Darwin, Earthworms & Circadian Rhythms: A Fertile Field for Science Fair Experiments

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○ Darwin & Earthworms

Strange noises, including shouts, whistling, and music, from the playing of a bassoon and a piano, echoed from Down House in Downe, England. However, it was not a festive 19th century social occasion; instead, a careful scientific experiment was being conducted by the ever-curious naturalist Charles Darwin (1809 - 1882). Cooperative earthworms, *Lumbricus terrestris*, were being tested to see if they would react to sound. He also investigated their sensitivity to light, vibrations, odors, and touch. For his work, he kept earthworms in soil-filled flowerpots in his library. Darwin suffered from chronic insomnia, a condition that no doubt led to his making so many observations of the nocturnal earthworm.

Intrigued with their everyday behavior, Darwin wondered just how smart earthworms were. After watching earthworms plug up their burrows with petioles, leaves, pebbles, or twigs wedged in subtly different ways, Darwin concluded that they possessed a surprising level of intelligence. After watching them manipulate pine leaves, Darwin commented "In order, therefore, that worms should do their work well, they must drag pine leaves into their burrows by their bases, where the two needles are conjoined. But how they are guided in this work is a perplexing question" (Darwin, 1881). For one experiment, Charles Darwin and his son Francis cut the sharp ends from the pine needles, but found that the earthworms still preferred to drag the needles into their burrows by the bases. "It appeared both to my son and myself as if the worms instantly perceived as soon as they had seized a leaf in the proper manner" (Darwin, 1881). How the eyeless earthworm gains an "overview" of individual leaves so as to be able to maneuver them was not readily understood, but it was thought to depend mainly on the earthworm's tactile sense.

In Charles Darwin's book, *The Formation of Vegetable Mould through the Action of Worms*, there are measurements and calculations that the Darwins made of the weight of the nightly manure castings produced by earthworms, and consequently how much of an accumulation of this "vegetable mould" would build up over the years. Such detailed observations and careful attempts to numerically quantify various natural processes are found throughout Darwin's writings. The earthworm's relatively simple digestive system, consisting of a mouth, pharynx, esophagus, crop, gizzard, and intestine, enables it to grind vegetable matter and other stray material that it ingests to produce the clay-like manure castings egested from its anus and deposited on the ground's surface. This relentless activity mixes and enriches the topsoil to create fertile humus.

Generally, earthworms live less than three years, but some have been known to live as long as six years in captivity. The age at which earthworms reach sexual maturity varies greatly based on season, environmental temperature, food availability, and moisture content of the soil. (Further information, as well as good photographs and drawings of dissected earthworms, can be found online with a Google™ search.)

Utilizing earthworms for experiments allows students to follow in Charles Darwin's legendary footsteps and also to enter into the realm of scientific thought.

The reproduction of earthworms is of particular interest. Both male and female reproductive organs are found in the same individual. In general, organisms are classified as diecious (two houses) if male and female reproductive organs are in separate individuals, and monecious (one house) if both types of organs are in one individual, as is the case for earthworms. Even though earthworms are monecious, they cannot self-fertilize. The common earthworm comes to the surface of the ground to mate on damp summer nights. Reproductive readiness is evident by the swollen clitellum, an enlarged region seen partway down the length of the body from segments, or metameres, 31 or 32 to 37. During mating, they position themselves so that the flat, ventral parts of their bodies are together, and their head ends point in opposite directions, which is necessary so that the genital pores match up. The clitellum of each worm secretes mucus to form a slime tube around the earthworms. Both earthworms release sperm, and the sperm travel posteriorly down a sperm groove in each worm's ventral surface, to the location where sperm are passed into the seminal receptacles of the mate. After a simultaneous exchange of sperm occurs, the sperm are next stored in spermathecae, and fertilization itself is delayed until after the two earthworms have gone their separate ways. Fertilization occurs when the stored sperm and the eggs are released later into a mucous ring secreted by the clitellum. Eventually, this mucus ring passes off the head end of the worm and forms a cocoon that is left in the soil. In certain situations, it is said offspring may be produced through parthenogenesis, i.e., the egg undergoes cleavage without fertilization.

○ Unusual Earthworm Relatives

As you read about earthworms, you will find references to some of their unusual relatives. Amazingly, there are giant earthworms, such as *Megascolides australis*, native to southeastern Australia, with a diameter of 3 cm and reaching a length of 3 m (Pearse et al., 1977). The giant earthworms, as well as the common earthworm, *Lumbricus terrestris*, taxonomically belong to the Class Clitellata in the Phylum Annelida. In fact, the Phylum Annelida has many thousands of species, all of which have segmented, or more correctly, metameric, bodies. Leeches (e.g., *Hirudo medicinalis*) in the Class Hirudinea are well-known annelids for their supposed medicinal applications in the practice of blood-letting, an ancient method once claimed to remove poisons from the blood, and, according to Hippocrates, useful in balancing the body's humors. Today, leeches are enjoying a resurgence of attention from the medical community for their natural anticoagulants and their ability to remove dead tissue from gangrenous wounds.

Various species of annelids in the Class Polychaeta called Palolo worms are found in scattered locations around the world, but most famous is *Eunice viridis* in the Samoan Islands. (See the Smithsonian "The Palolo Spot" online.) Palolo worms can measure up to a meter in length. The female worms are dark green to blue green, while the males are white to yellow. These polychaete worms are marine and locally abundant but are rarely seen because they spend most of the year burrowed in the coral reefs. However, they are certainly noticed when they spawn in the months of October and November (spring in the Southern Hemisphere). The spawning occurs in the dark just before dawn, one day each month at the last quarter of the moon. For a few brief hours, the ocean is filled with writhing posterior ends of worms that break off and release the milky eggs and sperm. In *Biological Clocks: Their Functions in Nature*, J.L. Cloudsley-Thompson describes the phenomenon as "... when great funnels of worms burst to the surface and spread out until the whole area is a wriggling mass of green and brown." People go out into the ocean to collect the epitokes, which are the yolky egg-filled posterior ends shed by the spawning females. These nutrient-filled reproductive structures are considered a rare delicacy and are eaten either raw or cooked. The yearly spawning of the Palolo once played an integral part in the Somoan peoples' traditions. As in most species, Palolo gametes are fertile for only a brief period. The Palolo worm's survival therefore depends on a precise combination of daily, lunar, and seasonal physiological rhythms to time its reproduction. The common earthworm at first may seem less exciting than these exotic species, but earthworms have turned out to be fascinating to many scientists.

○ Early Student Research Projects

Professor Miriam F. Bennett and her students at Colby College carried out some of the most intriguing follow-up studies. They exposed earthworms to bright light at mid-day and again early in the evening to determine if at certain times of the day the earthworms would withdraw more quickly. They soon observed that a circadian (about 24 hour) rhythm was present in the earthworms' speed of withdrawal from light. Furthermore, by measuring the earthworms' reaction time year-round, they also discovered seasonal variations in the light-withdrawal speed. These worthwhile experiments required only a stopwatch, a flashlight, a darkened room, and a supply of earthworms. Also, the variations in crawling speed of earthworms moving up an incline were measured. The earthworms were placed in moistened grooves on a slanted board, and gently prodded to crawl forward and upward. The number of seconds taken to crawl 10 cm was recorded. Again, circadian rhythms were found in the speed of locomotion throughout the day and night. In another experiment, Professor Bennett and Mary Willis Finlay removed the "brains" (suprapharyngeal or cerebral ganglia) of earthworms, and discovered that these organs were crucial for maintaining the previously-described circadian rhythms. Unlike sham-operated earthworms, a control group where an incision was made and the suprapharyngeal ganglia were left intact, brainless earthworms no longer exhibited any difference in their speed of withdrawal from light at mid-day as compared with the evening speed. (Extraordinarily, the brainless, or suprapharyngealectomized, earthworms can survive and their brains, or suprapharyngeal ganglia, may regenerate within several weeks.)

In some experiments, a Helmholtz coil was used to encircle the earthworms so that when an electrical current was passed through the coil, the Earth's local geomagnetic field was attenuated or removed. Surprisingly, the earthworm's circadian rhythm of light-withdrawal virtually disappeared under these conditions (Bennett, 1974). This interesting line of research needs to be repeated and extended by a new generation of students who may wish to read the experimental details in Professor Miriam F. Bennett's fascinating 1974 book, *Living Clocks in the Animal World*.

○ Fundamentals of Biological Rhythms

Recently, Russell G. Foster and Leon Kreitzman wrote an excellent, up-to-date overview of what is now known about biological rhythms, *Rhythms of Life* (2004). Reading this well-written book will provide today's student with a substantial background in chronobiology, or the study of biological rhythms. The broad range of biological rhythms includes those with periods less than a second, such as brain waves, to those of many years in length, such as the flowering of various plants, e.g., the Century plant. The majority of past research has been devoted to understanding circadian rhythms, which have already been described as those roughly 24 hours in length. *Circadian* is derived from Latin words that mean "about a day." In fact, circadian rhythms are ubiquitous in living things, and their presence has been considered by some biologists to be a fundamental characteristic of life. Most biologists consider circadian rhythms to be endogenous, or the result of internal biochemical processes. Normally, the daily photoperiod entrains or synchronizes our somewhat too-long or too-short circadian rhythms to match the 24-hour rhythm of the environment. Interestingly, circadian rhythms tend to "freerun," or express their own period length, in an environment where there is continuous darkness or continuous light. For example, a cave explorer's body temperature rhythm will freerun (i.e., 24.1 or 24.2 hours) and gradually drift out of synchrony with the surface environment's light-dark cycle if the spelunker is deprived of time cues, such as the usual 24-hour light-dark cycle. In *Beyond Time*, the adventurer Michel Siffre chronicles his two-month stay in France's Scarasson Cavern in 1962 which, along with other isolation studies, made scientists aware of circadian rhythms in human vital signs.

The basic characteristics of any rhythm are its period, amplitude, and phase. The period is the duration of the rhythm, the amplitude measures the rhythm's strength of fluctuation, and the phase tells you when the peak occurs. Rhythms shorter than 24 hours are called *ultradian*, while those longer than 24 hours are called *infradian*. Circadian rhythms seem to be unaffected by temperature. Thus, unlike most physiological systems that involve biochemical processes, the rate of the underlying reactions do not double or triple with a 10° C increase in temperature; therefore, circadian rhythms are said to be temperature compensated. This property has been difficult to explain in biochemical terms. Obviously, if the period of circadian rhythms were substantially affected by temperature, they would not make very good clocks!

Depending on the animal, various pacemakers in the brain have been identified to synchronize circadian rhythms found on the cellular and tissue level. For example, the suprachiasmatic nucleus (SCN) in the hypothalamus of mammals serves this role. In mammals, the SCN receives information from a nerve tract connected to the retina, which is sensitive to changes in the photoperiod. Based on what we have seen, perhaps the suprapharyngeal ganglia in earthworms carries out similar functions to the SCN, but more research is needed to substantiate this possibility.

The time of the daily peak in locomotor activity of an organism is frequently described when circadian rhythms are discussed. For example, animals active during the daytime are called *diurnal*, those active at nighttime are called *nocturnal*, and those active at dawn and dusk are referred to as *crepuscular*. (Deer and rabbits are good examples of crepuscular animals.) Interestingly, humans are often thought of as being diurnal, but there are many variations on this theme based on one's gender, age, health, and occupation. Not everyone can tolerate working the night shift, and there is evidence that considerable self-selection occurs among people willing to become shift workers, depending on the individual health effects of the shift work. In general, younger people adapt more quickly to the effects of shift work, and even to jetlag. Their circadian rhythms seem to reset more easily. Also, young children like to get up early, while teenagers are somewhat nocturnal! For teenagers, test taking and learning in general can be optimized by such tasks being scheduled later in the day. If you perform a Google™ search on "teenage circadian rhythms," you will find a number of articles that discuss the substantial changes that occur at puberty in student circadian rhythms.

Figure 1. Placement of an earthworm into the plastic tubing of the earthworm activity wheel by University of Pittsburgh graduate student Amy M. Furda.

○ Materials & Methods & The "Worm Wheel"

We have enjoyed building on the research of the Darwins, Bennett, Richter, and various students. Like many scientists faced with somewhat limited time and resources, we sought readily available and inexpensive experimental animals that would be easy to maintain in the laboratory. Earthworms also appealed to many students as suitable animals for experimentation. We often collected earthworms from the campus lawn, using flashlights to collect them when they came to the surface of the ground to mate during damp summer nights. As everyone knows, earthworms can be kept in Styrofoam™ cups in a refrigerator for months on end. Rather than putting soil in the containers, we use Buss Bedding®, manufactured by Buss Manufacturing Co. in Lanark, Illinois, 61046, as an ideal earthworm substrate. Buss Bedding®, which resembles ground-up newspaper, is mixed with water to form a moist, clay-like, porous material. It serves as both a food and a burrowing material for these easily-desiccated creatures. (See online references for "Rearing Earthworms.")

Detailed studies of animal activity often entail the use of so-called activity wheels. An activity wheel is basically an apparatus that rotates whenever the animal moves. An activity wheel is very much like a hamster wheel in that the animal runs on a circular track around the circumference of a central disk. Unlike the hamster wheel, however, for experiments the animal is usually enclosed within the activity wheel and cannot leave. The overall locomotor activity is determined by counting or automatically recording the number of wheel rotations per hour, or other unit of time.

Dr. Curt P. Richter (1965), Professor of Psychobiology at Johns Hopkins Medical School, utilized the first activity wheels for science in order to monitor and analyze the running activity of wild rats that he and his students captured in the alleys of Baltimore. Professor Richter reported that wild rats produced better activity records than did their more docile cousins raised in captivity. Since those early years, modified activity wheels have been designed for a variety of organisms, including cockroaches, fruit flies, lizards, slugs, and, of course, earthworms.

Our activity wheel that detects the earthworm's movement consists of an old-fashion plastic audiotape reel, 18 cm in diameter, connected to a Vernier® rotary motion sensor, which is attached to a ring stand. Next, a piece of 1.5 cm diameter clear plastic tubing is cut to fit completely around the outer groove on the audiotape reel. A few small holes are poked into the plastic tubing to allow for the passage of air. The tubing is loosely filled with moist Buss Bedding®, and the earthworm is then placed into the tube, as demonstrated by Amy M. Furda (Figure 1). Then, the tubing is wrapped around the audiotape reel, and secured with a few pieces of tape. For some experiments, the entire setup is placed into a light-tight cabinet or box so that a timed photoperiod, or continuous light or darkness, can be provided.

Vernier® Equipment

At Bethany College, students use Vernier® Logger Pro 3® software and computer interface equipment to record the circadian rhythms of earthworms. Complete information on the use of this excellent equipment is available online at www.vernier.com. The activity wheel or "worm wheel" is employed to automatically record the locomotor activity of individual earthworms by way of an attached Vernier® rotary motion detector. After we designed our apparatus, we later found that a similar earthworm activity wheel had been invented previously (Abramson, 1994). The Vernier® Logger Pro 3® software allows one to record activity data in thousands of "bins" or intervals of time, such as one minute bins, over several days, with either PC or Mac computers. These records can be viewed as a chart (e.g., Figure 2) and printed out, or the data can become the basis for time series analysis to look for rhythms.

Details from Neurophysiology

A review of two physiological concepts is needed to understand some of our experiments. First, neurotransmitters are chemicals released by nerve cells called neurons to carry impulses across a gap, or synapse, to the next neuron. Dozens of different neurotransmitters have been identified. Second, serotonin is a common neurotransmitter associated with the inactive, or sleep, period in many animals. We hypothesized that indirectly increasing the serotonin level in the suprapharyngeal ganglia of earthworms might affect their circadian rhythms. For example, we injected 5-hydroxy-tryptophan (a precursor to serotonin) into earthworms and thereby dampened their circadian rhythm of locomotion (Burns et al., 1991). This effect was not observed in earthworms that previously had their suprapharyngeal ganglia removed. Additionally, certain drugs are effective in decreasing the normal reuptake of serotonin by the secreting neuron. These are called SSRIs, or selective serotonin reuptake inhibitors. They allow serotonin to remain in the synapse, which enhances its action. Prozac® (fluoxetine) is a widely-used SSRI to treat clinical depression and other disorders that seem to involve neurons producing too little serotonin. We found that Prozac® dampened the circadian rhythm of locomotion and also slowed the crawling of earthworms. The suprapharyngeal ganglia had to be intact for these effects to occur (Burns, et al., 1992). (The Prozac® used was a gift from the Eli Lilly Co.) Overall, these results support the idea that these circadian rhythms involve serotonergic processes and are either generated and/or synchronized by the suprapharyngeal ganglia in earthworms.

How To Take the Brains Out

As mentioned, for some of our experiments, earthworms had their suprapharyngeal ganglia removed, which is accomplished by using a razor blade to cut a single midline incision into the dorsal surface from the earthworm's first metamere to its fourth metamere. After the incision is made and the wound gapes open, two small, bilaterally symmetrical, white, pear-shaped suprapharyngeal ganglia are visible. With a pair of iridectomy scissors, the ganglia are cut and then removed. Middle school students can learn to suprapharyngeal ganglionectomize earthworms after a few tries, even without the use of a dissection microscope. Earthworms can be anesthetized by exposing them to cold for a few minutes before the operations. The earthworm is then placed in its own container, a Styrofoam™ cup with Buss Bedding®. A lid is put on; the cup is labeled and stored in a refrigerator. After surgery, earthworms are allowed to heal for at least a day or two before further use.

Preliminary Results

Interestingly, when Paul J. Scurti compared the locomotor activity records of suprapharyngeal ganglionectomized earthworms to those with intact ganglia, the suprapharyngeal ganglionectomized earthworms were found to have longer freerunning rhythms. Preliminary analysis, using Fast Fourier Transform analysis, showed that earthworms with intact ganglia, on average, had a rhythm of locomotion of about 23 hours in length, whereas suprapharyngeal ganglionectomized earthworms, for unknown reasons, had rhythms of locomotion of about 40 hours in length.

The mathematical analysis of the data with Fast Fourier Transforms and the utilization of other techniques to characterize rhythms are of interest. Online sites can be found for Vernier®, Actiview®, SPSS®, and other brands of useful time series analysis software.

○ Conclusion

Earthworms make ideal experimental animals for students to test in the laboratory. Although earthworms may appear to be primitive, they are governed by both circadian and seasonal rhythms, just as more advanced organisms are. They possess an intelligence that enables them to decide the placement and angle of implements with which to plug their burrows and sense organs that allow them to react to a variety of stimuli. Utilizing earthworms for experiments allows students to follow in Charles Darwin's legendary footsteps and also to enter into the realm of scientific thought.

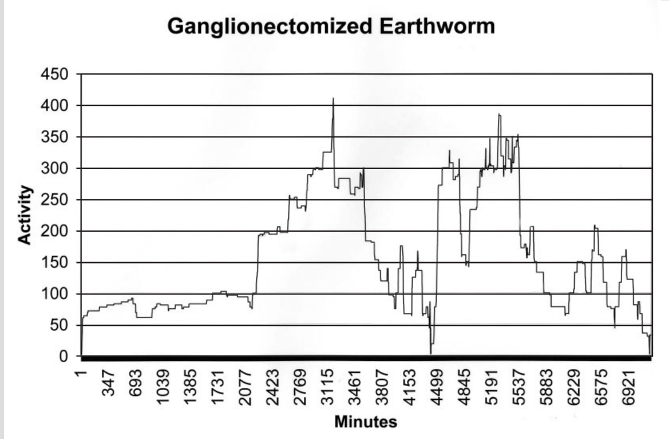

Figure 2. Chart showing the relative locomotor activity of an earthworm over several days (1440 minutes per 24 h) in the earthworm activity wheel (1440 minutes per 24 h)(after supra-esophageal ganglionectomy).

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