared in a water container cleaned once/week. Error bars represent +/- 1 SE (n=15). (c) Bar plots of mean dorsal vessel pulsation and segmental reflex rate of *L. variegatus* reared in a 24-hour light treatment (control) vs *L. variegatus* reared in a 24-hour dark treatment (darkness). Error bars represent +/- 1 SE (n=15). (The variances used in computing the error bars in these plots do not take into account the effect of container. However, these effects are accounted for in the formal statistical analysis.)



(ANOVA F(1, 4) =12.8791, p = 0.023). (The analysis also indicated that container-to-container variability was quite small. This was the case for all subsequent statistical analyses of quantitative data and, therefore, will not be formally addressed elsewhere).

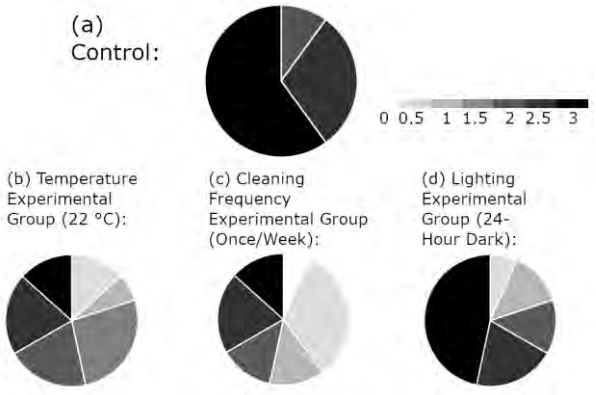
Figure 4a also shows that the mean segmental reflex rate of *L. variegatus* worms housed at the lower 10°C temperature was 16.4±3.0 reflexes per minute as compared to 20.7±4.6 reflexes per minute for worms kept at 22°C. This difference in

segmental reflex rate was statistically significant at 5% (ANOVA F(1, 4) = 10.3959, p = 0.0321).

For the circulation assay, the *L. variegatus* worms housed at 10°C yielded a mean index of 2.75 while worms housed at 22 °C had an average index value of 1.83 (Figure 5a,5b). This difference in circulatory index between the two temperatures was statistically significant at 5% (p-value = 0.0003). (Note that in performing the Fisher Exact Test (FET) we ignored the effect of container. A careful examination of the data along with the fact that there was negligible variability due to container in all of the quantitative data analyses using the nested model, indicates that our use of the FET is appropriate).

Figure 5. Circulation Assay of *L. variegatus* Reared under Varying Environmental Conditions

Pie chart shows the results of the circulation assay of (a) control *L. variegatus* reared under a water temperature of 10°C, twice/week cleaning frequency and a 24-hour Light treatment (b) experimental *L. variegatus* reared at 22°C water temperature, (c) experimental *L. variegatus* reared at once/week cleaning frequency, (d) experimental *L. variegatus* reared under a 24-hour dark treatment. Each shade represents a different circulation assay score. The brighter the shade the lower the score. Lower assay scores (1.5 or below) indicate poor circulation as indicated by amount of red coloration. (n=15 for experimental groups, n=30 for the control)



Cleaning Frequency

The mean dorsal vessel pulsation rate of *L. variegatus* housed at a twice/week cleaning frequency was 21.7±4.0 pulsations per minute, but this decreased to 17.7±2.7 pulsations per minute for worms housed at once/week cleaning frequency (Figure 4b). This difference in pulsation rate was statistically significant at 5% (ANOVA F(1, 4) = 9.7035, p = 0.0357). The mean segmental reflex rate of *L. variegatus* housed at twice/week cleaning frequency was 16.4±3.0 reflexes per minute while worms housed at once/week cleaning frequency had an average segmental reflex rate of 9.2±2.3 pulsations per minute (Figure 4b). This difference in segmental reflex rate was statistically significant at 5% (ANOVA F(1, 4) = 53.3647, p = 0.0019).

For the circulation assay, *L. variegatus* housed at twice/week cleaning frequency had a mean circulation index score of 2.75 as compared to an average index of 1.3 for worms housed at once/week cleaning frequency (Figure 5a,5c). This difference in circulatory index was statistically significant at 5% (p-value = 0.0001).

Lighting Condition

Figure 4c shows that the mean dorsal vessel pulsation rate of *L. variegatus* housed in a 24-hour light treatment was 21.7±4.0 pulsations per minute and decreased to 18.3±2.8 pulsations per minute for worms housed in a 24-hour dark treatment. This difference in pulsation rate under different lighting conditions was not statistically significant at 5% although it was quite close (ANOVA F(1, 4) = 7.6974, p = 0.0501). Additionally, Figure 4c shows that the mean segmental reflex rate of *L. variegatus* housed in a 24-hour light treatment was 16.4±3.0 reflexes per minute which increased to 25.7±1.5 pulsations per minute in worms in a 24-hour dark treatment, a difference that was statistically significant at 5% (ANOVA F(1, 4) = 110.6768, p = 0.0005).

For the circulation assay, *L. variegatus* worms housed in a 24-hour light treatment had a mean index of 2.75 while worms housed in a 24-hour dark treatment had a circulatory index of 1 (Figure 5a,5d). This difference in circulation index was not statistically significant at 5% (p-value = 0.1485).

A summary of the statistical test results for all of the assays is shown in Table 1.

Discussion

We found a statistically significant increase in mean dorsal vessel pulsation (of about 30%) and segmental reflex rates (of about 26%) in *L. variegatus* groups reared at 22°C, relative to *L. variegatus* groups reared at 10°C (Figure 4a). Moreover, circulation assays showed that a 22°C environmental temperature had a deleterious effect on circulatory performance (figures 3a-3b). Therefore, the hypothesis that dorsal vessel pulsation rate and segmental reflex rate in *L. variegatus* would be significantly related to water temperature was supported. This is further supported by studies that showed that the dorsal vessel pulsation rate of *L. variegatus* tends to increase as environmental water temperature increases (Fillafer & Schneider, 2013). Moreover, circulation assay data showed a statistically significant effect for temperature on circulatory performance (figures 3a, 3b). Hence, our data indicate that it is highly advisable to house *L. variegatus* at temperatures that characterize the typical habitats of this species (4°C - 15°C), particularly, when metabolic activity is the focus of study or educational task.

We found a statistically significant decrease of ~20% in mean dorsal vessel pulsation and a ~44% decrease in segmental reflex rates in *L. variegatus* groups reared in water containers cleaned once/week, relative to *L. variegatus* groups reared in water containers cleaned twice/week (Figure 4b). Furthermore, circulation assays showed that less frequent cleaning of environmental water negatively affects circulatory performance. We believe that these observed effects are biologically meaningful. Therefore, the hypothesis that there would be a significant change in dorsal vessel pulsation and segmental reflex rate in L. variegatus reared in water containers cleaned twice/week relative to *L. variegatus* reared in water containers cleaned once/week, which is more representative of the microorganism-rich natural habitats of *L. variegatus* as recorded by Brinkhurst and Gelder (1991), was supported. Our data indicate that water cleaning sanitation is a critical environmental factor to *L. variegatus*

Table 1. Summary of Statistical Testing Results. P-values indicating lack of statistical significance are underlined. DVP = Dorsal Vessel Pulsation. SS = Segmental Shortening. ‡: between treatments. †: within containers.

	Temperature	Cleaning Frequency	Lighting
DVP Rate: p-value (F-value) $dF^{‡} = 1$ $dF^{†} = 4$	0.0230 (12.8791)	0.0357 (9.7035)	0.0501 (7.6974)
SS Rate: p-value (F-value) $dF^{‡} = 1$ $dF^{†} = 4$	0.0321 (10.3959)	0.0019 (53.3647)	0.0005 (110.6768)
Circulation assay: Fisher-Exact Test: p-value	0.0003	0.0001	0.1485

Table 2. Suggested *L. variegatus* husbandry & environmental guidelines for water temperature, lighting and water cleaning frequency, and relative importance for studies of circulation/metabolism and neuromuscular behaviors.

	Influence on Circulation/Metabolism	Influence on Neuromuscular Reflexes	Suggested Husbandry/Environmental Guidelines
Water Temperature	High Effect	Low-Moderate Effect	Ideally, (4-15) C * Must not exceed 37.2 C
Cleaning Frequency	Moderate Effect	Moderate Effect	twice/week
Lighting condition	Low-Moderate Effect	High Effect	24-hour dark treatment for optimal metabolic function. 24-hour light treatment for minimal photosensitive reflex stimulation.

survival. Interestingly, our own oxygen availability pilot study showed that less frequent cleaning frequencies significantly reduced oxygen concentration inside water containers relative to the oxygen concentration inside more frequently cleaned water containers. This suggested that decreased competition over resources with aerobic microorganisms, which are more likely to be removed with more frequent cleaning schedules, may have contributed to increased vascular and neuromuscular function. In addition, we note the greater effect of cleaning frequency on segmental reflex rate than on dorsal vessel pulsation rate which implies greater sensitivity of neuromuscular function to this factor than that of metabolic function. Therefore, we recommend that researchers consider prioritizing more frequent cleaning schedules regardless of the focus of a study or educational task, particularly, if neuromuscular function is being inspected.

A ~57% statistically significant increase in segmental reflex rate was observed due to the 24-hour dark treatment (Figure 4c). Therefore, the hypothesis that segmental reflex rate in *L. variegatus* would be significantly related to lighting condition was supported. A modest (~16%) decrease in dorsal vessel pulsation rate was observed in the 24-hour dark treatment (p-value = 0.0501).Thus, this can be viewed as providing marginal support for the hypothesis that dorsal vessel pulsation rate in *L. variegatus* would be significantly related to lighting condition. Further work is needed to better elucidate the nature of this relationship. Furthermore, we did not observe a statistically significant effect of lighting on circulatory performance as indicated by circulation assays, indicating that lighting is not a critical factor for *L. variegatus* survival. This finding is supported by the ecological observation that *L. variegatus* can be found in shallow water surfaces, as well as burrowing in soil layers, two habitats with different light conditions (Jamieson, 1981). Further work could examine whether *L. variegatus* circulatory systems can adapt to a variety of environmental lighting conditions. Our data indicate that circulatory activity is less sensitive to changes in environmental light levels than neuromuscular reflex rate (Figure 4c). It may be that there has been stronger evolutionary selection on morphology and behaviors that allow blackworms to escape predators, as compared to selection on cardiovascular function. Predation evasion has historically been shown to be a significant evolutionary driver (Endler, 1986). It seems reasonable to assume that segmental reflex rate is an indicator of swimming behaviors that allow blackworms to escape predators, (i.e., worms with faster neuromuscular reflexes should be better able to evade predators). The 16% decline in dorsal blood vessel pulsation rate in 24h dark relative to well-lit environments indicates that circulatory responses are much less sensitive to light levels. It is likely that the anaerobic, short-term bursts in neuromuscular activity that occur during predator evasion do not require a substantial change in gas exchange. Our data are thus consistent with the postulation that black worm segmental reflexes have been more sensitive to evolutionary selection pressures than cardiovascular function. Regardless, we conclude that lighting condition is a significant environmental factor to control, at least for maintaining *L. variegatus* for the purpose of monitoring their neuromuscular function, due to the significant effect of lighting on segmental reflex rate.

There are certain limitations to this study that must be acknowledged. This study was conducted in a residence hall

room due to the COVID19 pandemic. Therefore, the data sets collected could have been confounded by fluctuating environmental conditions, such as temperature and humidity. Moreover, the insufficient space of a dorm room limited the ability to use a larger sample size. This fact must be taken into consideration as well. Additionally, due to the fact that the same controls were used for all of the experimental groups within each of the quantitative and qualitative data collections, there is a lack of independence among the three tests of experimental treatments within each type. All these hurdles shed light on the need for flexibility required by research conducted in low-resource settings, such as dorm rooms. Such flexibility, which may incorporate making compromises regarding the use of independent controls for instance, demands the need for the researcher to be extremely diligent and vigilant about study design and implementation in order to produce rigorous, high-quality data. Yet, despite these challenges, it can be observed that studies concerning *L. variegatus* can be undertaken in a range of settings (including dorm rooms) so long as high levels of diligence and vigilance are observed.

In conclusion, our data strongly indicate that *L. variegatus* circulatory and motor functions can be significantly affected by environmental conditions, such as lighting, temperature and water cleaning frequencies. Based on these data and previous research, we have devised suggested guidelines for optimal *L. variegatus* environmental conditions and husbandry approach (Table 2). Our findings emphasize how careful control of environmental conditions will allow instructors, students and researchers to collect robust data on *L. variegatus* which further elucidates how their vascular and neuromuscular reflexive behavior change with varying environmental parameters, such as lighting, water temperature and water cleaning frequencies.

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Appendix A. Circulation Assay of *L. variegatus* Reared under Varying Environmental Conditions. Pie chart shows the results of the circulation assay of *L. variegatus* reared under a water temperature of (a) 10°C (discarded from analysis as discussed above) and (b) 22°C, (c) twice/week cleaning frequency and (d) once/week cleaning frequency, (e) a 24-hour Light treatment and (f) 24-hour dark treatment. Each pattern represents a different circulation assay score. Lower assay scores (1.5 or below) indicate poor circulation as indicated by amount of red coloration. (n=15). Note that (a) has a significantly different distribution than (c) and (e) despite the fact that they all represent control conditions.