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Introduction

This focus of this exercise is the phenomenon of segmental regeneration in a selected annelid worm, *Lumbriculus variegatus* (Class Oligochaeta, Order Lumbriculida, Family Lumbriculidae). Specific objectives are to illustrate that (1) segmental regeneration is a developmental problem and process involving major outgrowths of new body segments that must interface with preexisting, older segments, (2) the process follows a developmental plan, termed segmental pattern formation, that is predictable from both qualitative and quantitative standpoints, and (3) regeneration in this particular species is a clear-cut example of morphallactic reorganization.

Lumbriculus variegatus is a freshwater oligochaete known to have remarkable powers of segmental regeneration (Hyman, 1916). These worms should not be confused with *Tubifex* or other tubificid worms (Order Tubificida) whose regenerative abilities are not nearly so marked. The regenerative powers of *Lumbriculus* are undoubtedly related to its normal mode of reproduction, namely asexual fragmentation.

The high rate of survival in body fragments, the rapid rate of segment regeneration, and the clarity in distinguishing new segments from old make *Lumbriculus* an exceptionally good experimental organism for classroom studies of regeneration, pattern formation, and morphallactic reorganization. Over the past few years, the author has introduced numerous high school and college biology teachers from the Iowa region to this species. They now culture it in their schools, routinely use it for student exercises in regeneration, and uniformly report that regeneration in this species is more reliable as well as much easier to manipulate and quantify than in smaller, unsegmented worms such as planaria.

Despite its potential for student laboratory use, *Lumbriculus* is not yet commercially available from biological supply companies. However, instructors can gain easy access to these worms by one of several avenues. First, worms are available as so-called "California blackworms" at tropical fish stores or pet shops, where they are commonly and inexpensively sold in bulk for living fish food. Second, some instructors may prefer to locate natural populations of *Lumbriculus* in the field, since they are widely distributed throughout North America (especially in leaf litter along the shallow margins of marshes and ponds). Third, live specimens may be obtained from others who have established cultures of them. It is easily cultured and large populations may be established within a couple months from small numbers of stock worms.

Methods for establishing and maintaining laboratory cultures of *Lumbriculus* are given in Appendix A. If you have additional questions relating to sources, field collection, laboratory culture, or biology of *Lumbriculus*, please feel free to contact the author (C.D.D.).

The exercise is designed to illustrate principles of pattern formation at phenomenological and organismal levels. Although intended for use in general college biology courses, the exercise may also be appropriate for intermediate-level courses in invertebrate zoology or developmental biology.

Time blocks from several successive weeks will be required. Initial observations and amputations of worms, made during the first week, can easily be completed in a two-hour block. Then, smaller blocks (about 30 minutes/week for several additional weeks) will be required for students to make follow-up observations of regeneration progress in their specimens and to pool class data. If desired, additional class time may be used to study other features of *Lumbriculus* biology, such as (1) patterns of circulation (frequency and direction of dorsal and lateral blood vessel pulsations), (2) feeding behavior and intestinal peristalsis, (3) respiratory posture and rapid escape behavior when worms are immersed in containers with natural sediments (Drewes and Fournier, 1989), and (4) patterns of locomotor behavior. Examples of the latter are forward and rearward peristalsis that occur when worms are placed on moistened paper and lightly stimulated by touching their tail and head ends, respectively. Another type of locomotion is helical swimming that is evoked in response to tail touch when worms are under water and atop a smooth, hard substrate.

Instructors should note that for most aquatic oligochaete species there is little or no previously published research regarding many aspects of their physiology, behavior, ecology and development. Thus, spin-off investigations of anatomical, physiological, or biochemical correlates of regeneration may represent good opportunities for students to make original contributions to understanding developmental or general biology of oligochaetes.

Materials

Live *Lumbriculus variegatus* (at least 3–5 worms/student) [These are sold (in bulk) in pet shops and tropical fish stores as “California blackworms” for live fish food.]

Small aquaria or large finger bowls containing worms

Aquarium pump with tubing and adjustable air flow

Medicine dropper (preferred), or large-bore disposable plastic pipette

Aged, dechlorinated tap water (or non-carbonated spring water)

Single edge razor blades (new)

Filter paper discs or squares

Disposable petri dishes for cutting fragments

Dissecting microscope with illuminator (side lighting preferred)

Numerous small-volume containers for storing isolated fragments

[Possible containers include very small petri dishes, 6-well or 24-well tissue culture dishes with covers, or 1.5 ml or larger capped disposable centrifuge tubes; one student may create 10–20 fragments, each requiring an individual container or well with tight-fitting cover.]

Thin plastic ruler with millimeter markings

Black paper (dark background for viewing segment regeneration)

Large bore pipette (turkey baster type)

Distilled water (no chemical additives)

Brown paper towel

Scissors for cutting paper

Sinking fish food pellets

Notes for the Instructor

Main technical factors that will insure students' success are their: (1) abilities to distinguish differences between anterior and posterior ends of whole worms and worm fragments; (2) avoidance of damage (mechanical, thermal, or chemical) to worms and fragments during handling and observation; (3) prevention of desiccation of fragments during storage and observation; (4) precision in excising and isolating fragments from known body locations; and (5) correct placement and replacement of worms in labeled storage wells. An additional factor that could limit success in counting segments is the quality of dissecting microscopes and light sources.

Examples of regenerated fragments, showing clear-cut demarcations between original segments (pigmented) and new head and tail segments (unpigmented), are shown in Appendix B. These photographic images were obtained using a video microscopy set-up (dissecting microscope and video camera), a VHS videocassette recorder, and an inexpensive black and white television monitor (5 inch diagonal screen). Images were displayed in the freeze-frame (pause) mode and photographed with a Polaroid oscilloscope camera.

Although video documentation is useful, it may lack sufficient resolution for counting segments. The most accurate method of segment counting is by direct observation under high power of a dissecting microscope.

An example of students' actual data for tail regeneration in posterior fragments is shown in Appendix C. It shows that the number of newly regenerated tail segments is directly related to the original length of these fragments. Note that due to normal variation in tail segment regeneration, the trend may only be evident when a wide range of fragment lengths are used. In comparison, the pattern of head segment regeneration is nearly invariant; that is, regeneration of eight new head segments occurs in nearly all cases, regardless of fragment length or origin. This pattern of head regeneration therefore provides the basis for morphallactic reorganization of the original fragment, assuming it originated from mid-body or posterior locations along the body axis.

Occasionally, however, "errors" in this developmental pattern occur. For example, only six or seven new head segments may form instead of eight. Sometimes, a regenerating bud may become branched, forming a two-tailed or two-headed worm (Appendix B). On rare occasions (usually in short anterior fragments), a head may form on both cut faces of a fragment; the resulting aberrant head is termed heteromorphic. Heteromorphic tails are also possible in short posterior fragments. Finally, for no obvious reason, segmental regeneration may fail to occur at an amputation site.

Student Outline

Introduction

Embryonic development is an orderly and stereotyped process during which an organism's cells differentiate and its body gradually acquires adult-like characteristics. If the adult organism is bilaterally symmetrical (as in many invertebrates and all vertebrates), then at some time during embryogenesis a body plan is established along three different body axes: (1) an anterior-posterior axis that establishes head and tail ends, (2) a dorsal-ventral axis that establishes front and back sides, and (3) a left-right axis. During development, the fate of differentiating cells and tissues will vary, depending on their exact position within these axes. Cells closest to the head end, under the influence of chemical factors (called morphogens) and physical constraints, will develop proper, head-like features. Cells in the middle will be similarly influenced to develop features appropriate to

a mid-body position, etc. This complex developmental process of acquiring a characteristic body plan with position-specific features is referred to as pattern formation (Malacinski and Bryant, 1984).

In some invertebrates (such as hydra, planaria, and certain segmented worms) the process of pattern formation may also be played out during the regeneration of body parts. Loss of body parts, such as a head or tail, can occur following accidental damage or predation, or it can occur normally in conjunction with certain types of asexual reproduction, one of these being fragmentation. Fragmentation is a self-produced, mechanical breaking of the organism into two or more pieces, followed by regenerative growth of missing parts in each fragment.

If a worm loses a part of its posterior end by fragmentation, how does the worm “know” whether to regrow a new head or tail at the wound site? How does it “know” how long the replacement part should be? In relatively large and segmented organisms, such as annelid worms, these questions don’t have clear answers because underlying mechanisms of development are not completely understood. However, we can begin to understand these mechanisms if we carefully observe the regeneration process following various surgical manipulations and then try to determine the “rules” and general patterns that govern the process.

When worms regrow missing body parts, one of two general regeneration patterns seems to be followed (Berrill, 1952; Karp and Berrill, 1981). One pattern, seen in the marine polychaete, *Clymenella*, is segment regeneration by compensatory growth; that is, the number of regenerated segments exactly equals the number removed. So, for example, when five (or ten) head segments are amputated, then exactly five (or ten) head segments regenerate in their place. Thus, all newly regenerated segments acquire precisely the same positional and numerical identity as the segments that were removed (Barnes et al., 1993).

An alternative pattern, seen in the marine polychaete, *Sabella*, is that the number of regenerated head segments is a constant. So, regardless of whether 5 or 25 anterior segments are amputated, no more than three new head segments regenerate in their place. As this happens, a developmental reorganization of the segments adjacent to the new head occurs; that is, adjacent segments become transformed anatomically and physiologically to match their new positional identity along the body axes. This reorganization, or respecification, that may occur without cell division, is called morphallaxis (Barnes et al., 1993).

Objectives

In this exercise you will determine which general pattern of segmental regeneration (compensation or morphallaxis) occurs in the freshwater oligochaete, *Lumbriculus variegatus*. To do this, you will study segment regeneration in worm fragments obtained from a known body region and with a known number of original segments. Regenerative growth is then observed at the cut anterior face and/or posterior face of each fragment. One key experiment will be the systematic comparison of head and tail regeneration in amputated fragments that *have the same number of segments, but differ with respect to their original position* within the longitudinal body axis. You will also determine whether short and long fragments from the same body region have differing capacities for head and tail regeneration, and whether there is a minimal size fragment that can survive and support head and tail regeneration.

Procedures*Maintaining and handling worms*

Inspect individual worms to insure that body segments appear healthy, normal, and relatively uniform in size and pigmentation. Single worms always should be transferred to and from containers by drawing them up quickly into a medicine dropper, along with a small amount of water. If a worm becomes attached to the inside of the dropper and cannot be expelled, simply draw additional water into the dropper and gently shake the worm towards the tip so it can be readily expelled. (NOTE: *When transferring worms, avoid using disposable glass transfer pipettes; their sharp edges could damage worms. Never handle worms with forceps or hooks*).

Step 1: Cutting worms into anterior and posterior halves

Begin, by placing a filter paper disc in the bottom of a petri dish or other shallow container. Moisten the paper to saturation with dechlorinated tap water. Using the medicine dropper, transfer a worm to the middle of the moist filter paper. Next, tilt the dish to one side and withdraw excess water, leaving the worm approximately in the middle of the paper. Wait until the worm is approximately straightened and then position the razor blade above it, at the middle of the body. (Note: the blade edge should be held parallel to the dish but perpendicular to the long axis of the worm). Quickly press the blade through the worm and flush against the paper, holding the blade down for a couple seconds. The worm should separate into two halves with little or no bleeding (Figure 2.1).

Carefully note which half contains the head end, and which contains the tail end. To do this, you may need to use a dissecting microscope and note the following: (a) the head end of the anterior half is blunter and more darkly pigmented in comparison to the more slender and paler tail end of the posterior half; and (b) the anterior half is generally more active in terms of searching movements and locomotion than the posterior half.

Now, remove the posterior half from the dish by flushing it off the paper and toward the side of the dish using a few squirts of dechlorinated tap water from the eyedropper. Draw up this half with the eyedropper, transfer it to another container, and save it for later use.

Step 2: Obtaining a long anterior fragment

The anterior half should now be positioned for additional amputations, as shown for worm A in Figure 2.1. The idea is to obtain an anterior fragment that is about 30–40 segments in length from which at least 15 head segments have been removed. Count the number of segments in the desired fragment and in the amputated head. Next, remove the fragment from the dish by flushing it with water and drawing it up into an eyedropper. Then, transfer it to one of the storage containers or wells, and label the container/well as fragment LA (long anterior). In your notebook record the number of segments in fragment LA, and the length of the amputated head. Pieces that remain from trimming may be discarded.

Step 3: Obtaining a long posterior fragment

Transfer the posterior half of the worm back to the cutting surface. Refer again to worm A in Figure 2.1 and make appropriate amputations, as in step 1, that yield a 30 to 40 segment long *posterior* fragment. Count segments in it, transfer it to a labeled container/well, label it fragment LP, and record its number of segments.

