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Corraling Wiggling Worms—Collecting Data for a Multi-Week Laboratory on the Effect of Various Treatments on the Pulsation Rate of the Dorsal Vessel of California Blackworms (*Lumbriculus variegatus*)

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The California blackworm, *Lumbriculus variegatus*, is a common model organism for instructional laboratory experiments examining the effect of environmental conditions and chemical treatments on cardiovascular function. Our introductory biology students test the effects of temperature, neurotransmitters, and common drugs on the pulsation rate of the dorsal blood vessel in the blackworm in a multi-week guided inquiry-based laboratory. We have found that by restraining the active worms in micropipets, students can accurately measure pulsation rate with relative ease. Using this technique, students are able to collect and analyze robust data within the time constraints of a typical laboratory period.

Keywords: blackworm, physiology, pulsation rate, dorsal blood vessel

Introduction

Blackworms, *Lumbriculus variegatus*, have been promoted as experimental animals in general biology laboratories for several reasons—large numbers can be purchased at minimal cost, they are readily available, they are easy to keep alive, and because they are invertebrates, they do not fall under the testing protocol restrictions commonly imposed for vertebrate research. Blackworms are useful for testing the physiological effects of chemical substances because they have a thin and permeable body wall that readily takes up chemical substances from their aqueous environment, resulting in immediate physiological changes. One physiological change that is highly evident is the change in pulsation rate of the worm's dorsal vessel ("heart"), a vessel that runs the length of the worm. Pulses begin at the posterior end of the worm and progress to its anterior end. By counting the number of pulses that pass through a particular mid-body segment during a known period of time (for example, a 30-40 second time period), one can accurately measure the pulse rate. To most accurately measure this rate, the worm is best viewed from its dorsal aspect (through the relatively thin body wall here), because thicker muscle layers, pigmentation, the gut and other tissues greatly obscure the pulsations when viewed from other aspects, i.e. lateral or ventral.

Due to the active nature of these worms, keeping the dorsal aspect of a single worm segment in view long enough in order to make an accurate determination of pulsation rate is problematic. Sometimes a free unrestrained worm will remain in position long enough to make an accurate count, but since this is a rare event some means of restraint or manipulation is needed. Drewes (2004) and Boher (2006) both proposed a slotted slide to restrain the worm, however, in our experience this system allows for excessive movement of the worm. Though physical prodding can keep the worm moving within the slot so that the dorsal vessel might eventually come into view, with the slotted slide there is no way to reliably manipulate the worm for optimal viewing, i.e. to keep its dorsal vessel positioned so that it is always facing the eye of the observer. Our solution to this problem is to place the worm in a glass tube (micropipet), a system proposed and used by others (Dix, 2008; Drewes website; Reynolds, personal communication).

The advantages of the micropipet (capillary tube) are two-fold: (1) it can be rolled to keep the dorsal vessel in view and (2) it helps reduce the worm's movement, i.e. "corralling" these "wiggling" worms. In this system a single worm is placed within the micropipet. Though this does not stop their movement (some worms fold back on themselves and continue to move about), their activity has been reduced significantly when compared to the slotted slide. More importantly, this glass tube allows the observer to rotate the worm

(by rolling the tube), therefore allowing for the dorsal vessel to be kept in view longer as the worm invariably moves about within the tube. Both student frustration and probable reporting of inaccurate data has been reduced through our use of this restraining system.

Context for this Technique: A Multi-week Introductory Biology Lab

Our introductory biology students begin their multi-week guided inquiry-based lab experience in Week 1 by learning our blackworm system while conducting an experiment testing the effect of temperature (i.e. ice-water bath compared to room temperature water). During this first week, students also design an experiment to test the effect of a neurotransmitter, drug, or environmental chemical on pulsation rate. Experimental design is flexible; however, our instructors typically guide students (working in groups of four) towards a paired design (Excel™ t-Test: Paired Two Sample for Means) in which each pair of students, in the group of four, tests one level of a variable against a control group. During Week 2, students conduct their experiment and analyze their data. During Week 3, each group delivers an oral presentation. Some instructors additionally assign an abstract and graph.

Student Outline

Week 1

Part 1: Learn how to restrain the worm and measure pulse rate

1. To restrain the worm for optimal viewing, place a single worm in a 40 µL glass micropipet tube by first drawing up the worm (along with some "worm water") in a 1 mL transfer pipet. Push the worm into the micropipet by placing the end of the transfer pipet against the micropipet and gently squeezing the bulb.
2. Place the tube into the bottom of a 60 mm x 15 mm plastic Petri dish; place the Petri dish on a dissecting microscope and view the worm using incident light (reflected light; lamp above). The tube should be positioned such that its ends are blocked by the side wall of Petri dish.
3. While observing the worm under the dissecting microscope, select a single mid-body segment to view from its dorsal aspect. Keep the dorsal vessel in view by rolling the micropipet as necessary to keep it "up" towards the objective end of the dissecting microscope.
4. Count the pulses passing through this segment for a minimum of 30 seconds (up to 60 seconds if the dorsal view of a particular segment can be maintained). Record both the pulse and the time.

Part 2: Test the effect of temperature on pulsation rate

Using the above procedure, test the effect of temperature on the pulsation rate. To minimize the effect of the variation between individual worms, use a "paired design": measure the pulse rate of each worm at both treatment temperatures, then calculate the change in pulse rate for each individual worm.

1. First, measure the rate of an individual worm at room temperature (~20°C). Record the number of pulses and the number of seconds in the data sheet (Table 1), and enter these values into the Excel worksheet (Fig. 1) to calculate pulse/minute.
2. Next, measure the rate of that same worm cooled with ice water (~2°C). First, add "worm water" ice to the Petri dish and nestle some ice along the micropipet. Incubate for 60 seconds, then measure and record pulse number and time as above.
3. Repeat steps 1 and 2 with four more worms.
4. Using Excel™:
   a. Calculate the % change in pulse rate for each worm.
   b. Calculate the mean % change and the standard error of the mean for each treatment.
   c. Perform a "paired t-test."

Table 1. Data sheet to record the data and analysis for test of temperature effect on pulsation.

	Control Temperature: _____				Treatment Temperature: _____			
Worm	Pulse	Time	Rate		Pulse	Time	Rate	% change
Mean →					Mean →			
Standard Error →					Standard Error →			
					Mean % Change →			

Part 3: Design an experiment to test the effect of a chemical variable on pulsation rate

Adapt the procedure above to design an experiment to test the effect of a biological molecule or environmental chemical on the pulsation rate:

1. Develop a testable hypothesis based on the observation "The pulse rate of blackworms varies," and the resulting questions such as, "What might be the cause—might it be some chemical in the environment of the worm? What are the effects of environmental or regulatory chemicals on the dorsal vessel pulse rates of the California blackworm?"

   State a hypothesis for a particular chemical. Available chemicals include:

   a. sodium chloride (0.6% and 0.3%)
   b. epinephrine [20 parts per million (ppm) and 10 ppm]
   c. acetylcholine chloride (0.1 mg/mL and 0.2 mg/mL)
   d. ethanol (1% and 0.5%)
   e. L-nicotine (0.0125 mM and 0.00625 mM)
   f. caffeine (1 mM and 2.5 mM)

2. Think about how you will test this hypothesis, and then make a *prediction*, i.e. the outcome that would be expected if the hypothesis is true. To illustrate: "If the extracellular concentration of sodium chloride affects the depolarization of regulatory 'pacemaker' cells (e.g. in humans), and I add sodium chloride to the blackworm's environment, then the blackworm's dorsal vessel pulse rate will increase compared to the pulse rate in the control water."
3. What is your independent variable?
4. What is your dependent variable?
5. Sketch out your experimental design in detail. (**Note:** To sufficiently expose the worms to the experimental treatment, you will need to remove the worm from the micropipet between control and treatment. To minimize any handling effects on the worms, test only the control condition and one concentration of treatment chemical on each worm. Therefore, if testing two concentrations of a given treatment, you will need two groups of worms.)

Week 2

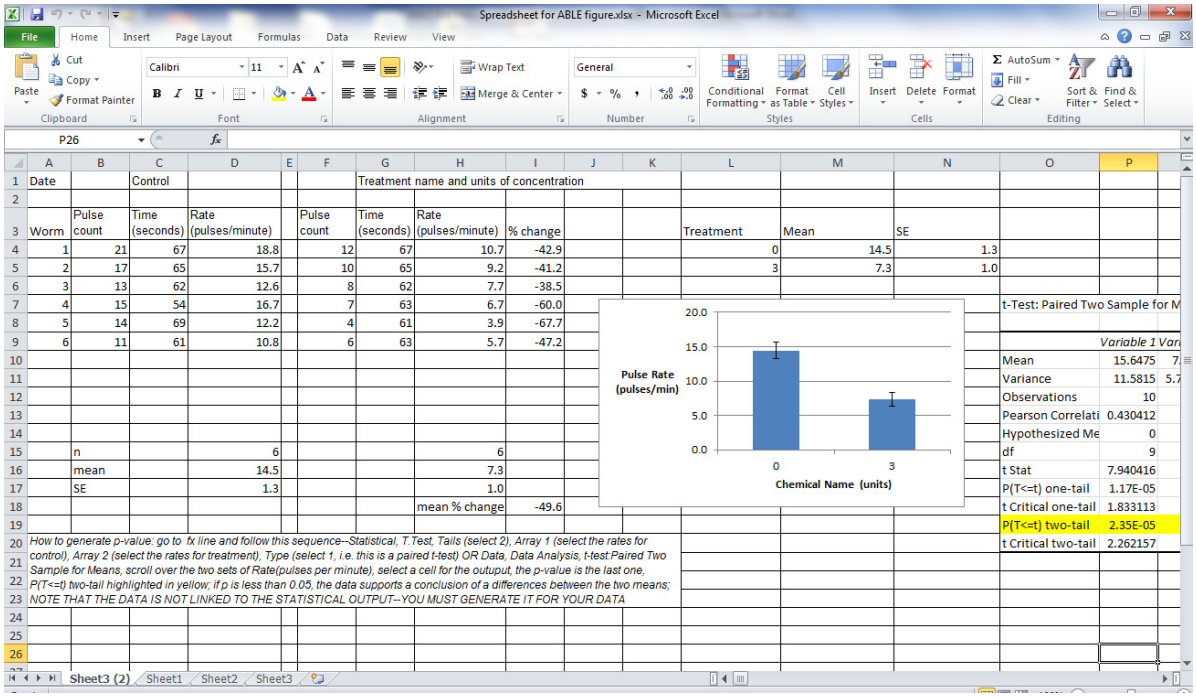
Part 1: Conduct experiment

1. Measure the rate of one worm and record pulse number and time.
2. Using a transfer pipet, gently expel the worm from the micropipet into a weigh boat containing the treatment solution, and incubate for 60 seconds.
3. Return the worm to the micropipet. Measure and record pulse number and time as above.
4. Repeat steps 1-3 with four additional worms.
5. Using five new worms, test effect of second treatment concentration as above.

Part 2: Analyze, describe, and interpret the data collected.

1. Using Excel™ (Fig. 1):
   a. Perform a "paired t-test."
   b. Calculate the % change in pulse rate for each worm.
   c. Calculate the mean % change and the standard error of the mean for each treatment.

2. Graph the results (comparison of control and treatment and/or comparison of mean percent change for the two treatments resulting from the group experiment).
3. Interpret your results. Does the data support your hypothesis?
4. Following the general guidelines for scientific reporting, create a PowerPoint™ presentation.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	Date		Control				Treatment name and units of concentration									
2																
3	Worm	Pulse count	Time (seconds)	Rate (pulses/minute)		Pulse count	Time (seconds)	Rate (pulses/minute)	% change			Treatment	Mean	SE		
4	1	21	67	18.8		12	67	10.7	-42.9			0	14.5	1.3		
5	2	17	65	15.7		10	65	9.2	-41.2			3	7.3	1.0		
6	3	13	62	12.6		8	62	7.7	-38.5							
7	4	15	54	16.7		7	63	6.7	-60.0						t-Test: Paired Two Sample for M	
8	5	14	69	12.2		4	61	3.9	-67.7							
9	6	11	61	10.8		6	63	5.7	-47.2						*Variable 1*	*Var*
10															Mean	15.6475
11															Variance	11.5815
12															Observations	10
13															Pearson Correlati	0.430412
14															Hypothesized Me	0
15		n		6				6							df	9
16		mean		14.5				7.3							t Stat	7.940416
17		SE		1.3				1.0							P(T<=t) one-tail	1.17E-05
18								mean % change	-49.6						t Critical one-tail	1.833113
19															P(T<=t) two-tail	2.35E-05
20	*How to generate p-value: go to fx line and follow this sequence--Statistical, T.Test, Tails (select 2), Array 1 (select the rates for*														t Critical two-tail	2.262157
21	*control), Array 2 (select the rates for treatment), Type (select 1, i.e. this is a paired t-test) OR Data, Data Analysis, t-test:Paired Two*															
22	*Sample for Means, scroll over the two sets of Rate(pulses per minute), select a cell for the outuput, the p-value is the last one,*															
23	*P(T<=t) two-tail highlighted in yellow; if p is less than 0.05, the data supports a conclusion of a differences between the two means;*															
24	*NOTE THAT THE DATA IS NOT LINKED TO THE STATISTICAL OUTPUT--YOU MUST GENERATE IT FOR YOUR DATA*															
25																
26																

Figure 1. Screen capture of the data analysis template provided to student. Mean and standard error was linked to the graph template. Note directions for the paired t-test.

Notes for the Instructor

Materials Used at the Miniworkshop

- Blackworms: Aquatic Foods, Fresno, CA (**http://www.aquaticfoods.com/**)
- "Worm water": holding water and water used to prepare treatment solutions; made by adding 0.06 g Instant Ocean Sea Salt (Aquarium Systems, Inc., UPC 0 51378 01100 6) to 1 L of deionized water
- 40 µL glass micropipet tube, Drummond Scientific Company, Microcap capillary tube, 1-000-0400; length 52 mm, 0.055 inch O.D., and 0.0390 inch I.D.
- 1 mL transfer pipet, Fisher Scientific, 13-711-9AM
- Petri dish, 60 mm x 15 mm, Fisher Scientific, 0875713A
- Nudging tool: made by taping a small piece of 1/8 inch rubber band, cut at an angle, to a 6 inch round wooden laboratory applicator stick; directions for making and using this "widget" at **http://www.ableweb.org/volumes/vol-26/19-Drewes.pdf**
- 3 mL transfer pipet to transfer bulk worms
- Stopwatch
- Dissecting microscope capable of producing incident light

Methods

What follows is a description of our testing of this protocol, using the methods and data analysis consistent with what we expect of our students in the laboratory classroom. We recruited some of our trusted lab aides to assist us (the authors) in collecting data that we could report with confidence. Also included is data from two years of tests (2011 and 2012).

Worm Management: Bulk blackworms were obtained from Aquatic Foods (Fresno, CA). To prevent the death and decay associated with overcrowding, these bulk worms were divided by placing approximately 1 teaspoon of worms in 1 L of "worm water" in a plastic shoebox. The holding water ("worm water") was made by adding 0.06 g Instant Ocean Sea Salt (Aquarium Systems, Inc., UPC 0 51378 01100 6) to 1 L of deionized water, and has mineral content similar to spring water. Worms were maintained in a refrigerator at about 4°C (without food). We have found that worms housed this way can be held in excellent condition for at least three weeks.

Restraining the Worm: To restrain the worm for optimal viewing, a single worm was placed in a 40 µL glass micropipet tube (Drummond Scientific Company, Microcap capillary tube, 1-000-0400) by first drawing up the worm (along with some water) into a 1 mL transfer pipet (Fisher Scientific, 13-711-9AM). The worm was then pushed into a micropipet by placing one end of the micropipet against the opening of the transfer pipet. This particular size of tube was chosen because it was large enough (0.99 mm or 0.0390 inch I.D.) to allow for the worm to readily be pushed into it (smaller tubes were tried but did not receive the worm easily enough to be acceptable). The tube, with the worm inside, was then placed into the bottom of a 60 mm x 15 mm plastic Petri dish (Fisher Scientific, 0875713A), used to hold treatment solutions. To keep the dorsal vessel in view, the micropipet was rolled by hand as necessary to keep the dorsal vessel "up" towards the objective end of the dissecting microscope (Meiji EMZ). Optimal viewing was attained by using a microscope with variable magnification and incident light (reflected light; lamp above) rather than transmitted light (lamp below). Backlighting produced by the transmitted setting tended to obscure the dorsal vessel.

Determining Pulsation Rate: The pulsation rate for all treatments was determined by rolling the tube to keep the worm in view for a minimum of 30 seconds (and up to 1 minute if the dorsal view of a particular segment could be maintained). During this period the number of pulses passing through a particular mid-body segment was counted. This count yielded a pulsation rate per minute.

Manipulation Effect: To determine whether the act of repeatedly transferring worms to the micropipet is a confounding variable (i.e. produces a measured change in pulsation rate regardless of treatment condition), we compared pulse rates in worms after their first transfer with that after their second transfer, using control water at room temperature. This was done by measuring the initial pulse rate of each worm in a micropipet, then transferring the worm to holding water for 2 minutes before returning the worm to the micropipet for a second pulse measurement.

Temperature Effect: For the temperature effect (week 1 of the multi-week lab during which students learn the system), a room temperature (20°C) rate was compared to treatment with ice water (~2°C). This was a paired test using worms (n=10) that had been taken from the refrigerator and held at room temperature for about 1 hour, followed by transfer to room temperature water. An ice bath treatment was created by placing a few pieces of ice in the Petri dish adjacent to the worm-containing micropipet. Ice was made up using the "worm water" described above (0.06 g Instant Ocean/L of DI water).

Chemical Effects: The following chemical treatments and levels were tested: epinephrine hydrochloride (Sigma Life Science, E4642) at 20 parts per million (ppm); ethanol (AAPER Alcohol and Chemical Company, ethyl alcohol USP, absolute-200 proof) at 0.5% and 1%; sodium chloride (Fisher Scientific, BP358) at 0.6%; L-nicotine (99+%, Acros Organics, 181420250) at 0.0125 mM; acetylcholine chloride (Sigma Life Science, A6625) at 0.1 mg/mL; and caffeine (Sigma, C-8960) at 1 mM. Worms were immersed in about 1 mL of chemical treatment for 2 minutes before being returned to the micropipet. Because previous observations suggested that the chemical treatments caused physiological decline of the worms and because each handling was perceived to be physically damaging, each individual worm was tested under the control condition and at one level of a particular chemical treatment only, rather than at both levels. Temperature was 20-22°C for all chemical treatment experiments.

Statistical analysis: All statistical analysis was conducted using Excel™ 2007 (Microsoft™; Redmond, WA). The effect of each treatment was determined using a paired t-test (Excel™ t-Test: Paired Two Sample for Means), comparing pulse rate in the control condition with that in the treatment condition. Since the two levels of ethanol were tested on different groups of worms, separate paired tests were used for each. To test for significant differences between the two levels of ethanol a two sample t-test was used (Excel™ t-Test: Two-Sample Assuming Equal Variances. For all treatments, n = 10.

Results

Corralling the Worms: Though worms still move about significantly within the micropipet, including turning back on themselves and occasionally attempting to crawl out of the micropipet, they are sufficiently contained, so that, with patience, one can accurately measure pulsation rate. Most chemical treatments noticeably increased activity.

Manipulation Effect: There was no consistent effect of handling on pulsation rate. Of 10 worms tested, pulsation rate was higher during the second measurement than the first in six worms but lower in four worms, resulting in a mean change among individuals of 12.3 ± 11.0% ($\bar{x}$ ± SE; Table 2). This difference was not statistically significant (p=0.46; paired t-test).

Temperature Effect: Pulse rate decreased significantly (p<0.001) during cold exposure, from 13.4 ± 1.1 ($\bar{x}$ ± SE) pulses/minute at 20°C to 6.9 ± 0.7 in the ice bath (~2°C), a mean change of -47.7 ± 3.9% relative to the room temperature worms (Table 2). Because the ice bath also slowed the movement of the worms considerably, pulse rate was most easily measured in this treatment.

Chemical Effects: All chemical treatments significantly increased pulsation rate (Table 2). Of the neurotransmitters, epinephrine (epinephrine hydrochloride; 20 ppm) increased pulse rate by a mean of 29.5 ± 4.5%, and acetylcholine (0.1 mg/mL) increased pulse rate by a mean of 43.7 ± 11.3% (p=0.003). Nicotine (0.0125 mM) increased pulse rate by a mean of 52.9 ± 12.9% (p=0.0007), and caffeine (1 mM) increased pulse rate by a mean of 85.3 ± 22.8% (p=0.002). Sodium chloride (0.6%) also increased pulse rate, by a mean of 62.1 ± 13.5% (p=0.0008).

Treatment with both levels of ethanol also increased pulse rate. Treatment with 0.5% ethanol increased pulse rate by a mean of 49 ± 24.4% in year 1 (p=0.048), and by a mean of 69.3 ± 20.1% in year 2 (p=0.003). Treatment with 1% ethanol (tested only in year 1) increased pulse rate by a mean of 26.8 ± 7.8% (p=0.002). Comparison of the two levels of ethanol indicated no statistically significant difference (p=0.40).

All chemical treatments were consistent; in each experiment, the chemical treatment increased pulse rate in at least eight of the ten worms tested. Pulsation rate increased in all ten worms treated with caffeine, nicotine, and epinephrine, in nine of ten tested with sodium chloride, acetylcholine, and 1% ethanol.

Table 2. Changes in pulsation rate of the dorsal vessel in California blackworms (*Lumbriculus variegatus*) in response to select treatments.

		Control (pulse/min)*	Treatment (pulse/min)*	Mean Change (%)*	p-value#
Temperature (20°C → 2°C)		13.4 ± 1.1	6.9 ± 0.7	-47.7 ± 3.9	0.000008
Transfer effect		17.4 ± 2.1	18.5 ± 1.7	12.3 ± 11.0	0.46
Epinephrine (20 ppm)		11.5 ± 0.6	14.9 ± 0.8	29.5 ± 4.5	0.00012
Ethanol (0.5%)	Year 1	20.4 ± 2.2	26.2 ± 1.1	49.0 ± 24.4	0.048
	Year 2	10.4 ± 0.9	16.1 ± 0.9	69.3 ± 20.1	0.003
Ethanol (1%)		23.7 ± 1.7	29.7 ± 2.0	26.8 ± 7.8	0.002
Caffeine (1 mM)		11.7 ± 1.2	19.8 ± 1.7	85.3 ± 22.8	0.002
Sodium chloride (0.6%)		12.4 ± 1.1	19.2 ± 1.3	62.1 ± 13.5	0.0008
Nicotine (0.0125 mM)		10.6 ± 0.9	15.8 ± 1.3	52.9 ± 12.9	0.0007
Acetylcholine (0.1 mg/mL)		12.1 ± 0.6	16.9 ± 0.9	43.7 ± 11.3	0.003

*mean ± SE; # paired two sample, two-way t-Test

Conclusions

While it may be impossible to completely eliminate unwanted movement of worms, we found that the micropipet technique is an improvement over other less restraining methods such as the (open) slotted slide.

Responses to the chemical treatments we tested were generally consistent with those either directly observed by others or suggested as probable outcomes. One notable exception was the ethanol experiments. Our results for ethanol did not agree with the outcome suggested by Bohrer (2006) and Johnson (2009), who both stated that a decrease in pulsation rate was expected. In one case (Johnson), a much lower concentration of ethanol was used (0.02%), and could explain the different result, however in the other (Bohrer), the concentrations suggested/used (0.26% to 2.6%) were more in line with ours (0.5% and 1%), and do not offer a simple explanation for an opposite result. To avoid lethality with the nicotine treatment, we suggest a lower concentration (not greater than 0.025 mM) than has been suggested by some. All chemical treatments (nicotine, sodium chloride, acetylcholine, caffeine, epinephrine, and 0.5% and 1% ethanol) caused significant (p<0.05) increases in pulsation rate; however, there was no statistically significant difference between

the two ethanol treatments ($p>0.40$).

We believe that beginning this multi-week lab experience with the temperature experiment is the best way to introduce our mixed-majors students to the worms because any frustration they initially experience with these active wiggling worms at room temperature quickly dissipates with the ice treatment. This is important because, as instructors, we want students to have success in gathering their data and to enjoy the scientific process without being overly frustrated by gathering data. On the other hand, the chemical treatments during the second week are more challenging for students, as all of these treatments are stimulatory, causing increased activity (movement of worms) relative to normal worm activity. Though we do not require our students to eliminate the transfer effect as a potential confounding variable, this might be introduced to the lab experience (or at least discussed).
We tested 10 worms per experimental treatment, but based on our results, a smaller sample size (e.g., five) is likely to be sufficiently robust. From the student's standpoint, a smaller sample size is probably the best strategy because some student pairs will not be as adept as others or by chance may have sampled more active worms, and will necessarily require more time to obtain accurate results.

Additional Comments

Students typically work in groups of four with each pair (lab partners) testing a single treatment level against a control value. Two instruments are used by students to record data, analyze the data, and graph the results: (1) a recording sheet in their lab manual (Table 1) and (2) an Excel™ template which combined data-reporting, statistical analysis, and graphing (Fig. 1). The data sheet was designed for a sample size of five to insure that students would have sufficient time to both collect and analyze their data during the confines of the lab period (2 hours and 45 minutes). Students are advised to bring their own laptops to lab.

The Excel™ spreadsheet template generates means, standard errors, percent change, and with the means and standard errors linked to a graph template. Directions for performing a t-test in Excel™ are also provided, although a thorough discussion of variation and statistical testing is not expected by instructors due to time constraints. It has been observed that students who bring some knowledge of statistics to the class appear to be excited about applying their knowledge in a real-world application.

Some instructors elect to have students perform less statistical analysis while others require their students to learn more about using Excel™, in particular, the graphing, and therefore do not provide the template in its full form to their students.

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