Chapter 2

Heads or Tails? Patterns of Segmental Regeneration in a Freshwater Oligochaete

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Contents

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 Fourtner, 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.

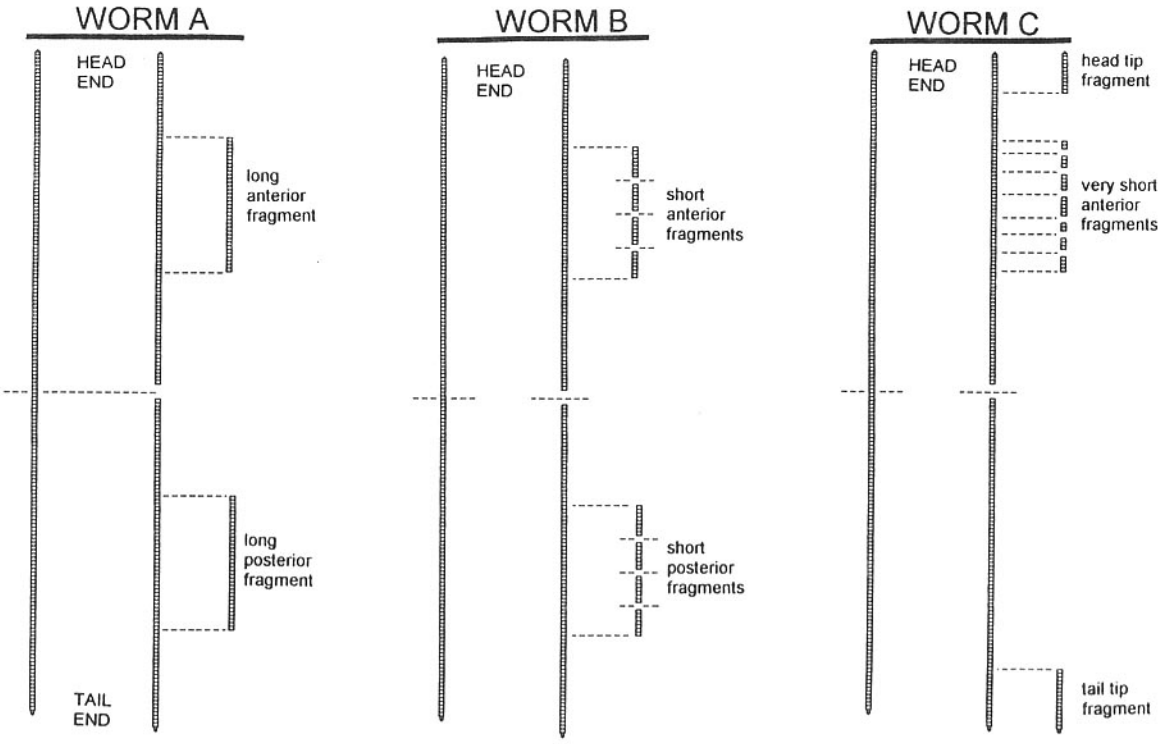

Figure 2.1. Amputation sites for regeneration experiments. Sites for desired cuts are shown by the horizontal dashed lines.

Step 4: Obtaining short anterior and posterior fragments

Obtain a new worm. Place it on the moist paper and cut it into two equal pieces as previously described. Referring to worm B in Figure 2.1, obtain several short anterior fragments that are approximately 5–8 segments in length. Again, count and record the number of segments in each. Transfer them to individual containers/wells labeling them as SA1, SA2, SA3, etc. Now, in the same way, obtain several short posterior fragments labeling them SP1, SP2, SP3, etc.

Step 5: Obtaining other fragments

Using another worm, attempt to isolate very short fragments (perhaps 2–4 segments in length) from anterior, middle, and/or posterior positions. Give each fragment a code name and record numbers of segments for each code name.

Use the same worm to obtain head tip and tail tip fragments, as shown for worm C in Figure 2.1. Code and count segments in these.

Step 6: Preparing worms for storage

After isolating all fragments, add dechlorinated tap water to storage container/wells, so that each is about half full. Then add a tiny piece of organic debris to each; a 1 mm ∞ 2 mm piece of brown paper towel that sinks to the bottom should suffice. The debris provides an additional surface for the regenerating worm to contact and seems to improve survival.

Secure all storage containers with a snug or tight cover; it is essential to prevent spillage and evaporation from containers. Keep containers in the dark at room temperature. *Do not add any food* to containers while regeneration experiments are in progress.

Step 7: Observing regeneration and counting segments

Using a dissecting microscope, make repeated observations of segment regeneration in each identified fragment, starting at weekly or biweekly intervals and continuing for at least three successive weeks. Initial observations of the fragments can often be done with fragments still in their storage containers, thus minimizing handling. Note the appearance and relative length of regenerating head and tail buds. To do this, you must determine which is the anterior and which is the posterior end of each fragment. Even before regeneration occurs this is easily done by noting that blood flow in the dorsal blood vessel always moves from posterior to anterior along the body. Make weekly measures of the actual length (in mm) of each bud and dimensions of new and old segments.

Although some delineation of new segments in head and tail buds may be apparent as early as one week, final counts of new segments (especially in the new tail) are usually difficult until about two weeks after amputation. For precise counting of segments it is often helpful to remove the fragment from its storage container and transfer it to a petri dish containing a piece of wet black paper (pre-soaked in dechlorinated tap water). Carefully adjust lighting to optimize resolution of new segments.

If fragments are too active to count segments, it may be helpful to slightly cool them, thereby reducing body movements. If cooling is attempted, be extremely careful not to allow the worm to directly contact any ice or ice water. *Thermal shock due to excessive cold (i.e., near 0°C) or too rapid cooling may easily kill fragments*. For best results, cool the bottom of the dish containing the moistened black paper and worm, by placing the dish on a wet paper towel that covers a small amount of crushed ice. Then, using a dissecting microscope and optimal lighting for segment definition, count segments.

Step 8: Questions, data reduction, and analysis

a. Describe the week-by-week progress of head regeneration, noting head bud length (in mm), formation of new blood vessels, pigmentation, and size proportions of new and old segments. What differences, if any, were evident between LA, LP, SA, and SP fragments? What is your conclusion about the pattern of regeneration (compensation versus morphallaxis) in *Lumbriculus*? Explain how your results support this conclusion.
b. Describe the week-by-week progress of tail regeneration, noting tail bud length, formation of new blood vessels, pigmentation, and size proportions of new and old segments. Do LA, LP, SA, PA fragments have the same capacity for tail regeneration? Prepare a graph showing the relationship between the final number of new tail segments (vertical coordinate) versus number of segments in original fragment (horizontal coordinate). Pool class data, using separate symbols for each fragment type. Explain how these results relate to the pattern of regeneration.
c. What is the minimal fragment size that can survive and normally regenerate? Explain results.
d. How did patterns of regeneration differ in fragments with one amputated end (i.e., head tip and tail tip fragments) as compared to fragments with both amputated ends ends? How could worms that ended up very short after regeneration (e.g., tail tip regenerates) eventually regain segment numbers and body length to match full-sized worms?

e. Did any fragments exhibit abnormal patterns of regeneration? How could abnormal development arise?
f. What are possible survival advantages in using asexual fragmentation as a means of reproduction in this species? What are possible disadvantages?

Glossary

Morphallaxis —the regeneration of a structure by the respecification of pre-existing cells; cell division need not occur for the development of new structures.

Pattern formation —the mechanisms that generate the spatial organization of a developing organism, or the regeneration of its parts.

Positional information —the concept that cells are able to recognize their relative positions within a coordinate system and respond by regulating the pattern of their development (derived from Malacinski and Bryant, 1984).

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APPENDIX A
Laboratory culture and handling of worms

If worms are purchased from a pet shop, retailers usually keep them in plastic bags, with minimal water volume, in a refrigerator. A handful of worms (at least several hundred worms) may cost only a dollar or two. Before starting a colony, worms should be carefully sorted by placing them in a large shallow pan (a white enamel pan works well) containing dechlorinated tap water. Using an eyedropper, select intact, uniformly pigmented, and active worms for regeneration experiments or for starting a new colony. Discard any worms or fragments that appear damaged, immobile, or unhealthy.

To start a sustainable laboratory colony, place the following in a small aquarium or large finger bowl: (a) aged, dechlorinated tap water to a depth of about 5 cm, (b) thin strips of brown paper towel, to lightly cover the bottom of the aquarium, and (c) about a hundred healthy worms per container. The aquarium should be continuously aerated by very gentle bubbling. Filtering is not advised or necessary.

Add an amount of food that can be completely eaten within a day; look for its disappearance. For a hundred worms this may be approximately one or two small food pellets (sinking fish food) every few days. Do not overfeed. Water lost to evaporation should be replaced by adding distilled water, rather than aged tap water. This prevents build-up of mineral content in the aquarium. It is suggested that at least two or three separate aquarium cultures be established in parallel, in case of accidental contamination and die-off in one.

After a few weeks, the paper towel will begin to disintegrate and new strips may be added periodically. If the aquarium water becomes cloudy, yellow, or extremely smelly, then water may be changed by carefully tipping the container and decanting (or siphoning off) most of the water, while leaving worms and debris in the bottom. Then, add clean, aged, dechlorinated tap water back to the original level. Repeat this procedure, if necessary.

Colonies of worms can be left unfed for extremely long periods, as long as gentle aeration and water level are maintained. During starvation worms will “degrow,” using their own body mass as a food reserve. Regrowth quickly occurs when feeding is resumed.

Following these maintenance and culture procedures, worms will reproduce continuously and survive indefinitely. As the colony becomes larger its oxygen demand increases; thus it is critical that aeration is continuous and uninterrupted, or there may be mass die-off. Also, it is possible that the colony may eventually become too large, in which case surplus worms can be removed and used as live food for other freshwater animals (e.g., fish, crayfish, fiddler crabs, some leeches), or as “starters” for worm colonies in additional aquaria.

To remove a large number of worms at one time from the aquarium, allow worms to collect near a food pellet and quickly withdraw them using a large bore pipette (turkey baster). This mass then can be transferred to a clean dish or finger bowl containing aged tap water.

APPENDIX B

Examples of head and tail regenerates

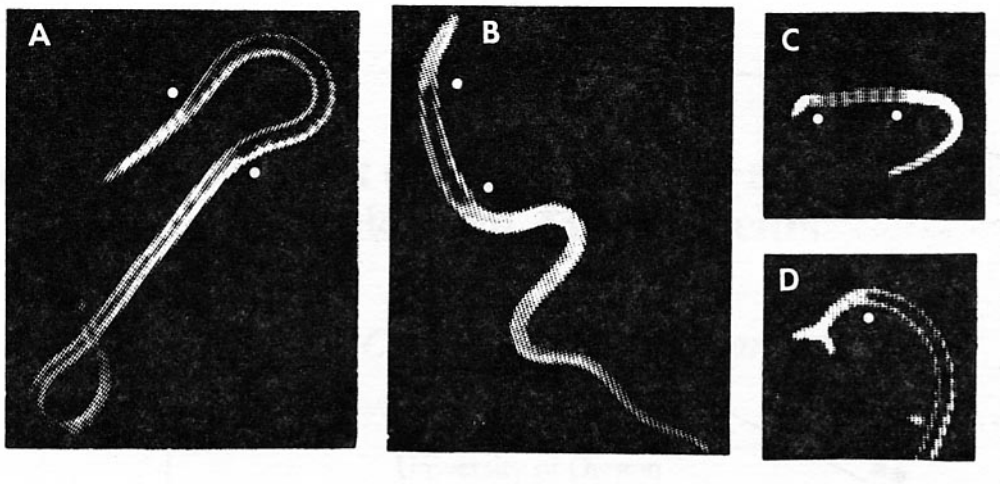

Figure 2.2. (A) Head and tail regeneration after three weeks in a 28-segment-long fragment, originating from the posterior half of a donor worm. Original segments are indicated between the two dots. Eight new head segments were regenerated on the anterior end of the fragment; this is the usual number of regenerated head segments in either anterior or posterior fragments of all lengths. (B) Head and tail regeneration after three weeks in a six-segment-long fragment, originating from the anterior half of a donor worm. Original segments are indicated between the two dots, a distance of about 2.5 mm. (C) Head and tail regeneration after two and a half weeks in a six-segment-long fragment, originating from the posterior half of a donor worm. The six original segments are more darkly pigmented and indicated between the two dots. (D) An abnormal anterior regenerate with two heads. The regenerated anterior end consisted of five common segments and three segments in each of the two head branches. Demarcation between old and new segments is shown at the white dot.

APPENDIX C

Tail segment regeneration in Lumbriculus

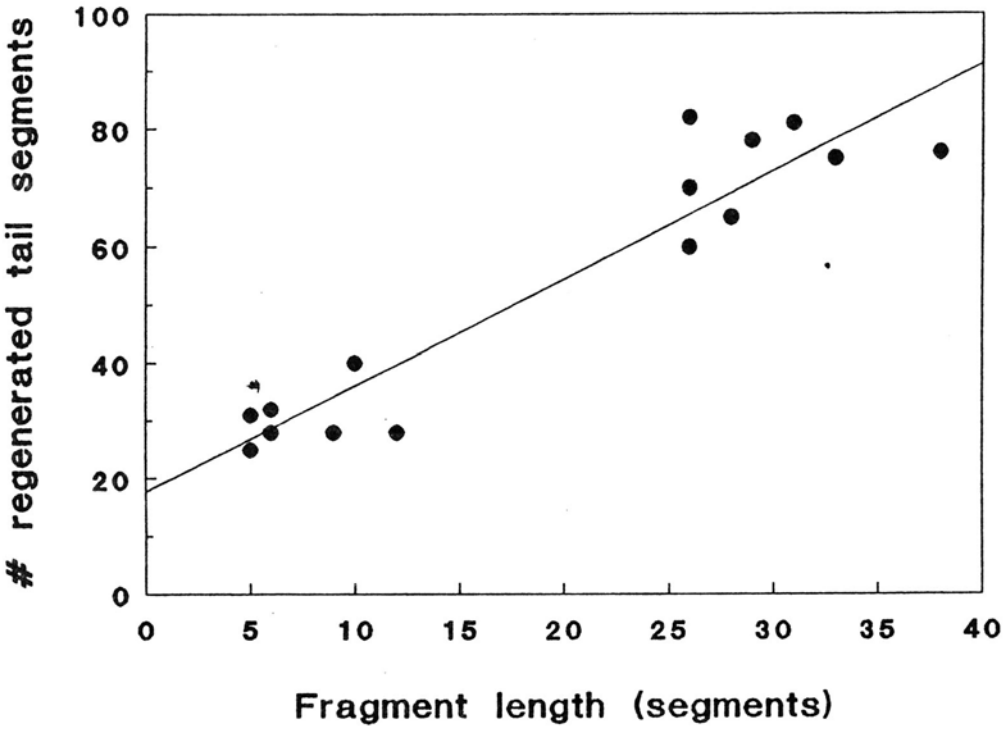

Figure 2.3. Relationship between number of regenerated tail segments and length of fragments. All fragments were derived from posterior halves of donor worms.