RADIOLE REGENERATION AND BRANCHIAL CROWN STRUCTURE OF THE FEATHER DUSTER WORM, *SCHIZOBRANCHIA INSIGNIS*

by

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Dr. Richard Emlet

The annelid feather duster worm, *Schizobranchia insignis*, is an abundant marine invertebrate found subtidally and in the low intertidal of the northeastern Pacific Ocean. The Phylum Annelida is frequently utilized as a model of regeneration due to the pronounced array observed. The main purpose of this study was to examine the radiole regeneration of *S. insignis* and to perform a branchial crown survey of the understudied species. Previous research has examined the regeneration of the entire posterior and anterior region of the worm; however, limited information is available on the regeneration of radioles, the anterior appendages used for feeding and respiration. By cutting radioles of *S. insignis* and observing the patterns of regeneration, I identified twelve distinctive morphological stages (A-L) with detailed descriptions of the external and internal features. The radiole regeneration of *Eudistylia vancouveri*, a species from a closely related genus of *S. insignis*, was also examined and found to have a less complex, non-branching regrowth process. During the radiole regeneration of *S. insignis*, the initiation of bifurcations is prioritized over the increase of internode length. The branchial crown of *S. insignis* exhibits a positive, non-linear power relationship between the number of radioles and drained body weight. Radioles of *S. insignis* with more bifurcations are generally longer and located more dorsally in the branchial crown. Lastly, the length of pinnules decreases as they travel distally on the radiole, unlike in other feather duster worm species. As the only sabellid with branched radioles, my study of *S. insignis* provides a unique look into appendage regrowth and branchial crown features.

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Introduction

Regeneration is the restoration of an organism's body part that is lost or damaged (Bely and Nyberg 2009). Regeneration occurs in a wide variety of marine and terrestrial organisms at different developmental stages throughout embryonic development and in full-grown adults (Bely 2010). Based on phylogenetic distribution, regeneration likely originated in early animals, potentially coinciding with origins of multicellularity. The presence of regenerative capabilities has been influenced by several evolutionary and ecological factors; however, many animal lineages have lost the ability to regenerate despite this trait's potential benefits (Bely and Nyberg 2009). The loss of an organism's appendage can result in detrimental effects, including decreased nutrient reserve due to a limited feeding surface area, reduced locomotory performance, and limited reproductive output and growth (Lawrence 2010). For regeneration to occur, the fitness-related cost caused by the harmful effect of appendage loss must be greater than the relative cost of the regenerative process. In addition, to restore the functionality of the appendage, the fitness-related cost of the appendage cannot be so disadvantageous that the animal dies (Bely and Nyberg 2009). While regeneration is exhibited in numerous organisms, the Phylum Annelida demonstrates a pronounced array of regenerative abilities, which makes this group of organisms an especially fertile ground for comparative and mechanistic studies of regeneration (Bely 2010).

A majority of annelid regeneration studies have focused on entire posterior or anterior regeneration patterns. Most species of the family sabellidae demonstrate the ability to perform posterior and anterior regeneration from any segment; a few even exhibit the phenomenal ability to regenerate their entire body from a small, mid-body segment. Studies on sabellid regeneration indicate anterior regeneration challenges lead to higher mortality. The regeneration of anterior appendages, required for feeding and respiration, result from an intricate reconstruction of the mouth and the entire branchial crown (Bely 2010; Murray et al. 2013).

Regeneration is commonly used as a tool for scientists to study features of development, such as blastema formation, cell differentiation, and wound healing. According to the web of science archives, within the last 20 years, the number of papers published yearly on regeneration has increased from the 100s to 10,000s (Maginnis 2006). Nevertheless, even with increased popularity, the field still lacks information. For instance, when researchers discover an anomaly in regeneration, their findings are often not published because the lack of regeneration is viewed as an insufficient result. The lack of documentation will persist in the field, unless future studies are dedicated to recording all regeneration anomalies (Bely 2010).

Most regenerative studies have been limited to investigating the regeneration of entire body segments rather than the regrowth of a single appendage. For example, one study performed by Murray et al. (2013) focused on the full body regeneration of sabellids in hope of reducing worm harvesting for the aquarium trade. The authors briefly mentioned that the radiole regeneration of *Sabella pavonina* and *Sabellastarte* sp. was noticed due the addition of equidistantly spaced eyespots (Murray et al. 2013). Other studies have captured photos of radiole regeneration during their experimentation; however, their conclusions are limited to the statement that radioles became pigmented and grow in length until developing into a full branchial crown (Licciano et al. 2012;

Licciano et al. 2015). Few studies offer information on radiole regeneration which is more likely to occur due to minor radiole damage on a day-to-day basis.

Structural Background of Feather Duster Worms

Feather duster worms (Annelida: Polychaeta) are sedentary organisms found in temperate and tropical marine ecosystems. These organisms, in the families Sabellidae and Serpulidae, are characterized by their elaborate feather-like anterior appendages used for feeding and respiration. Feather duster worms in the family Sabellidae are surrounded by a non-calcareous tube that attaches to hard substrata in the worm’s environment (Rouse and Fitzhugh 2001; Pechenik 2015). The non-calcareous tube is composed of mucus secreted from a specialized gland at the organism’s anterior end; sometimes surrounding sediment is incorporated into the tube. This non-calcareous tube provides protection from predators, from wave action, and from exposure to the low intertidal for the worm’s body and their feeding and respiratory structures. The tube also grows sporadically throughout the worm’s life (Giangrande et al. 2013; Murray et al. 2013; Merz 2015). To keep their tube environment waste-free, feather duster worms have a ciliated fecal groove that runs ventrally in the middle of the abdomen, then shifts to the mid-dorsal region in the thorax (Fitzhugh 1989; Rouse and Fitzhugh 2001).

While the feather duster worm’s thorax and abdominal region remain concealed within the tube, the branchial crown extends out as a feeding and respiratory structure for the worm (Figure 1; Fitzhugh 1989; Rouse and Pleijel 2001; Rouse and Fitzhugh 2001). When startled by a physical disturbance or shadows, feather duster worms utilize specialized chaete (uncini) and muscle contractions to quickly retract their branchial crown into their tube (Rouse and Fitzhugh 2001; Kicklighter and Hay 2006; Merz 2015).

A feather duster worm's branchial crown is composed of feather-like radioles that extend from branchial lobes to capture particles in the water column (Figure 1). Depending on the species and body size, a feather duster worm can have between two and several hundred radioles (Rouse and Fitzhugh 2001). Radioles have two descending rows of pinnules located on the oral side of a radiole. Pinnules are lined with laterofrontal cilia which generate a feeding current moving water from below the branchial crown and through the radioles and exiting above the branchial crown (Figure 2; Person and Mathew 1967; Strathmann et al. 1984). Feather duster worms are commonly found in dilute, nutrient-poor waters, so they must rely on an effective filtering mechanism to process large quantities of water (Tamaru et al. 2011). Captured food particles are transported to the base of the branchial crown to the mouth opening, located in the center of the crown, through a ciliated food groove located on the oral side of the radiole (Figure 3B). In addition to food capture, pinnules add surface area used in respiration (Rouse and Pleijel 2001). Radioles are tentacular extensions arising from the borders of the bilateral branchial lobes that hold the proximal-most position on the crown (Figure 1; Harrison 1991; Capa et al. 2011). Each of the two branchial lobes is a distinct semicircular tissue mass attached to the anterior end of the worm. The interior edges of each lobe form the dorsal and ventral lips (Figure 3; Fitzhugh 1989). The dorsal lip is a paired, ciliated projection that sorts particles collected by the branchial crown and transfers them to the mouth. Different shaped dorsal lips result from various feeding preferences, environments, and evolutionary histories (Fitzhugh 1989; Capa et al. 2011).

Another characteristic unique to sabellid worms is they have a central, cartilaginous, support structure continuous throughout the branchial lobes known as the branchial skeleton (Randel and Bick 2012). Extending from the branchial skeleton into each radiole is the radiolar skeleton composed of large, vacuolated axial cells that provide support and flexibility to the radioles (Person and Mathew 1967; Fitzhugh 1989; Cole and Hall 2004; Capa et al. 2011). The extension of the skeleton into the pinnules is referred to as the pinnular skeleton, which usually consists of a single row of cells stacked on top of one another (Figure 4A; Orrhage 1980; Cole and Hall 2004). A skeletal sheath of collagen fibers surrounds the radiolar skeleton and provides support. In contrast, there is no evidence for a skeletal sheath surrounding the pinnular skeleton (Fitzhugh 1989). Each radiole contains a blind blood vessel that branches off from the brachial blood vessel (Figure 4B). Each pinnules also contains a centrally-located blood vessel that branches from the blood vessel of the radiole. Blood in feather duster worm is green due to the presence of chlorocruorin (Orrhage 1980; Rouse and Fitzhugh 2001; Capa et al. 2011).

Background of *Schizobranchia insignis* and related genera

Schizobranchia insignis is a sabellid identified by its dichotomously branched, non-spiraled radioles. Found in intertidal and rocky habitats between Alaska and Northern California, this species has various branchial crown colorations of pinkish-red, black-white, or tan (Bush 1905; Hartman 1969; Fitzhugh 1989). *Eudistylia vancouveri* is a sabellid whose radioles exhibit a spiral configuration. The branchial crown is composed of radially striped, dark red and green radioles, some of which are white-tipped. *E. vancouveri* occurs in the middle to low intertidal on rocky beaches, sandy mudflats, or man-made structures from Alaska to central California. Though infrequent, in some large individuals, the radioles have been known to dichotomously branch (Kinberg 1866; Hartman 1969; Fitzhugh 1989; Giangrande 1991). *E. vancouveri* can also be found in prodigious clusters called hummocks where their tubes build up on top of each other and form bush-like structures (Rudy et al. 2013). In sabellids, all branchial crowns are utilized as feeding structures; however, the importance of the crown for respiration varies between species. The branchial crown of *S. insignis*, for instance, accounts for 60% of the organism's total oxygen uptake (Dales 1961). *E. vancouveri* branchial crown, on the other hand, is responsible for 75% of the worm's oxygen uptake (Giangrande 1991). When decapitated, both *S. insignis* and *E. vancouveri* can respire using tube irrigation, signifying that they can persist without their branchial crown.

S. insignis and *E. vancouveri* are categorized as similar genera for several reasons. First, both have unpaired, compound radiolar eyes found on the proximal half of their radioles. Both species have dorsal, marginal branchial base flanges, which are thin lateral margins that extend from the outer corners of the radioles (Fitzhugh 1989). The two species are also both gonochoristic, broadcast spawners that release gametes from the abdomen (Fitzhugh 1989; Rouse and Fitzhugh 1994; Beesley et al. 2000). *S. insignis* and *E. vancouveri* can also extend their tube when growing but are unable to grow a new tube if removed from their original tube (Merz 2015).

In regards to regenerative capabilities, *S. insignis*, according to Dales (1961) can regenerate their anterior end. No further research on the regenerative patterns of the species has been performed. There is also little information on the regeneration of *E. vancouveri*. After copper-induced tissue injury, *E. vancouveri* can regenerate their branchial crown, but no specific details of regeneration were given (Young and Roesijadi 1983). Annelids rely on their appendages for feeding, respiration, and movement; therefore, understanding the regenerative properties of *S. insignis* and *E. vancouveri* is significant. Some research, such as Murray et al. (2013), has investigated the initial moments of radiole development, but the analysis of appendages regeneration, especially radioles, still requires thorough examination.

Experimental Questions

The purpose of the current study was to examine the radiole regeneration and branchial crown configuration demonstrated by the feather duster worm, *S. insignis*. I performed qualitative and quantitative analyses of the organism's external radiole regeneration to answer the following: How does a radiole initially begin to regenerate after damage? Is there a noticeable difference in regeneration between a proximally cut radiole and a medially cut radiole? Do pinnules develop as the radiole regenerates? How does the radiole regeneration of the closely related species, *E. vancouveri*, compare to *S. insignis*? After regeneration, do radioles recover to the same branching patterns and length as exhibited before damage? During regeneration, does a radiole begin by increasing in length or by branching first?

I also investigated the presence of internal structures during the radiole regeneration of *S. insignis* to determine when blood vessel and support structures appear during regrowth. Lastly, in conclusion to the regenerative study, I performed a survey of the branchial crown of the *S. insignis*. I examined the relationship between feather duster worm size (drained weight) and number of radioles. In addition, I investigated the relationship between branch length, branch internode number, and pinnule length. To understand more about the *S. insignis* branching pattern, I compared the radiole number and size relationship between two separate populations: one occurred in the low intertidal, exposed to the ocean swell and the other was a subtidal group found attached to the floating docks in the boat basin.

By understanding the background of radiole regeneration, scientists can add to the overall field of regeneration and utilize radiole deterioration to quantify the effect of climate change on the worm. Feather duster worms experience radiole damage due to predation from ominous crabs and fish species (Kicklighter and Hay 2006). In addition, the degradation of the branchial crown occurs due to stress caused by fluctuating water temperatures and salinity levels (Murray et al. 2012; Giangrande et al. 2013). Since feather duster worms, such as *S. insignis* and *E. vancouveri*, rely on their radioles for essential feeding and respiration behavior, it is important to understand more about the regeneration required to heal these damaged structures (Dales 1961). Regenerative abilities of these worms not only provide an immediate survival advantage to the individual but also can influence the entire population (Licciano et al. 2015). Understanding the patterns of a branchial crown is significant because such characteristics can help distinguish species and developmental stages. The influence of feather duster worm size on morphology has also barely been addressed (Lindsay 2010).

Materials and Methods

Location of Studies

Schizobranchia insignis and *Eudistylia vancouveri* were collected from the Charleston Boat Basin (43°20'49"N, 124°19'29"W) in Charleston, OR from June 2015 through July 2016. Feather duster worms are gregarious and were commonly found in fouling communities along the sides of docks in the outer boat basin (Figure 5). Smaller *S. insignis* were occasionally collected from the underside of previously established settlement plates or inflatable dock fenders in the inner boat basin (Figure 5). For the branchial crown survey, *S. insignis* were also collected from an exposed site near the OIMB Boathouse (43°21'02"N 124°19'56"W). Unlike the fouling community of the Charleston Boat Basin, the tubes of these worms emerged from the holes left behind by burrowing clams. The radiole regeneration study and branchial crown survey were performed at the Oregon Institute of Marine Biology (OIMB) in Charleston, OR. Some of the branchial crown survey was conducted in Dr. Kelly Sutherland's lab at the University of Oregon in Eugene, OR.

Animal Collection and Maintenance

Feather duster worms with intact tubes were detached from the dock by hand or with a metal scraper. During each study, 10-20 worms were placed in an aerated 10-gallon tank with constant running raw seawater, as per suggestions in Dales (1961). *S. insignis* and *E. vancouveri* were held upright in test-tube trays weighed down by attaching a stone plate on the underside with zip ties (Figure 6). During all the studies, *S. insignis* and *E. vancouveri* were provided about 2 liters of *Isochrysis galbana* every

3-4 days, as suggested by Tamaru et al. (2011) and Giangrande et al. (2013). To distinguish worms from each other, floss and colored threads were tied around the organism's non-calcareous tube. Feather duster worms were given 2-3 days to acclimate to the laboratory environment prior to experimentation. To evaluate whether cutting radioles caused mortality, I maintained uncut worms as controls in the same aquarium and tube rack (Bely 2010).

Observational Study of Radiole Regeneration

External Analysis

In July 2015, I cut a single radiole off twelve unanesthetized *S. insignis* with angled, sharp scissors. To perform the cut and view the later damage, I placed the worm in a petri dish under an Olympus SZH10 dissecting microscope. All cut radioles were located near the organism's dorsal lip (Figure 3B). I made proximal, medial, and distal radiole cuts, resulting in four replicates of each cut type. However, I found it impossible to follow the regeneration of distal cuts because uncut radioles in the branchial crown obscured the damaged radiole. For twelve consecutive days, I examined the feather duster worms' regeneration, took detailed observations of the wound, and captured photographs with a Point Grey grasshopper express GX-FW-28S5C camera and Astro IDCC Version 4.09.03 computer software. After twelve days, I checked on the *S. insignis*'s regenerating radiole every 2-3 days until reaching twenty-one days' post-damage. To observe the cut radioles, *S. insignis* needed to be removed from their organic tubes during every observation. I accomplished this by sending water through their tube with a dropper, pushing them out with a probe, or using my hands to fold their tube and slide the worm upwards (Tamaru et al. 2011). If removed entirely from their tube, the survivorship of *S. insignis* dramatically decreased.

In August 2015, I cut a single radiole off eight *S. insignis* following the previously established cutting procedure to provide more replicates for the study on short-term regeneration. I made proximal and medial cuts, and I observed regeneration for twelve consecutive days.

In November 2015, I cut a single radiole by the dorsal lip off twelve *S. insignis*. I returned the worms to the laboratory environment for four weeks following this procedure, and I heavily reduced my interaction with the worms to determine whether stress from daily observation affects the survival rate. At the end of the four weeks, I recorded observations and captured photographs for all worms on the same day utilizing the same methods as the short-term study described above. Upon completing the study on short-term and long-term radiole regrowth, I utilized photographs and observations to create an outline of twelve stages (A-L) that a single radiole undergoes during regeneration. If a worm began to shed radioles during the study, they were removed from the trial. However, if a small piece of the posterior end was damaged or blood loss was exhibited, I kept the *S. insignis* for later examination and removed once detrimental damage was confirmed.

In July 2016, I cut a single radiole off each of twelve *E. vancouveri* following the same procedure previously performed for *S. insignis*. I made proximal cuts and observed regeneration for four consecutive days, then I checked the worms every 3-5 days until the twenty-second day after the initial damage. Throughout the process, I recorded detailed observations and captured photographs of the regeneration. I compared the regeneration for *E. vancouveri* to the stages I established for the regeneration of *S. insignis*.

Internal Analysis

To analyze the regeneration of internal radiole structures, I cleared six radioles at various stages of regeneration (F, H-K) in December 2015. Clearing the samples involved soaking each radiole in a series of ethanol concentrations (30%, 50%, 70%, 95%, 100%), increasing the concentration in 10-minute increments. Subsequently, I soaked radioles of *S. insignis* in a 1:1 solution of benzyl alcohol and benzyl benzoate for three minutes to clear the tissue and make internal structures visible under the compound microscope. While examining the radioles' internal structures under a Zeiss Universal compound microscope, I took qualitative notes and photographs to document the presence of the radiolar skeleton and blood vessels. I used cross-polarized light to examine birefringence, which was caused by the presence of collagen fibrils, a component of the skeletal sheath. I correlated the emergence of certain internal features during regeneration with the twelve external regenerative stages (A-L) described.

Quantitative Study of Radiole Regeneration

To determine whether radioles of *S. insignis* returned to the same bifurcation number and length after regeneration, I cut a single radiole near the dorsal lip of ten *S. insignis* in June 2016. Before cutting a radiole, I recorded its location, counted the number of branches, and measured its length. Next, I returned the worms to the laboratory environment for five weeks. At the end of the designated period, I captured photographs of the regenerated radiole, measured overall length with ImageJ, and counted the number of bifurcations.

I cut a single radiole off eight *S. insignis* in June 2016 to investigate branch regrowth during regeneration. I placed the damaged worms in the laboratory for three weeks. Every 3-5 days, I captured photographs of the regenerating radioles with a Point Grey grasshopper camera and Astro IDCC computer software. Then, I used ImageJ software to measure the length of each internode (the length between each bifurcation, Figure 7). In July 2016, I cut 3-5 sequential radioles near the dorsal lip of six *S. insignis.* I followed the same procedure as the previous study to measure the length of each internode. I performed a single cut and multiple cut (3-5 radioles) study to determine whether the damage of numerous radioles on a single worm resulted in different internode lengths of *S. insignis*.

Survey of the Branchial Crown

For a portion of the branchial crown analysis, I collected ten *S. insignis* at the boat basin in December 2015. I measured and recorded the organism's body length and the maximum diameter of the animal without their tube (Figure 1). In addition, I allowed the organism to drain water for 5-6 minutes before taking weight measurements. I placed the organism in M (50% of 7.5% $MgCl_2$, 50% of seawater) for four hours to relax the feather duster worms prior to cutting. I carefully removed the feather duster worm's anterior end by cutting below the branchial lobes with a razor blade. I placed the anterior end of the worm into 30% ethanol for one hour before replacing the solution with 70% ethanol for 30 minutes. Individual branchial crowns were moved into small, labeled container filled with 70% ethanol and saved, for future analysis.

While at the Sutherland laboratory in February and March 2016, I counted the number of radioles, the number of branches per ten radioles, and measured the length of each internode for ten radioles on each *S. insignis* (Figure 7). Total radiole length is the distance from the base to the tip of a radiole. Photographs were captured with a Canon EOS 5D Mark II under a Nikon SMZ1000 dissecting microscope and measurements were taken with ImageJ. During this analysis, I avoided damaged radioles. At OIMB, the branchial crown survey of *S. insignis* for the boat basin continued in June 2016 with the analysis of twelve additional worms. I recorded the drained weight of the organism and captured photographs for later ImageJ analysis. In August 2016, to investigate the relationship between pinnule length, drained weight, and location on the radiole, I examined pinnules on four radioles of each of seven previously photographed worms.

In July 2016, I completed a branchial crown survey of *S. insignis* found at an exposed site near the OIMB Boathouse. Since the worms were buried deep in holes, I carefully removed twenty *S. insignis* of various sizes from the substratum with a hammer and crowbar. Subsequently, I measured their drained weight, captured photographs, and followed the same survey procedure used on the branchial crowns of *S. insignis* found in the boat basin. In August 2016, I added blue food coloring to room temperature seawater and pipetted it slowly into a 10-gallon tank with one feather duster worm to observe the filtering pattern of *S. insignis* branchial crowns.

Data Analysis

All quantitative data and branchial crown survey information were organized and compiled with Microsoft Excel 2013. Radiole and pinnule length measurements were all determined with ImageJ 1.60 software. All graphs were produced with SigmaPlot 12.5 and tables were generated with Microsoft Word 2013. All graphs used standard error and are represented with double-ended bars. Statistical analysis to determine branchial pattern conclusions were accomplished with R-Studio Version 0.99.467 and SigmaPlot 12.5. A non-linear, power regression was used to quantify the relationship between the number of radioles and the drained weight of *S. insignis*. A 1-way ANCOVA tested whether the number of radioles was significantly influenced by sample site. Both radiole number and drained weight data were transformed using square roots to meet the assumptions of normality and equal variance.

Results

Observational Study of Radiole Regeneration

Based on photographs and observations, I recognized twelve stages (A-L) that a single radiole of *Schizobranchia insignis* passes through during regeneration. Since both proximal and medial cuts were made, for consistency, branches that result from the first regenerative bifurcation are called primary branches. Similarly, the second regenerative bifurcation leads to secondary branches, the third bifurcation results in the tertiary branches, and so on. Radiole regeneration begins within a day of the initial damage. Immediately after being cut, a small amount of green blood is visibly released and a rough, white cartilaginous piece of tissue is visible at the radiole wound site (Figure 8A). In less the 24 hours, the wound is covered by a flat, lightly pigmented epithelium that hides the previously visible cartilage (Figure 8B). Between 1-2 days after damage, a rounded surface with either smooth or rough edges forms from the epithelium (Figure 8C). As the radiole progresses through regeneration, a small, raised mound appears. Thin and slightly transparent, the small, raised extension signifies the next step of regeneration occurring 3-4 days after initial damage (Figure 8D).

Around 5-6 days after injury, the regenerating radiole begins the first bifurcation. The tips of newly developing primary branches are distinguishable, because of a central furrow, but are attached together with a thin layer of tissue and are not free moving. The regenerated portion is lightly pigmented and has a smaller diameter than the undamaged region; the branch tips are smooth and rounded (Figure 8E). As the primary branches continue regeneration in day 6-7, the newly formed branches lengthen and begin to move independently from one another. The tips of the first bifurcation are rounded and pinnule development has begun as small, paired projections rising from the bifurcation to the middle of primary branches (Figure 8F). At 8-10 days, the initiation of the second bifurcation begins in a similar manner as the first. Secondary branches with smooth, rounded tips become visually distinguishable, separated by a furrow (Figure 8G).

The regeneration of the secondary branches progresses with the tissue separation and free movement of the branches about 11-15 days after the initial radiole cut. Pinnule growth is apparent in the form of two rows of small, translucent bumps located on the oral side of the radiole, halfway up the secondary branch. The secondary branches have either smooth, rounded tips if they will bifurcate again or pointed tips if they will not continue bifurcation (Figure 8H). With the initiation of the third bifurcation between days 15-18, the rounded tipped tertiary branches are visually distinguishable, but not separated or moving independently of one another (Figure 8I). Around 18 days after regeneration initiated, the tertiary branches are now distinguishable and no longer connected by tissue. Pinnule growth is apparent until the lower portion of the tertiary branch. On the previously regenerated primary and secondary branches, the length of the pinnules has continued to grow but not yet reached their full potential size (~1.20 mm). The tertiary branches end in both smooth, rounded tips or pointed tips depending on whether more bifurcation will occur (Figure 8J). Like the initiation of the previous bifurcation, the fourth begins with the formation of two branch tips separated by a medial furrow around 20 days' post-damage (Figure 8K). The completion of the fourth bifurcation with the separate, free moving quaternary branches with smooth tips marks the latest observed moment of radiole regeneration in the current study. Pinnule development is apparent below the fourth bifurcation with paired pinnules increasing in length from the youngest part of the new radiole towards the site of the cut (Figure 8L).

Although pinnule addition and growth in length is continuous throughout regeneration, their appearance was not used to characterize specific regeneration stages because I could not reliably see pinnules or measure the length of radioles during analysis. No definitive ocelli were present on the newly formed radioles after 20 days of regeneration. Throughout radiole regeneration, radiole diameter and pigmentation varied. By the completion of the third bifurcation, the diameter of the regenerating portion had begun to increase on the primary and secondary branches making the location of initial radiole damage difficult to distinguish (Figure 8J). Pigmentation, like diameter, increased over time. In later stages (J-L), while the newly formed tertiary and quaternary branches are lighter in color, the primary and secondary branches have become a darker, pink pigmentation (Figure 8).

The sixteen regenerating radioles of *S. insignis* examined in the short-term study do not follow a temporally fixed orderly sequence. The timing differed among worms because the damaged radioles of different worms advanced through the stages I established on different days (Table 1). In the long-term study, after 20 days, the regenerating radioles were not consistently at the same stage, though most had accomplished their fourth bifurcation. Several even appear to have completed bifurcating, signified by the pointed tips on four of the twelve worms (Table 2). Among the twenty-eight feather duster worms observed regenerating, the short-term study Trial

1 (n = 8) had a 50% mortality rate, while Trial 2 (n = 8) had a 13% mortality rate caused by the death of one *S. insignis* at the study's end. During the study of long-term regeneration (n = 12), no death occurred (Table 1; Table 2). Two of the four control worms in Trial 1 died by the end of the study (day 21). In Trial 2, one of the four control worms had shed the branchial crown and detached a portion of its posterior end by the study's end. The control worms in the short-term study were handled in the same manner as the experimental worms. Since mortality and branchial crown shedding resulted, radiole damage is not the only factor that contributes to mortality. Unlike in the short-term study, none of the control worms in the long-term study died, meaning that radiole mortality likely decreased due to reduced interaction.

Markers for internal regeneration lagged behind those for external regeneration. From Stage A-F, no markers of internal regeneration were seen. In intact radioles, a blood vessel is present when the radiolar skeleton is apparent and a birefringent skeletal sheath surrounds the radiolar skeleton. In Stage F (Figure 9A), the radiolar skeletons' vacuole-like cells are distinguishable below the cut. In Stage A-F, the skeletal sheath, radiolar skeleton, and blood vessel are all found only in the undamaged region, as shown schematically in Figure 10A. About 11-15 days after injury, a birefringent skeletal sheath, visible internally, extends from below the cut to the middle of the primary branch, created as a result of the first bifurcation (Figure 9B). Above the cut, but below the first bifurcation, the development of a blood vessel and radiolar skeleton are apparent (shown schematically in Figure 10B). As regeneration continues into days 15-18, the collagenous skeletal sheath extends past the second bifurcation. A green tinted vessel, signifying the presence of blood, and the radiolar skeleton remains visible below the first bifurcation (Figure 9C, shown schematically in Figure 10C). By days 18-20, the skeletal sheath lengths into the secondary branch. Blood vessels are extended into the primary branch and are observed bordering the clustered cells of the radiolar skeleton (Figure 9D, Figure 9E, Figure 10D).

External radiole regeneration in *Eudistylia vancouveri* demonstrates a similar pattern to *S. insignis*. After initial damage, at the open wound, a rough cartilaginous piece is visible (Figure 11A). One days later, the wound is covered by a rounded, smooth epithelium (Figure 11B). Subsequently, between days 2-7, the radiole develops a dome-like extension, which is smaller in diameter and less pigmented than the non-damaged region (Figure 11C). By day 12, the regenerating radiole has elongated and has a slightly curved, pointed tip. The regenerated portion of the radiole is still thinner and lighter in color than the undamaged section. Paired, pinnule growth is apparent on much of the radiole (Figure 11D). Twenty days post initial damage, the elongated regenerated radiole becomes longer, thicker, and a radially striped pattern of red and green becomes visible along the radiole. Pinnules on the lower portion of the regenerated segment have almost reached full length at this developmental stage (Figure 11E). No ocelli development was apparent during the examination period. Among the twelve observed *E. vancouveri*, the mortality rate was 25% with most deaths occurring 16-20 days into regeneration. None of the control worms died during the study.

Quantitative Study of Radiole Regeneration

To determine whether a damaged radiole would regenerate the same number of bifurcations, I examined the number of branches and the length of the radiole before cutting and after radiole regeneration. During the period of study, the worms did not finish regeneration; however, the radiole of one worm increased its overall bifurcation number by one. Cut radioles on three of the six surviving worms also reached their original number of bifurcations but had not recovered to their full previous length by the end of the experiment (Table 3).

During the single cut and multiple (3-5) cut studies, radiole regrowth trends indicated that radioles bifurcate before undergoing full length increase (Figure 12; Figure 13). This trend is supported by the fact that the second internode (IN2) of all worms did not grow more than ~0.5 mm or reach its full potential length of 2.34 ±0.661 mm (n = 150) before the initiation of the next bifurcation. In eight out of the ten *S. insignis*, proximally-located internodes added earlier in the study period had longer end lengths compared to internodes added during later bifurcations. Evidence for this trend is seen by the fact that IN1 commonly exhibited the longest total length at the end of the study (Figure 12; Figure 13). Another noticeable, quantitative regrowth trend was that when IN1 was cut above ~0.8 mm, the regeneration of IN1 was reduced or non-existent, which may indicate that radioles can recognize internode length. In both studies, if IN1 had little regrowth during the allotted study time, the bifurcation resulting in IN4 and IN5 occurred earlier and the internodes were visible for longer durations of the study (Figure 12B/C, Figure 13A/D/F).

Survey of the Branchial Crown

For both populations of *S. insignis,* a non-linear, power regression best fit the data for the relationship between the number of radioles and the worms drained weight (Figure 14). For worms from the boat basin and those from the exposed site, respectively, 50.5% and 36%, of the variability in the feather duster worm's radiole number are explained by the worm's drained weight. Feather duster worms collected at the exposed site appeared to have more radioles when they are smaller compared to those from the boat basin (Figure 14). The relationship between radiole number and drained weight was not statistically different between the two samples sites (1-way ANCOVA test: $F_{1,41} = 0.423$ $p > 0.05$).

I examined the branching number pattern of *S. insignis* found at both sample sites. The pattern demonstrates that dorsally located radioles have more branches, whereas ventrally located radioles have fewer branches (Figure 15). I also noted whether the worms in the survey had any regenerating radioles. In the boat basin population, 15 of the 20 surveyed worms had at least 1 radiole that exhibited regrowth; more specifically, a mean of 5.27 regenerating radioles ± 1.05 SE from the mean 44.73 total radiole number ± 1.75 SE (n = 15). In the exposed site population, all of the *S. insignis* examined had radioles undergoing regeneration, a mean of 20.52 regenerating radioles ± 7.41 SE from the mean 43.10 total radiole number ± 6.17 SE (n = 21).

When investigating the branchial crown, I observed radioles with an average length of 11.84 mm (± 2.88 SD, n = 150) and with 3 to 7 bifurcations. The average length of internodes decreased as their position was more distal along the radiole. Total average length of the radioles varied with worm size (drained weight) and the number of branches; however, it was clear that the number of bifurcations and total radiole length are positively and linearly related (Figure 16). On larger worms (> 1.0g), radioles with three or four bifurcations tend to be longer in length than radioles with the same number of bifurcations on smaller worms (< 1.0g) (Figure 17A/B). On the other hand, if a radiole had five bifurcations, the size (drained weight) of the worm did not significantly affect the length of the radiole (Figure 17C). The average pinnule length for *S. insignis* was 0.939 mm (±0.226 SD, n = 119), but pinnule length is related to location on the radiole. More distal internodes have short pinnules (Figure 18).

Discussion

Observational Study of Radiole Regeneration

The radioles of a feather duster worm are essential for long-term survival because they are the primary structures used for feeding and respiration. Even with their significance as anterior appendages, radiole regeneration is poorly documented. Murray et al. (2013) recorded the rudimentary formation of radioles, pinnule development, and the appearance of eyespots during an investigation into the cephalic regeneration of feather duster worms; however, limited attention was paid to the radioles themselves because the study focused on full regeneration of the anterior end.

In the current study, I qualitatively analyzed the external and internal features of radiole regeneration of the feather duster worm, *Schizobranchia insignis*. I categorized regeneration into a set of stages based on appearance of external features including bifurcations. *S. insignis* is recognized as the only sabellid with dichotomously branching radioles making their regeneration unique (Fitzhugh 1989). The external features of the radiole regeneration of the closely related sabellid, *Eudistylia vancouveri*, were also observed. Both species were previously recognized as being capable of anterior regeneration, but neither has been examined for radiole regeneration specifically (Dales 1961; Young and Roesijadi 1983).

Upon being damaged, over the course of two days, *S. insignis* began healing by covering up their open wound with a lightly pigmented, flat epithelium (Figure 8A/B). The bifurcation process of the primary branches involves the development of two attached, dome-like extensions that eventually separate and begin moving independently. All subsequent bifurcations follow a similar pattern with slight internode growth before new branches develop (Figure 8). General descriptions of regeneration without quantitative measurements define stages because regenerating radioles tend to tuck behind other radioles, almost as a form of protection. Therefore, quantifying regeneration by pinnule growth or radiole length change would have been difficult and taken considerable time.

S. insignis are recognized as having unpaired, compound eyespots located on the proximal portion of the radiole, but the development of eyespots was not observed during the first 20 days of external regeneration, unlike in previous feather duster worm regeneration studies (Murray et al. 2013). This different result may be due to the difference in species, length of the experiment, or perhaps regenerating radioles of *S. insignis* do not develop eyespots. Throughout the regenerative process, radioles increased their pigmentation and developed pinnules on their oral side, a trend also seen in previous studies (Licciano et al. 2012; Licciano et al. 2015). New pinnules form on regenerating radioles as described for radiole development: paired, pinnules begin as small, raised mounds that eventually extend to reach their full length.

The internal structure regeneration of *S. insignis* lags behind regeneration of external features. Based on the observed stages, the skeletal sheath is the first structure to develop and was seen in Stage H around 11-15 days after damage. The presence of the collagenous sheath provides necessary support for the radioles growth and continues to increase in thickness as regeneration advances. Blood vessels and the radiolar skeleton become apparent later as they extend from the undamaged portion to newly regenerated regions (Figure 9; Figure 10).

In terms of external regeneration, *S. insignis* does not follow a temporally fixed orderly progression (Table 1). Individual worms did not consistently pass through the same stages at the same time nor did worms remain at stages for the same increment of time. Since observation was not continuous, I concluded that all worms must have progressed through the same stages, even those with proximal and medial cuts. After 20 days' post-damage, all *S. insignis* generally reached a similar range of stages (H-K), but their difference signifies that multiple factors contribute to the progression and rate of radiole regeneration (Table 2). During regeneration, specifically at Stages H and J, pointed tips were observed on the newly formed branches (Figure 8). The pointed branches may signify that the radiole will not continue to branch, as no stage progression was observed after pointed tip development (Table 1). If the conclusion about pointed tips is true, eight of the twelve worms (67%) from the long-term study had not yet finished branching after 20 days (Table 2).

E. vancouveri is recognized as a closely related genus of *S. insignis*, so the external regeneration of the non-branching feather duster worm species was also qualitatively investigated. In some individuals of *E. vancouveri*, researchers have recorded dichotomously branching radioles (Kinberg 1866; Fitzhugh 1989). Because all of the regenerating radioles that I observed remained unbranched, the conclusion can be drawn that damage and regrowth do not necessarily cause the observed branching phenomenon. In addition, unlike the complex branching regeneration of *S. insignis*, *E. vancouveri* accomplishes a simple regrowth pattern involving radiole elongation, pinnule addition, and increased pigmentation (Figure 11). When *E. vancouveri* recover, the tip of the regenerated region becomes pointed within at least 12 days, supporting the previous observation on *S. insignis* that pointed tips indicate the end to branching.

Quantitative Study of Radiole Regeneration

When the feather duster worm species, *Myxicola infundibulum,* are amputated in the middle of the body, each cut half regenerates the missing half (Licciano et al. 2015). Throughout the regenerative annelid phylum, a similar regrowth capability is apparent, however, limited research has examined the regenerative recognition process of appendages (Bely 2006). For *S. insignis,* the species does not appear to recognize the previous length and bifurcation number when damaged. When investigated, worms did not accomplish full regeneration because none grew to achieve typical radiole length (Table 3). Nevertheless, worm 36 bifurcated one more time than seen before damage. This result indicates that the regrowth pattern of *S. insignis* radioles is not predetermined by the pre-damaged patterns.

In the family Sabellidae, anterior regeneration experimentation has been demonstrated to have the highest mortality because without the branchial crown the worms lack necessary feeding and respiration structures (Bely 2010; Murray et al. 2013). Throughout the current study, the mortality of *S. insignis* was relatively high with few organisms surviving more than five weeks in the laboratory environment. One main cause of mortality may have been the repeated handling for observation and stress that resulted from removing the organisms from their tube for examination. During the study on long-term regeneration when *S. insignis* remained untouched for four weeks, all worms survived unlike in the short-term studies, which had mortality rates as high as

50% (Table 1; Table 2). Several other factors that may have influenced mortality could have been varying water temperature, food supply, and oxygen levels.

When studying organism regeneration, Lawrence (2010) emphasized that the increase in appendage length is a more meticulous study method and allows for the quantification of regeneration. For *S. insignis,* radioles bifurcate before the preceding internode reached its final potential length. In addition, based on the internode averages, none of the internodes examined had reached their full growth potential by the end of the study, even though most had bifurcated 4-5 times (Figure 12; Figure 13). When examining a radioles ability to recognize previous radiole length and number of bifurcations, these trends are also replicated because five worms managed to bifurcate numerous times before achieving their predicted total length (Table 3).

Another trend observed when quantitatively examining the regrowth of radioles is that several internodes demonstrated periods of no change, emphasizing that regrowth is not consistent or continuous. In the case of IN2 through IN5, the subsequent internodes never outgrew in length the previous internode at any period (Figure 12; Figure 13). Such tendencies may suggest that as radioles regenerate, the proximal-most internode extends to longer lengths. When performing the branchial crown survey, I did confirm that for *S. insignis* internode length decreases as they arise more distally on the radiole. During the regrowth of IN1, if the cut portion of the radioles was greater than ~0.8 mm in length, then increase in length appeared to be limited or non-existent during the experiment. This observation perhaps indicates that radioles recognize a certain internode length and choose to spend their energy on additional bifurcations before increasing the length of their internode (Figure 12; Figure 13). When reduced IN1 regrowth was observed, IN4 and IN5 resulted from bifurcations earlier on in the allotted study period (Figure 12B/C, Figure 13A/D/F). In a few cases, periods of length decrease were observed. The trend can be explained by the difficulty of measuring the radiole at the same angle during every collection day (Figure 12B; Figure 13A/D).

Survey of the Branchial Crown

The structure of the branchial crown of *S. insignis* has been scarcely covered in literature and observations vary. For instance, Hartman (1969) stated that *S. insignis* range between 32 and 36 radioles. However, in Henderson and Strathmann (2000) survey of a wide variety of feather duster worms, two of their samples were *S. insignis* with 45 and 46 radioles. Radiole counts and branchial patterns are important for future studies addressing respiration and feeding because those abilities are highly dependent on the available surface area provided by the branchial crown. Understanding the branchial crown of *S. insignis* is also significant because various species of feather duster worm are distinguished by their radiole number. If the size-dependent increase in radiole number is not understood, then discerning two different species may be impossible (Bick and Randel 2005).

When investigating the relationship between the number of radioles and drained weight, two different populations were examined: boat basin site and exposed site. The drained weight of the feather duster worm was utilized because *S. insignis* can change their body length and diameter while maintaining their volume. Between the two populations, the worms found at the exposed site were smaller on average than those collected in the boat basin. The cause of this difference may be due to predation differences or that the small, burrowed holes of the exposed site may limit the habitat for larger worms. The relationship between the number of radioles and drained weight was not statistically different between the two samples sites; however, in both populations, radioles were added to the worm as it increased in weight, but the addition of radioles began to slow as the worm reached a larger size (Figure 14). The two sites differ in flow regime because the exposed site, unlike the boat basin, receives wave action from ocean swells. In addition, the exposed site's habitat is on an intertidal bench, while the boat basin's habitat is subtidal. With these difference, it is remarkable that there is so little difference in radiole number between the two populations.

The feather duster worm, *Euchone analis*, demonstrated a similar positive power relationship between radiole number and body length (Bick and Randal 2005). The positive trend, also exhibited by phoronids and various other fan worm species, is expected because as a feather duster worm increases in size they require more food and oxygen for survival (Abele et al. 1983; Henderson and Strathmann 2000). Both the boat basin and exposed site worms have smaller worms with a similar average number of total radioles. However, as the size of the *S. insignis* increases, the boat basin worms increase in radiole number at a greater rate than exposed site worms. Exposed site worms, on the other hand, appear to have more radioles to begin with at a smaller stage; therefore, their exponential increase is not as dramatic (Figure 14). While phoronids and *E. vancouveri* coil or spiral their feeding structures in larger specimens to compensate for the smaller surface area, *S. insignis* is the only feather duster worm that increases their surface area by branching (Abele et al. 1983; Fitzhugh 1989). The branching pattern, therefore, may account for the outliers in the data. For instance, the boxed data point in Figure 14 is from a boat basin worm that has fewer radioles than expected, but upon surveying the worm, it had radioles with 4-7 bifurcations adding ample surface area compared to most worms which had 3-5 bifurcations.

In addition to examining the relationship between radiole number and drained weight of both populations, I examined the overall branchial crown pattern. As larvae of *S. insignis* develop into feeding adults, the dorsal-most radioles develop first and are the oldest, and the radioles located near the ventral lip are the youngest (Rouse and Fitzhugh 1994; Pernet 2003). Generally, the dorsal-most radioles have higher branching numbers, whereas the youngest radioles located ventrally have lower radiole numbers (Figure 15).

While investigating the overall branching pattern of the two populations, I observed a large amount of regeneration in the population sample from the exposed site. In a natural environment, feather duster worms experience damage due to predation or as a result of stressful environmental changes that cause their radioles to shed (Kicklighter and Hay 2006; Murray et al. 2012). A higher percentage of radioles and worms in the exposed site were damaged. Potential reasons for this difference include the dissimilar flow regimes and the predator difference between the two sites. While identified as natural damage and regeneration, the observation may have indicated the addition of new radioles because trends confirmed that as worms increase in size, radiole addition does occur (Figure 14). In addition to natural regeneration, I observed two radioles on two separate worms with a trichotomous split on the first bifurcation. Though uncommon, abnormalities such as this can occur naturally during annelid development or regeneration (Andrews 1892).

In the branchial crown of *S. insignis*, radiole length was correlated with the number of bifurcations. As seen with phoronids, tentacle length changed with tentacle number, whereas tentacle diameter often remained unaffected by changes in body weight or length (Abele et al. 1983). For *S. insignis*, radioles with more bifurcations were longer than radioles with fewer bifurcations (Figure 16). For radioles with three to four bifurcations, their total length increases with worm size, giving larger worms more surface area to feed and respire (Figure 17A/B) On the other hand, radioles with five bifurcations appear to have a relatively constant length. This may be due to the limited growth of more distal internodes because the surface area gained by a small amount of internode growth in length is no longer worth the energy cost (Figure 17C).

In the literature, *S. insignis* is recorded as having between 1-6 branches and a crown of around 14 mm in length (Hartman 1969). The current study found an average crown length shorter than this value, potentially due to the difference in samples or because Hartman's study did not individually measure radiole internode lengths to calculate the average total length. A previous study on *Megalomma* spp., a species in the same subfamily as *S. insignis*, concluded that dorsally located radioles are longer (Tovar-Hernandex and Carrera-Parra 2011). For *S. insignis*, radioles with more bifurcations are generally longer in length and located dorsally (Figure 16). Lastly, past research has also suggested that longer, more branched radioles extend farther from the substratum, making them more vulnerable to high-velocity currents (Strathmann et al. 1984). A future study could investigate whether *S. insignis* in exposed habitats experience more damage on longer, dorsal radioles.

Pinnules add essential surface area and are the basic functional unit used by *S. insignis* for feeding and respiration. Fitzhugh (1989) acknowledges that pinnules located closest to the branchial lobes are longer and they become successively shorter as on more distal locations along the radiole. In some species, pinnules remain an average length across the radiole (Henderson and Strathmann 2000). Pinnules of *S. insignis* decrease in length from proximal to distal (Figure 18). Henderson and Strathmann (2000) noticed a positive relationship between weight and pinnule length when inspecting five feather duster worm species, including *S. insignis*. While in the current study, the number of pinnules per radiole and worm weight was not examined, previous research has exhibited the potential of determining how pinnule number changes based on body weight or radiole number (Henderson and Strathmann 2000).

Feather duster worms have developed a specific filtering mechanism to benefit from water flow. In the laboratory environment, *S. insignis* use their ciliated pinnules to create a current that moves up and out of the branchial crown thus drawing in unfiltered water from below the crown (Figure 2). Previous studies have concluded that the same incurrent and excurrent patterns are generated by beating cilia in other feather duster worm species (Mill 1978; Strathmann et al. 1984; Merz 1984). With a recognized filtering pattern, future studies could examine how the food consumption changes with the loss of radioles or bifurcations (Abele et al. 1983). In addition to feeding potential, *S. insignis* is known to pass six times as much water through their branchial crown as they pump through their tube (Mill 1978). Future experimentation could examine how dissolved oxygen absorption rates change when the number of radioles decreases or increases.

Conclusion

The current study examined the radiole regeneration and the branchial crown structure found in the unique, dichotomously branching branchial crown of the feather duster worm, *Schizobranchia insignis*. The information collected on external and internal radiole regeneration provides a set of stages to recognize as radioles regrow, and the study also enhances the information on annelid appendage regeneration. Radioles hold an essential feeding and respiratory function for *S. insignis*; therefore, their ability to recover from damage is significant to study. In their natural environment, radioles are damaged through predation and shed because of environmental stress. By comprehending the growth patterns of regenerating radioles, scientists more carefully analyze recovering feather duster worm communities. Species of feather duster worms are often characterized by the appearance of their branchial crown. The relationship between drained weight and number of radioles helps explain the differences observed with worm's size in *S. insignis*. In addition, understanding the relationship between the number of bifurcations, radiole length, and pinnule length adds to the understudied branchial crown descriptions used to distinguish *S. insignis* from other species.

Figures

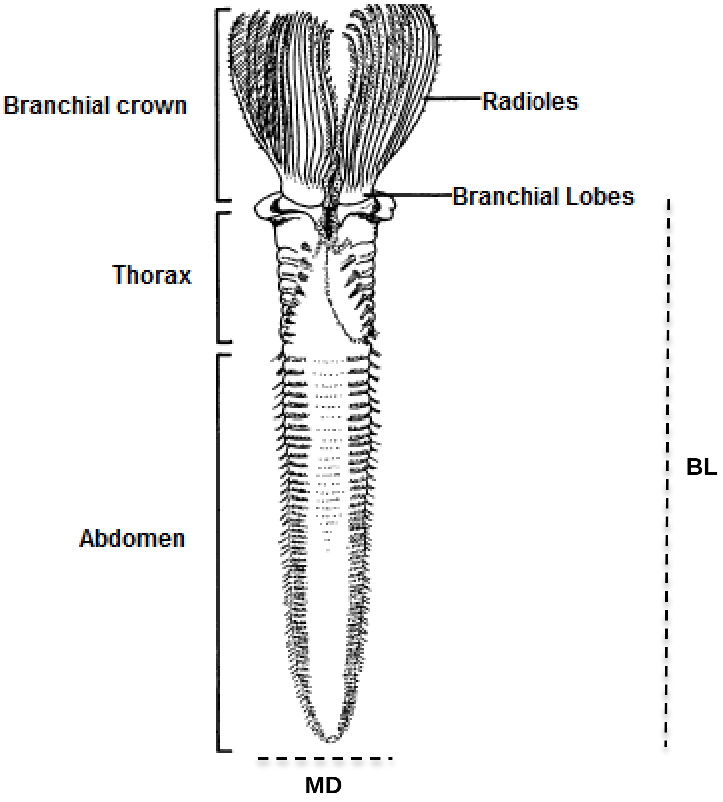


Figure 1. Overview of a Feather Duster Worm’s Anatomy

This worm (ventral view) is in the Class Polychaetae, Family Sabellidae. Figure from Fitzhugh (1989) with modified labeling. Measurement made for the branchial crown analysis are displayed with dashed lines. BL: body length MD: maximum diameter

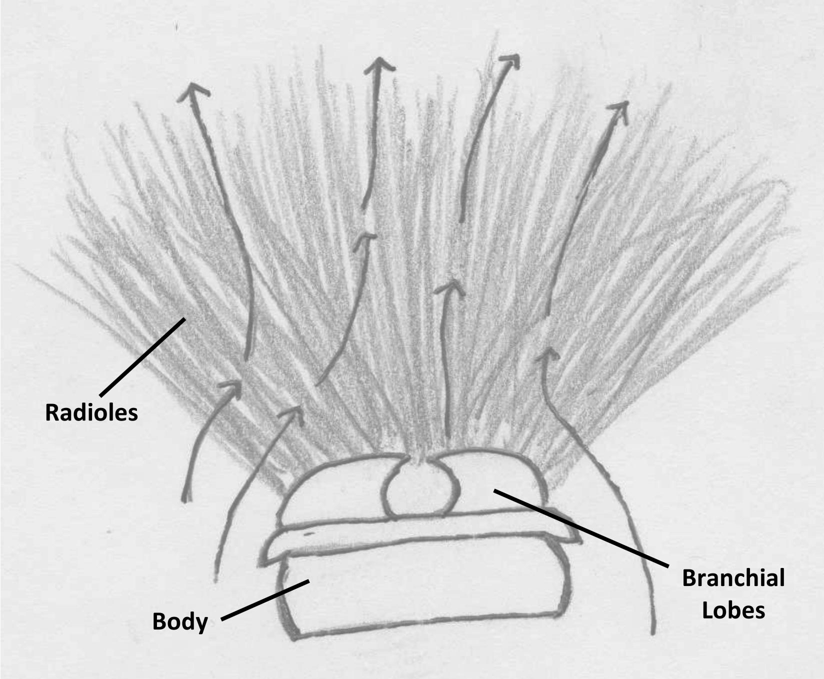


Figure 2. Diagram of the Filtering Method Used by *S. insignis*

Cilia on the pinnules create currents that moves water through the crown and past the pinnules were food particles are captured. Water currents (arrows) move from beneath the worm’s branchial crown upward past the radioles.

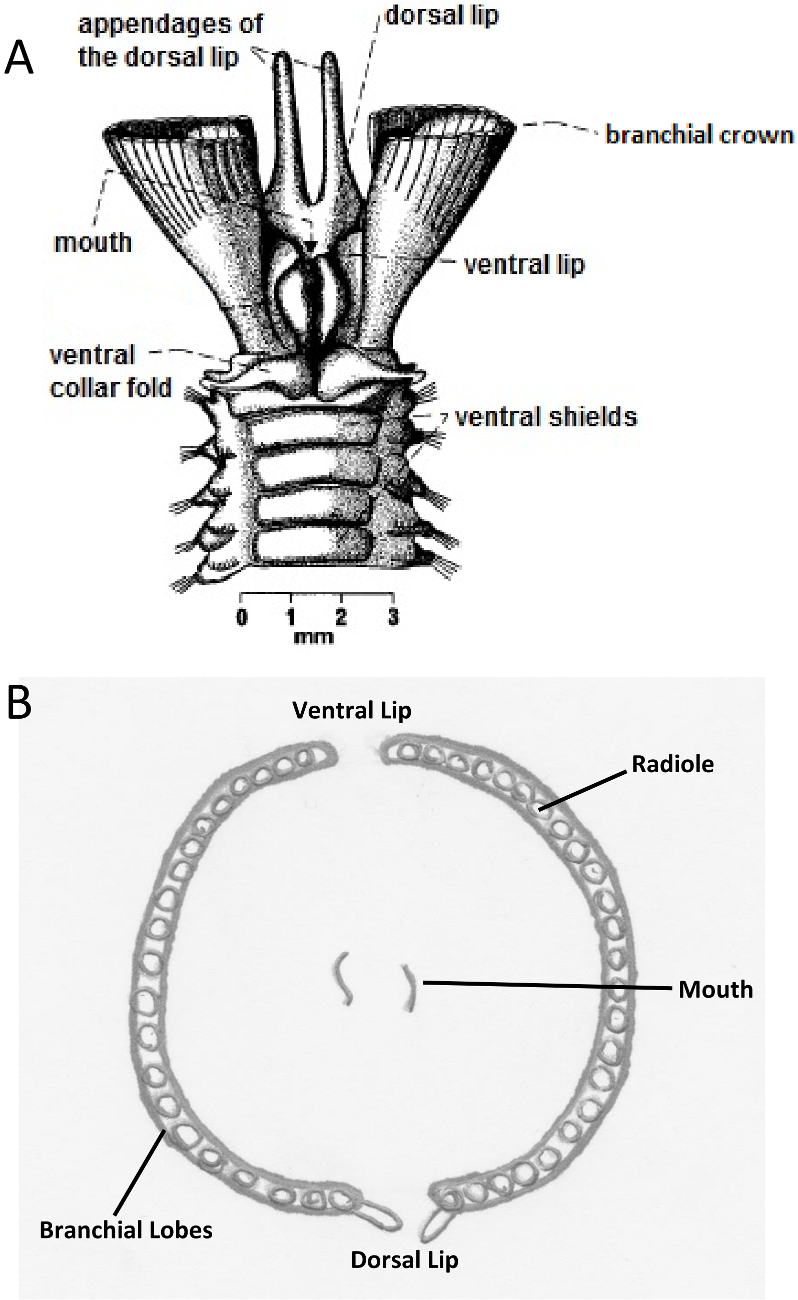


Figure 3. Ventral and Anterior View of Feather Duster Worm’s Anterior End

(A) Ventral view of a sabellid worm in the Class Polychaete, Family Sabellidae. Figure from Orrhage (1980) with modified labeling. (B) A generalized anterior view of the arrangement of radioles in a feather duster worm’s branchial crown. Full radioles are excluded for clarity.

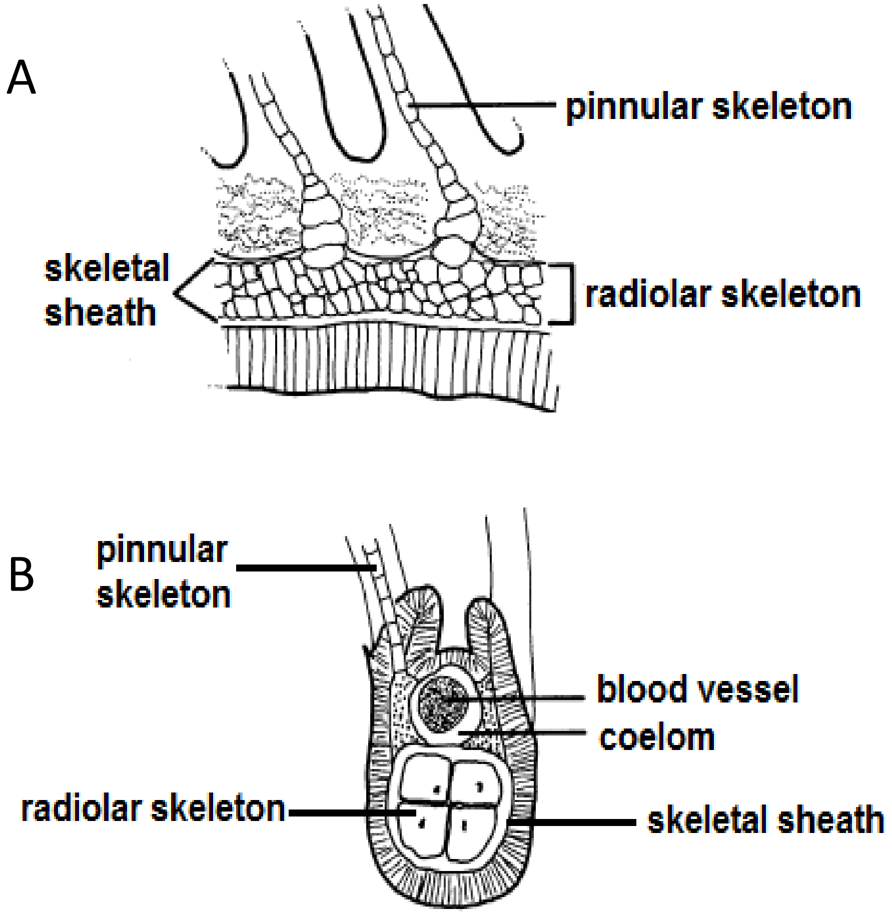


Figure 4. Overview of Internal Structures of a Feather Duster Worm Radiole

(A) Sagittal view of part of the radiole from a *Demonax microphthalmus*, showing internal structures used as markets in regeneration. (B) A cross-section of a radiole from *Sabella pavonina* showing internal structures. Both are figures from Fitzhugh (1989) with modified labeling.

Figure 5. Location of Samples Sites

S. insignis and *E. vancouveri* were collected from the sides of floating docks in the outer boat basin (OBB). Smaller worms were obtained from settlements plates and dock fender in the inner boat basin (IBB). For the survey of the branchial crowns, *S. insignis* were also collected from an exposed site (ES) located close to the entrance of Coos Estuary into the Pacific Ocean.

Figure 6. Laboratory Set Up for Radiole Regeneration Studies

In the laboratory, *S. insignis* and *E. vancouveri* were maintained in a test tube tray with their branchial crowns facing upward and set on the bottom of an aerated tank filled with unfiltered seawater.

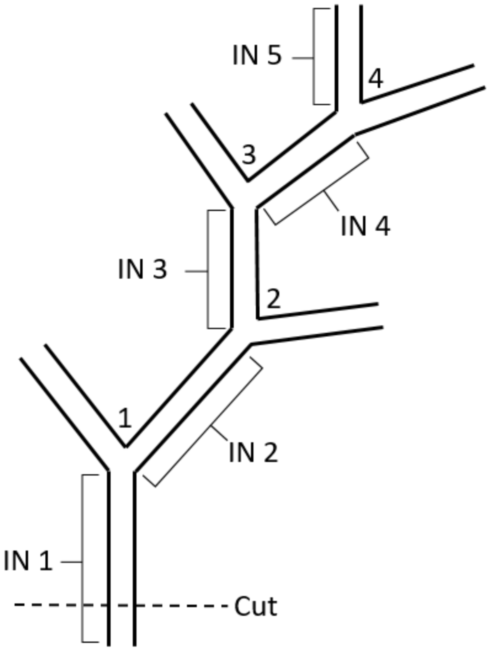
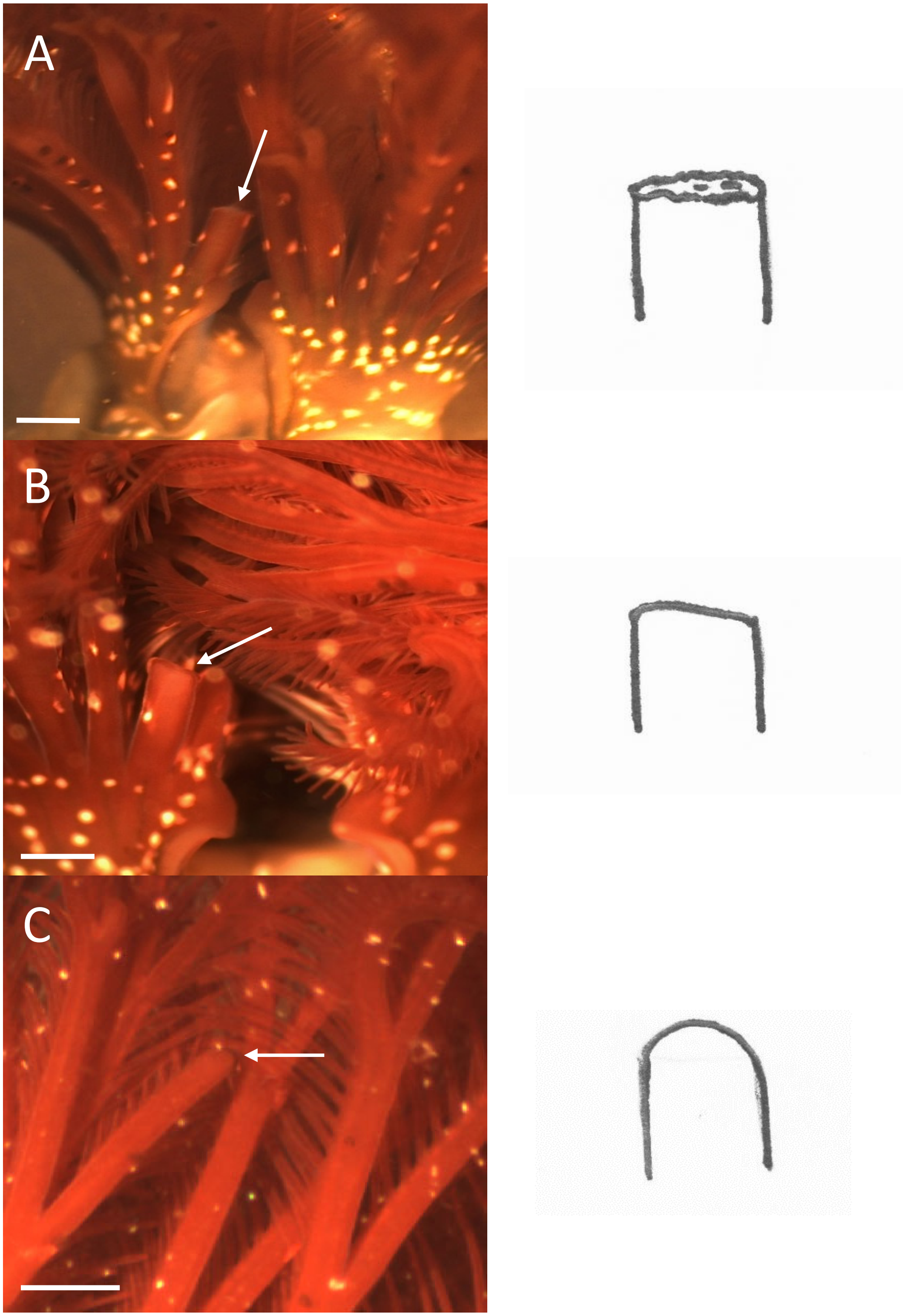
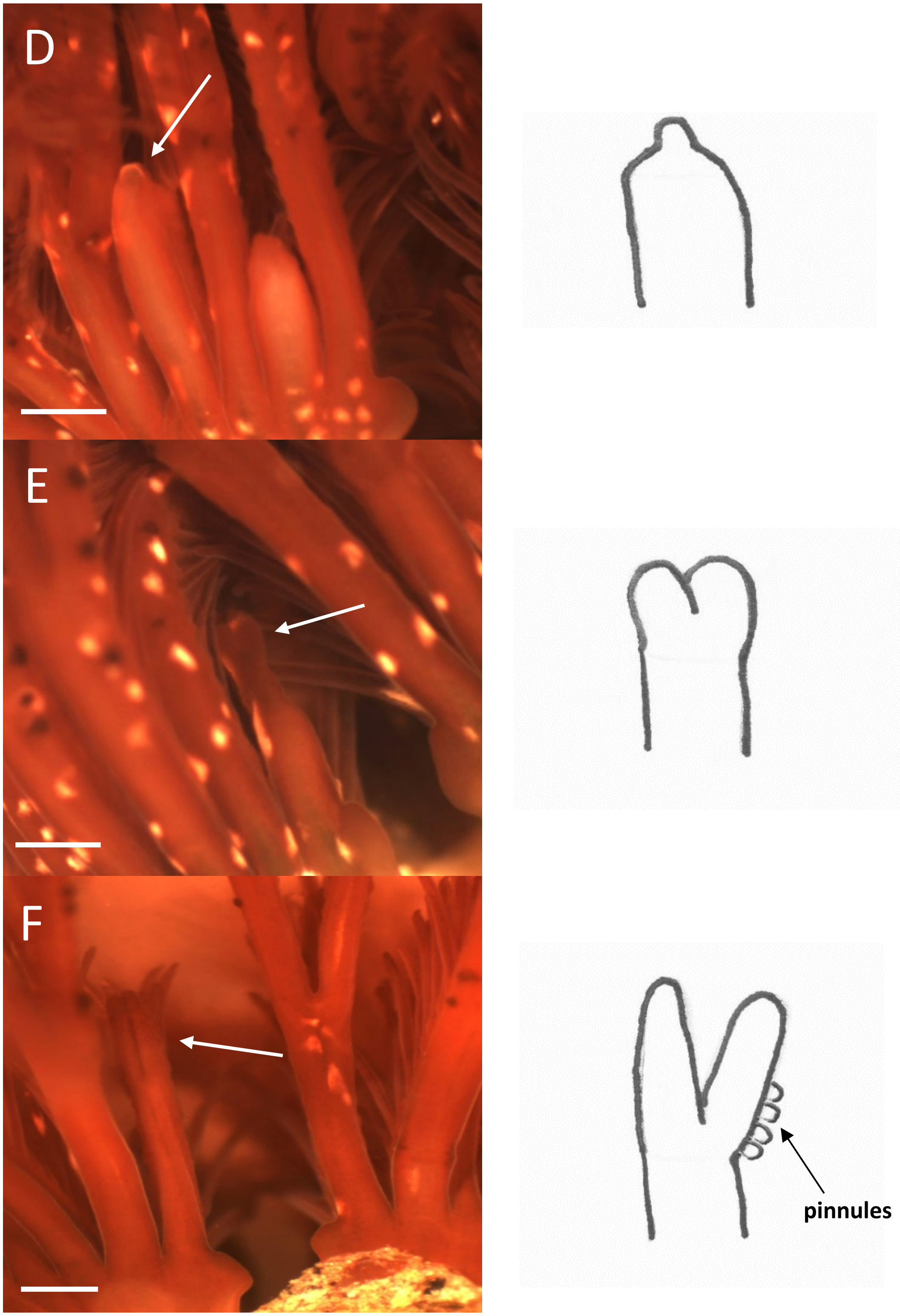
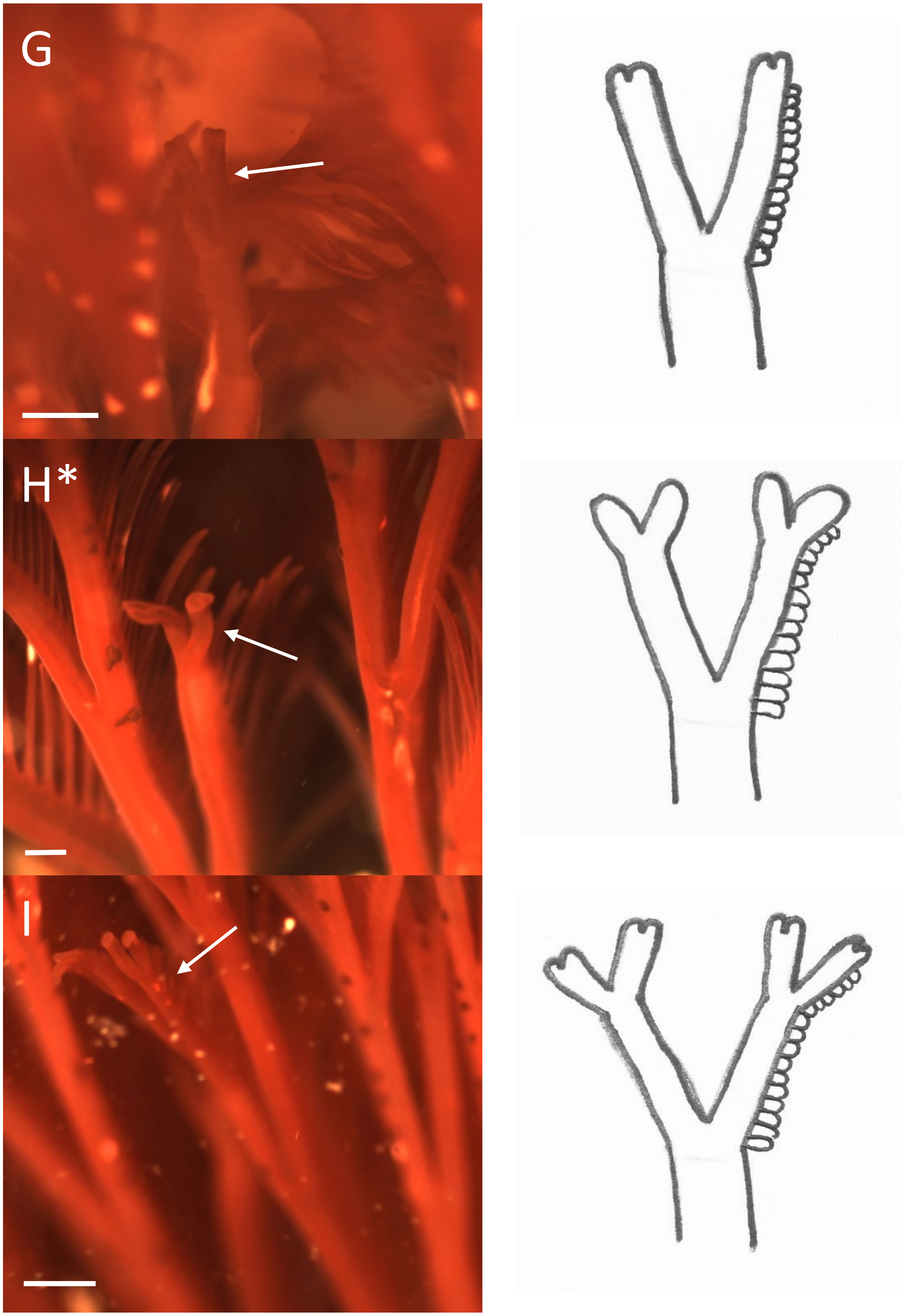


Figure 7. Visual Representation of Radiole Internodes and Bifurcations

A single internode (IN) is located between each bifurcation. Each branching point (bifurcation) is labeled either first, second, third, or fourth. The location of the proximal cut performed is displayed. When a medial cut was made, it was located on the radiole between the first and third bifurcation (IN2 to IN3).


A
B
C


D
E
F
pinnules


G
H*
I

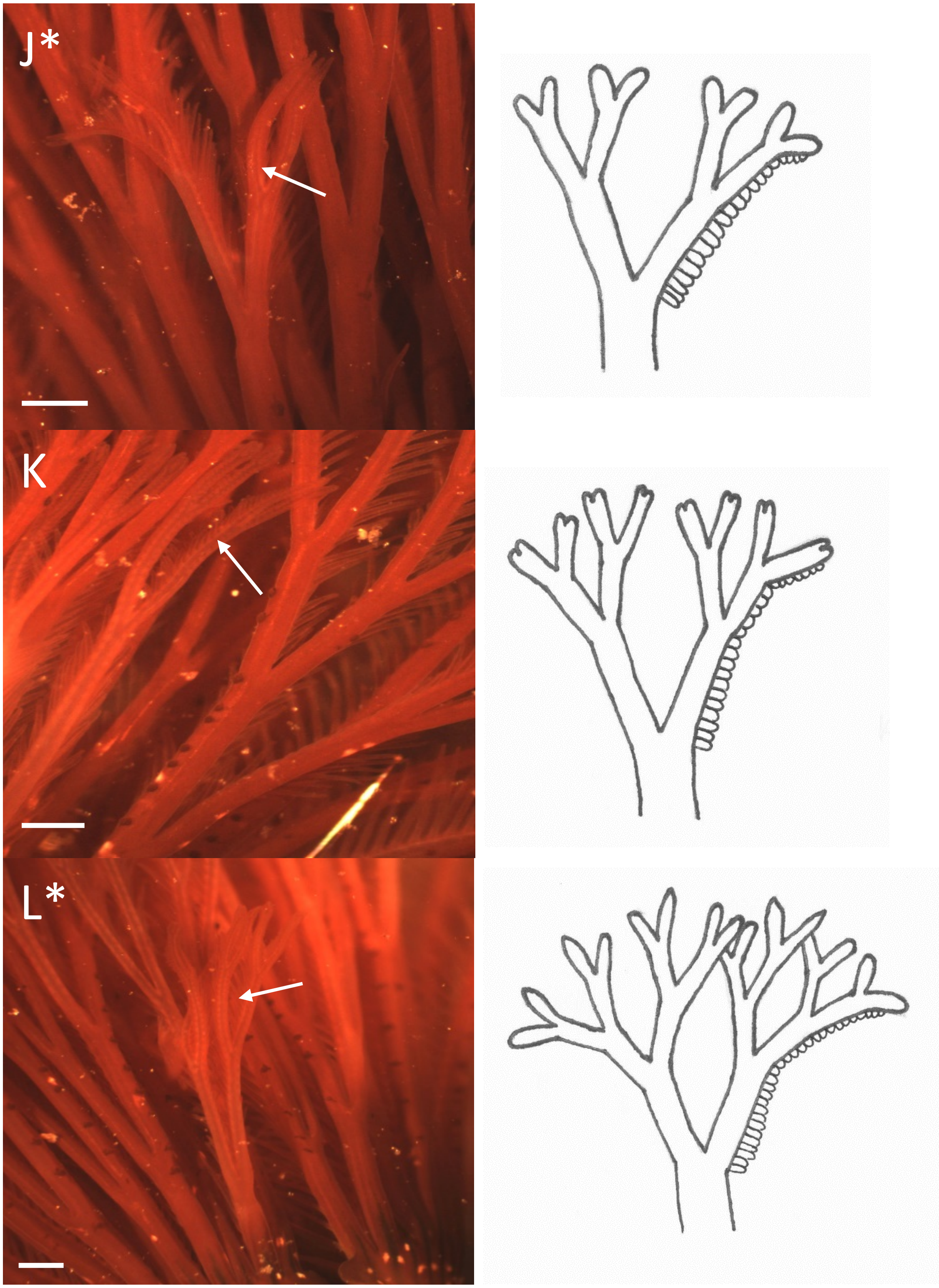
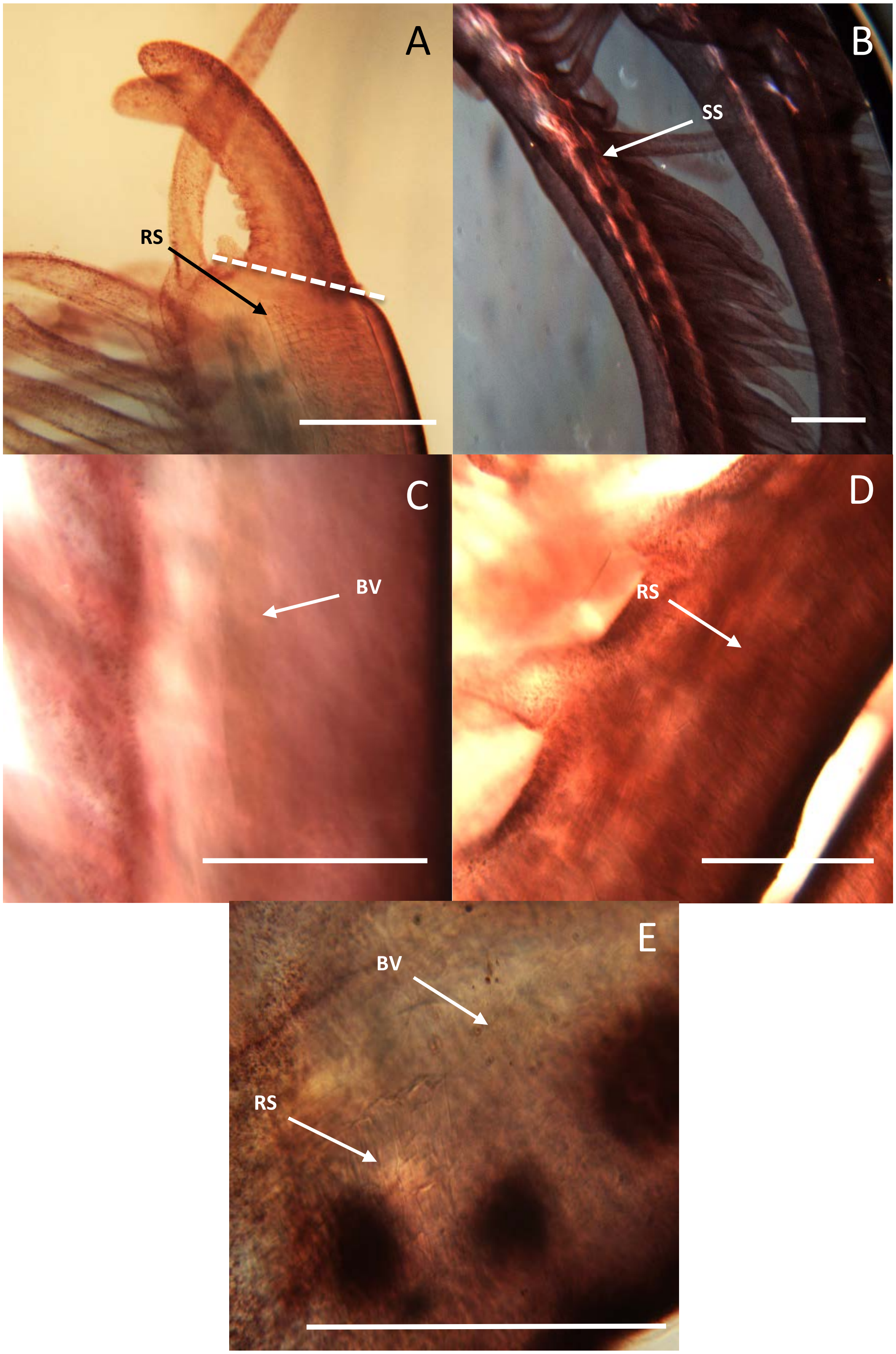

J*
K
L*

Figure 8. Photomicrographs of External Regeneration in Radioles of *S. insignis*

Twelve stages of radiole regeneration identified in *S. insignis* on an appendage cut near the dorsal lip are displayed. Stages A-L are shown in Figures A-L. The photographs of each stage were captured on different specimens and an arrow identifies the damaged radiole in each photo. For each stage, a sketch was drawn to show the general appearance in addition to each photograph. Above the cut, small bumps were drawn to signify the formation and growth of new, paired pinnules. Each radiole has pinnules coming out of two sides of the radioles oral side, but small bumps were drawn on one side of the radiole for clarity. Scale bar is 0.5 mm. Photos marked with * are stages which have also been viewed with pointed tips which signifies the end of branching. (A) Immediately after cutting, a rough piece of white cartilage is visible. (B) The wound site is flat and covered by an epithelium. (C) The epithelium develops into a rounded, smooth surface. (D) On the rounded surface, a singular dome-like extension appears. (E) Initiation of the first bifurcation has occurred as the primary branch tips are distinct, because they are separated by a medial furrow. (F) Primary branches become distinguishable, free-moving, and have developed rounded tips. (G) With the initiation of the second bifurcation come the distinct, but attached secondary branches. (H) Secondary branches are now distinguishable, free moving, and if still in the process of branching, have rounded tips. (I) Distinct tertiary branches result from the third bifurcation. (J) Tertiary branching is complete with the separation and movement of the previously attached branches. (K) The fourth bifurcation initiates the creation of the quaternary branches. (L) Completion of the quaternary branches is signified by the free-moving, distinguishable branches with either smooth or pointed tips.


A
RS
B
SS
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D
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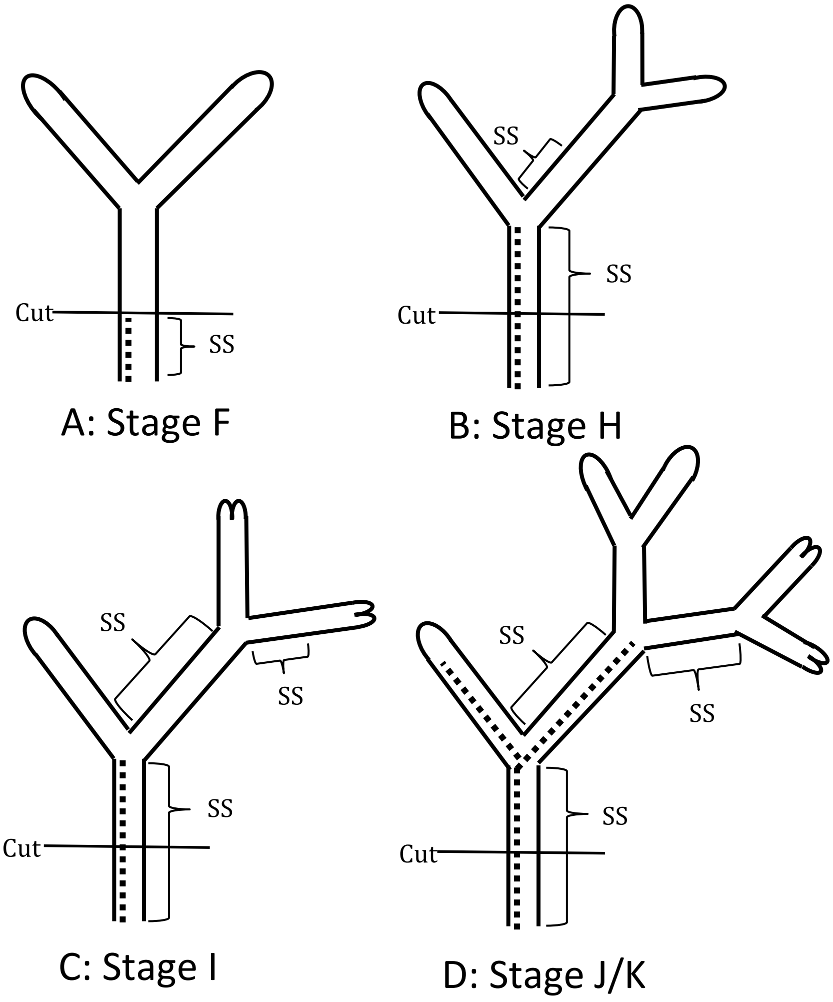
RS

Figure 9. Photomicrographs of Internal Regeneration in Radioles of *S. insignis*

Regeneration of the worm’s internal structures lagged behind external features. Photographs were captured on different specimens and not all external stages were examined for their internal featured. All scale bars = 0.25 mm. SS: skeletal sheath BV: blood vessel RS: radiolar skeleton. A dashed line indicates where the cut was made. (A) In Stage F, the vacuolated cells of the radiolar skeleton are only visible in the undamaged region of the radiole; no evidence of their presence exists in the regenerated portion. (B) At Stage H, the birefringence under polarized light signifies the presence of the collagenous skeletal sheath. (C) The greenish hue visible in the regenerating radiole by Stage I indicates a blood vessel. (D) The radiolar skeleton cells are visible in the regenerative region of the radiole at Stage J. (E) By Stage K, the blood vessel and radiolar skeleton are found adjacent to each other internally in the regenerating radiole.



A: Stage F

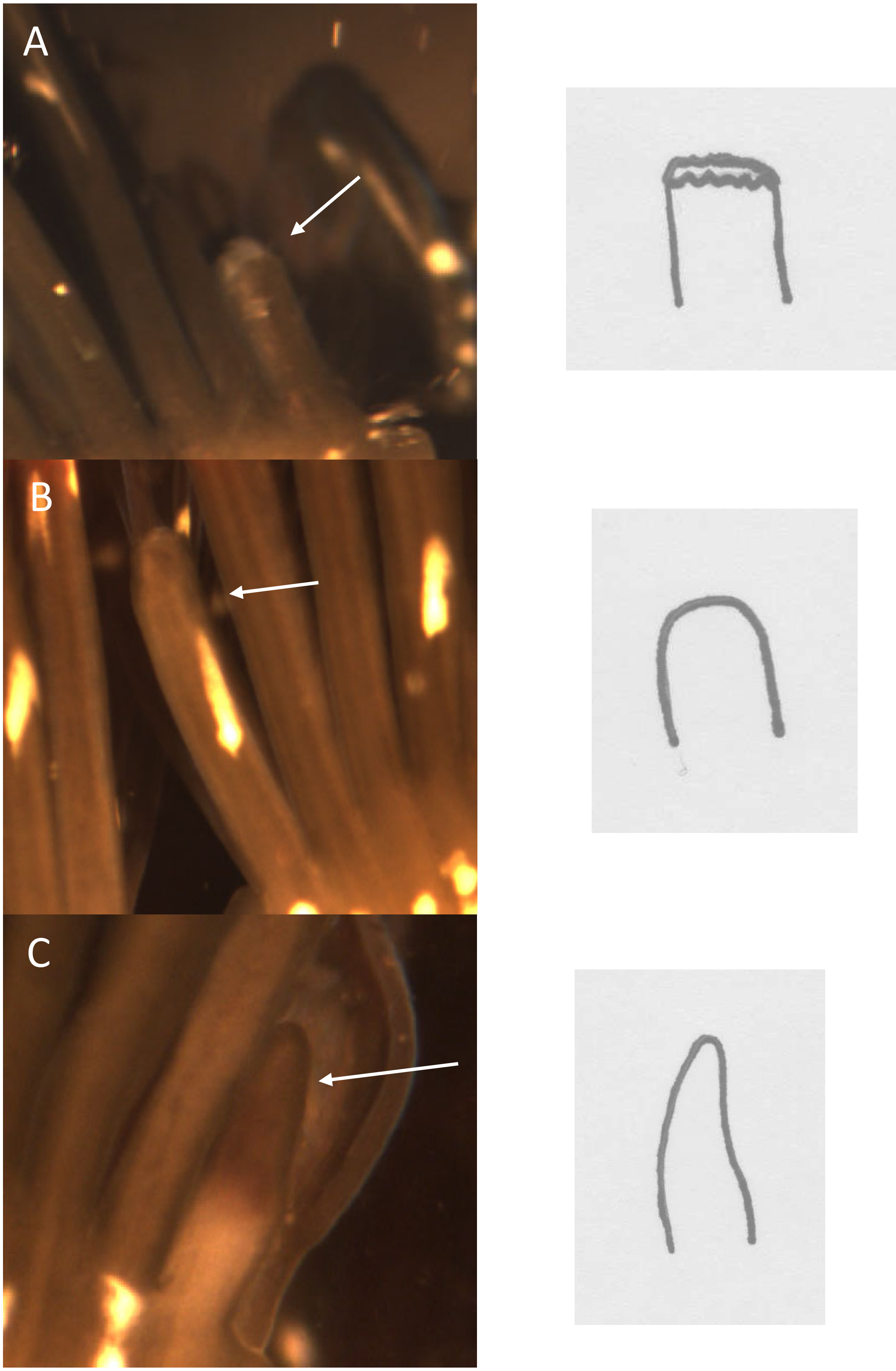
B: Stage H

C: Stage I

D: Stage J/K

Figure 10. Diagram of *S. insignis* Internal Radiole Regeneration Progression

The internal structure of twelve *S. insignis* was investigated to determine the progression of internal regeneration. Dashed line: radiolar skeleton and blood vessel. When the radiolar skeleton is present, the presence of a blood vessel was observed. SS: skeletal sheath. Pinnule bumps were omitted to simplify the schematic. (A) At Stage F, the radiolar skeleton, skeletal sheath, and the blind blood vessel are only located in the undamaged region. (B) By Stage H, the skeletal sheath has extended slightly past the first bifurcation, while the blood vessel and the radiolar skeleton are distinguishable above the cut but below the first bifurcation. (C) The skeletal sheath has regenerated past the second bifurcation by Stage I but the sheath stops abruptly midway up the secondary branch. A blood vessel and the radiolar skeleton remain visible only below the first bifurcation. (D) Around Stage J and Stage K, the skeletal sheath has extended to the end of the secondary branch; in addition, the blood vessel and radiolar skeleton have extended past the first bifurcation.


A
B
C

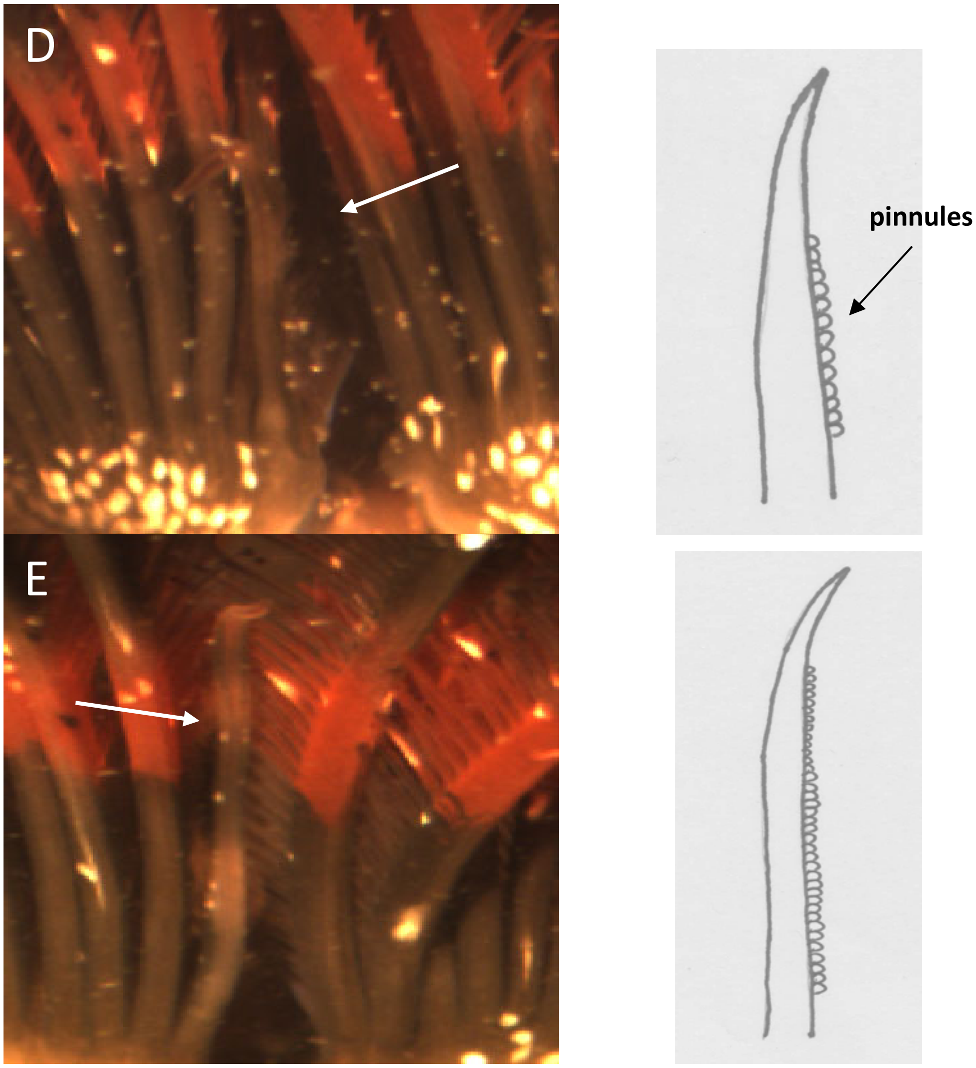

D
pinnules
E

Figure 11. Photomicrographs of External Regeneration in Radioles of *E. vancouveri*

The photographs taken on various days were captured on different *E. vancouveri* and the arrow identifies the cut radiole. For each stage, a sketch was drawn to show the general appearance in addition to each photograph. Small bumps were drawn to signify the formation and growth of new, paired pinnules at various stages. Each radiole has pinnules coming out of two sides of the radioles oral side, but simple, small bumps were drawn for clarity. (A) Immediately following the cut, the severed radiole has a rough, white piece of cartilage visible. (B) Within 1 day, the epithelium covers the wound which results in a rounded or smooth, flat surface. (C) Between 2-7 days after initial damage, a dome-like epithelium extension is apparent as the radiole begins to grow, and the undamaged portion of the radiole appears thicker than the newly regenerated region. (D) The now elongated regenerated region is slightly curved with a pointed tip by day 12. In addition, the radiole is transparent in color and thinner than the undamaged region. (E) Around day 16-20, the regenerative region continuous to elongate with a pointed tip and lightly visible radially stripped pattern.

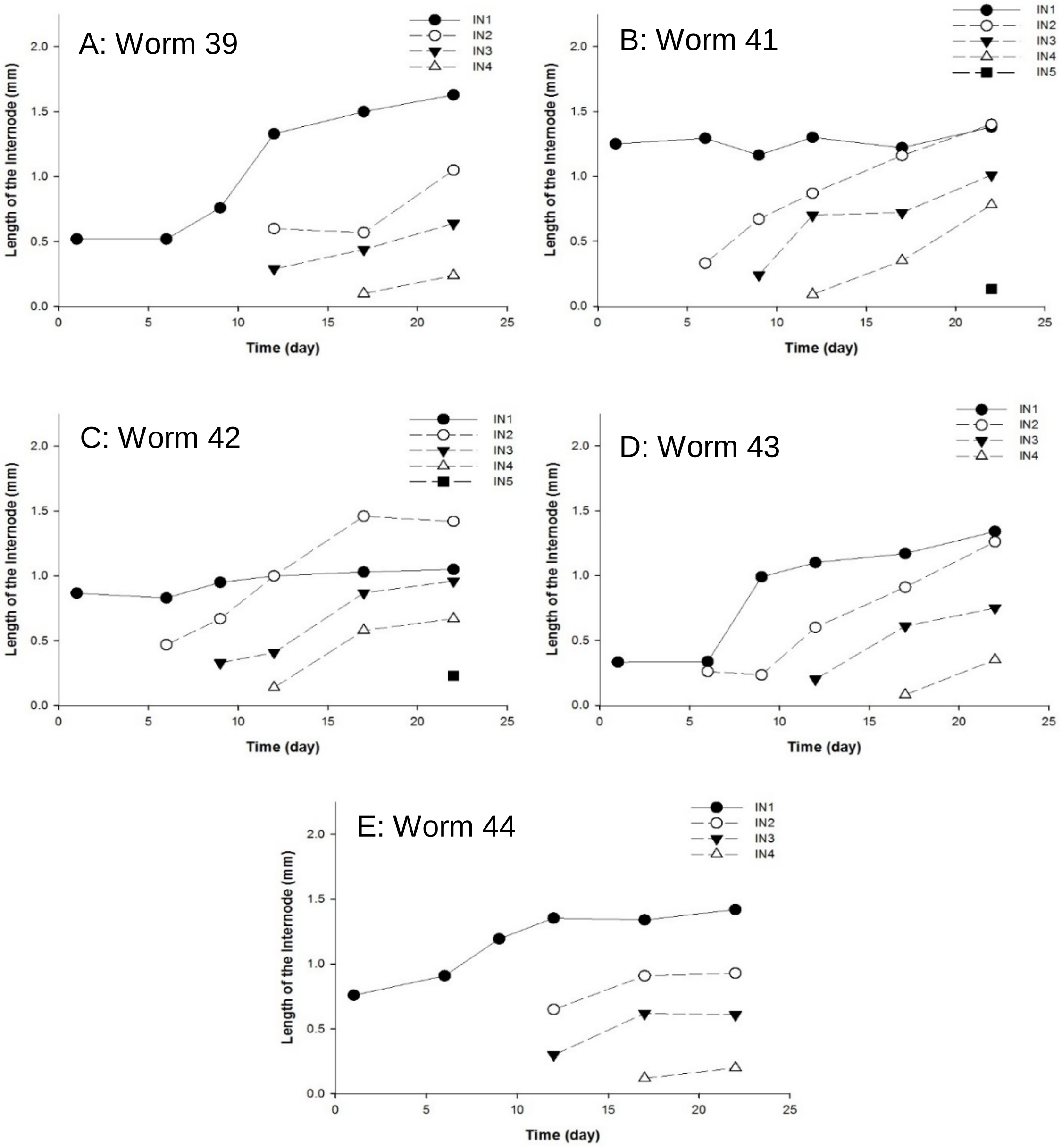


Figure 12. Regrowth in Length of Single Cut Regenerating Radiole

Each graph contains data from a worm with a single radiole cut. Length change was determined by measuring each internode of the regenerating radiole (see Figure 7 for internode labeling). IN1 is a measure of the total length rather than only the length from the cut. Eight *S. insignis* were measured on 3-5 day intervals to quantify regrowth over the course of radiole regeneration. Three of the worms died during experimentation.

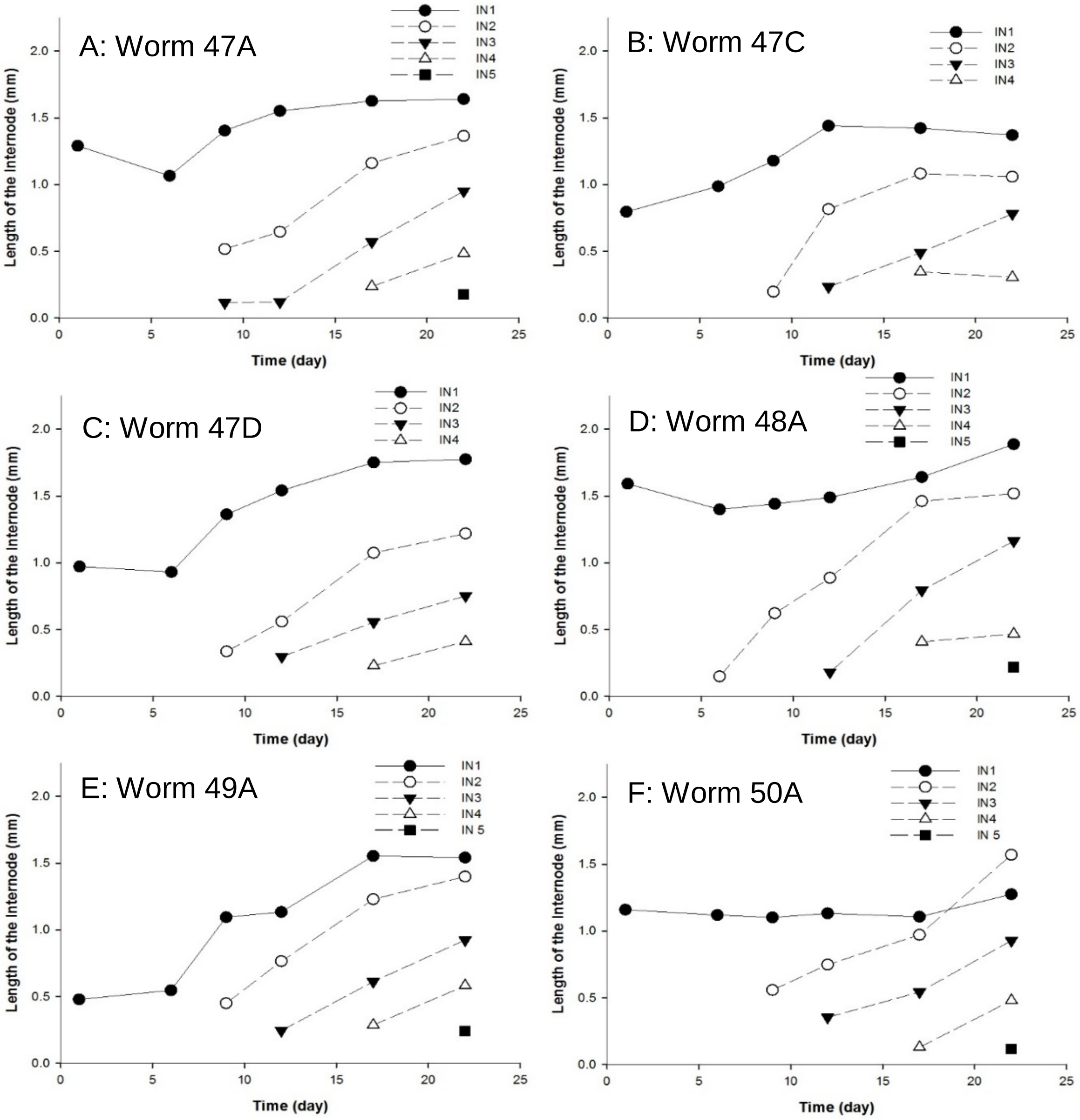


Figure 13. Regrowth in Length of Multiple Cut Regenerating Radioles

Each graph contains data from a worm with multiple cut radioles. Multiple cuts were performed to observe how the damage of multiple radioles would affect regrowth. Letters (A-D) stand for the various radioles cut on a single feather duster worm. Length change was determined by measuring internode growth (Figure 7). IN1 is a measure of the total length of the internode. Six *S. insignis* were measured on 3-5 day intervals to quantify regrowth during radiole regeneration. These graphs represent only five out of the twelve radioles examined for regrowth.

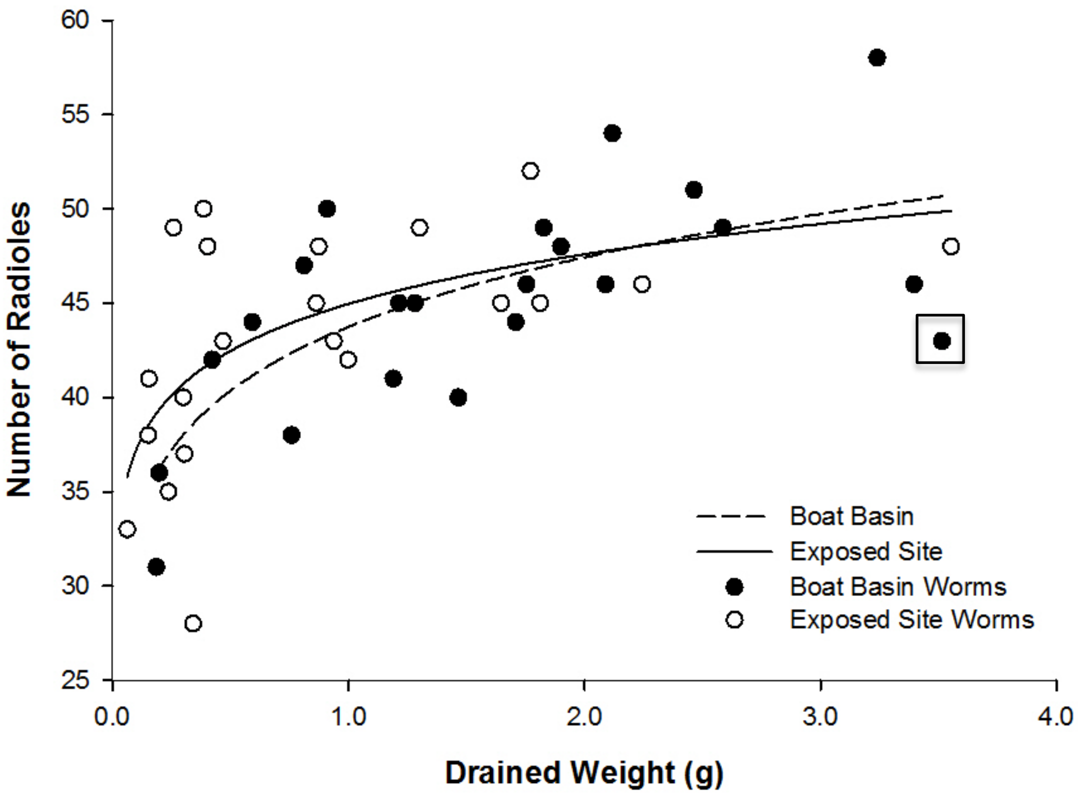


Figure 14. Number of Radioles vs Drained Weight of Both Samples Sites

A statistically significant non-linear, power relationship between the worm's drained weight (g) and number of radioles was exhibited by both populations of *S. insignis*. The boat basin worms exhibited radiole addition mathematically expressed by the allometric equation $y = 43.760x^{0.117}$ (n=22; $r^2 = 0.505$). The worms of *S. insignis* at the exposed site had a mathematical relationship of $y = 44.97x^{0.082}$ (n =20; $r^2 = 0.360$). The boxed data point strays from the fitted equation due to its lower than expected radiole number.

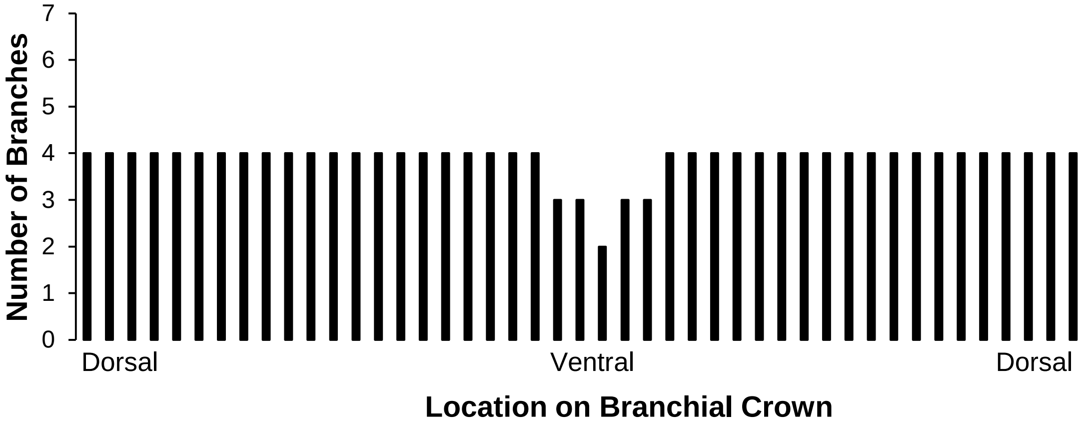


Figure 15. Branching Pattern of *S. insignis*

The branching pattern of a *S. insignis* found in boat basin site is displayed. Location on the branchial crown corresponds to the labeled diagram in Figure 3B.

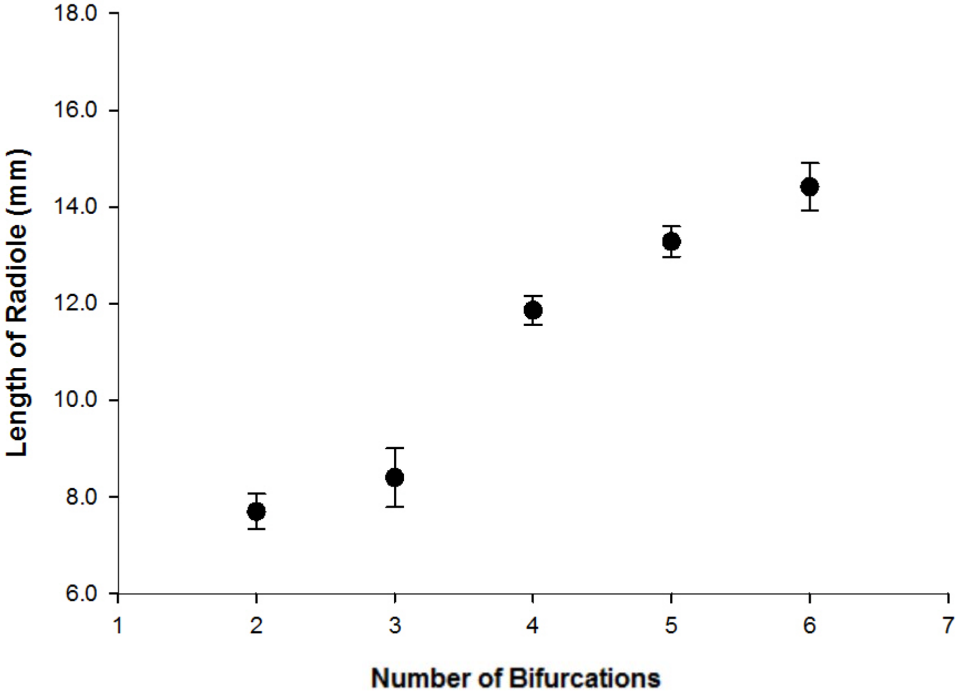
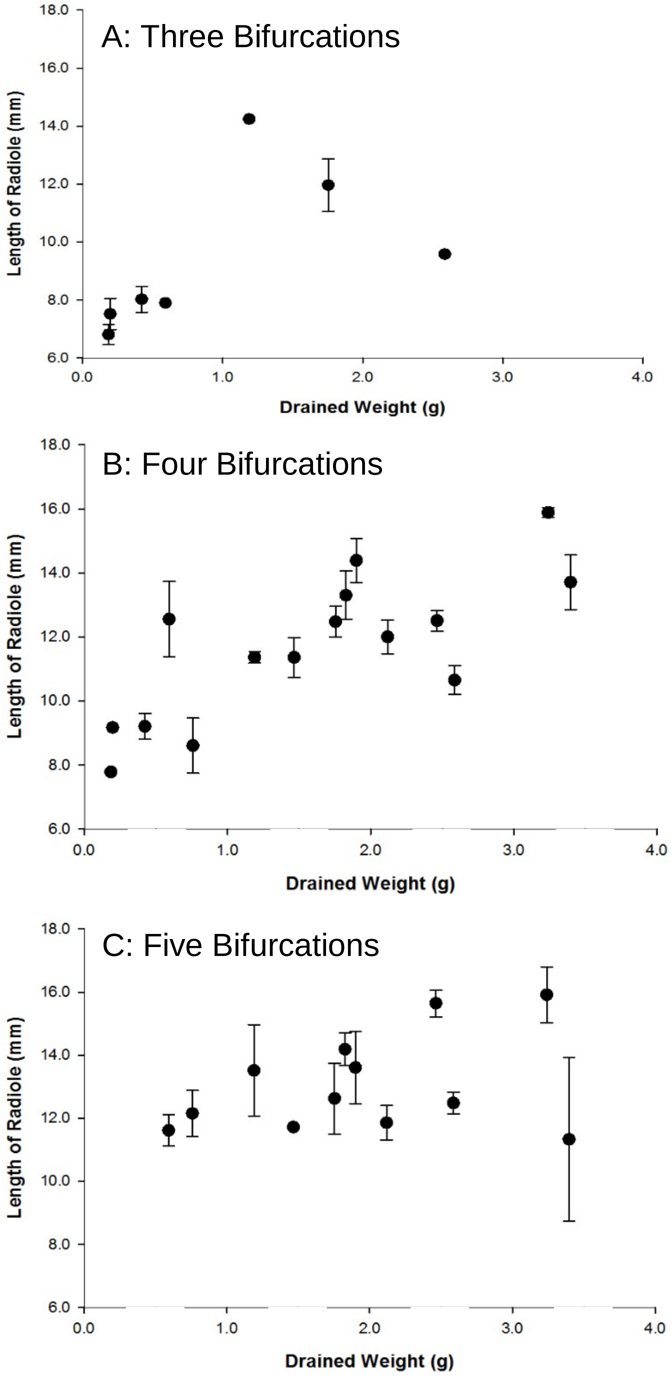


Figure 16. Length of *S. insignis* Radioles vs Number of Bifurcations

Ten radioles from each of fifteen individual *S. insignis* were examined to determine the relationship between the number of bifurcations and the total length of a radiole. Length is the distance from the base to the tip of a radiole. Error bars are +/- 1 standard error.


A: Three Bifurcations

B: Four Bifurcations

C: Five Bifurcations

Figure 17. Length of Radioles vs Drained Weight of *S. insignis*

The relationship between the drained weight of the worm and the length of their radioles was investigated. Graph A, B, and C group the data for radiole lengths according to the number of bifurcations (Figure 7). The data is based on ten radioles from each of fifteen *S. insignis*. Error bars are +/- 1 standard error.

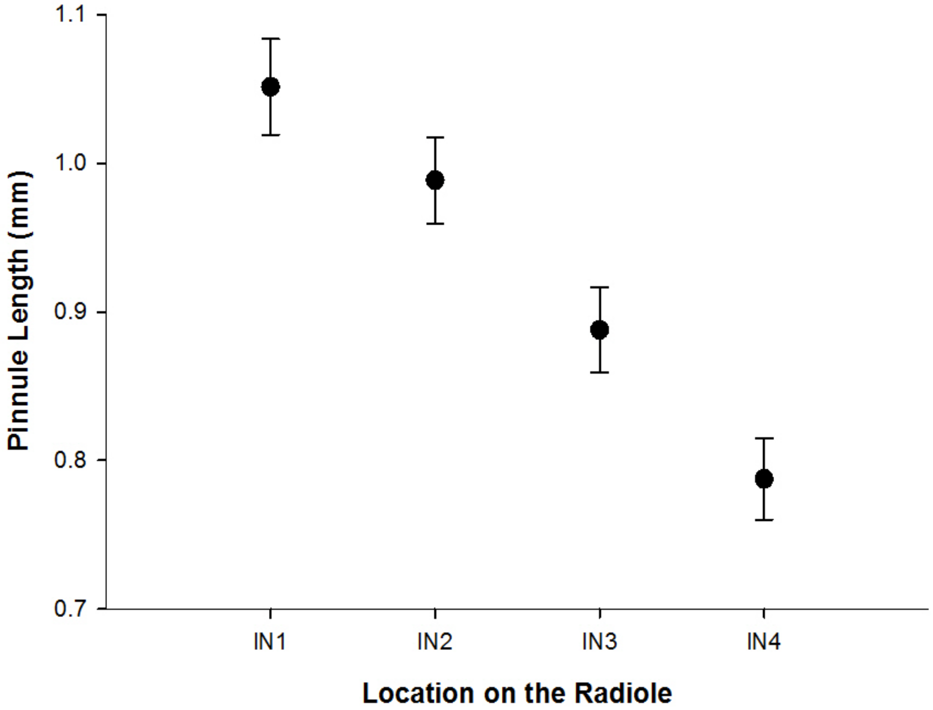


Figure 18. Length of Pinnules on *S. insignis* vs the Pinnule's Location on the Radiole

Four radioles were measured on each of seven *S. insignis* to determine the relationship between pinnule length and location on the radiole. Pinnule location was determined as internode number on the radiole (Figure 7). Error bars are +/- 1 standard error.

Tables

Worm	Day														
	1	2	3	4	5	6	7	8	9	10	11	12	14	16	19
(Trial 1)															
P1	A	C		C	C	C	E	F	F	F	G	G	H	H*	H*
P2	A	C		E	F	E	F	E	C	-	-	-	-	-	-
P3	A	C		-	-	-	-	-	-	-	-	-	-	-	-
P4	A	C		E	E	F	-	-	-	-	-	-	-	-	-
M5	A	B		E	F	F	F	F	F	G	H	I	I	I	J
M6	A	-		-	-	-	-	-	-	-	-	-	-	-	-
M7	A	C		E	F	F	F	F	F	F	G	H	H*	H*	H*
M8	A	A		F	F	F	F	F	H	H	H	H	H	I	J
(Trial 2)															
P9	A	B	C	C	C	C	E	F	F	G	H	H			
P10	A	B	C	C	E	E	F	F	F	H	H	I			
P11	A	C	C	E	F	F	G	G	H	H	H	I			
M12	A	C	C	D	D	E	E	F	F	F	-	-			
M13	A	B	E	E	F	F	G	H	H	H	I	I			
M14	A	C	C	C	E	E	F	F	G	G	G	H			
M15	A	B	D	D	E	F	F	G	G	H	H	H			
M16	A	C	C	D	D	E	E	F	G	G	G	H			

Table 1. Short-Term Study: Radiole Regeneration Progression

For the short-term study conducted as two trials, individuals of *S. insignis* passed through the various regeneration stages I established (Figure 8). The occurrence of a proximal (P) and medial (M) cut is distinguished in the names of the worms. Dashed lines indicated a worm's death. Stages marked with an * emphasize that the regenerated radioles had a pointed tip and were finished bifurcating.

Worm	Day	
	1	21
17	A	H
18	A	L
19	A	K
20	A	J*
21	A	H*
22	A	I
23	A	I
24	A	H*
25	A	J
26	A	J
27	A	J
28	A	H*

Table 2. Long-Term Study: Radiole Regeneration Final Stage

In a long-term regeneration study, individuals of *S. insignis* were observed at dissimilar final stages (Figure 8). Full recovery may not have been observed after the three weeks. Stages with a * emphasizes that the regenerated radioles had pointed tips and were finished bifurcating.

Worm	BR: Branch #	BR: Length (mm)	DR: Branch #	DR: Length (mm)
29	4	10.352	4	5.825
30	4	14.320	-	-
31	4	8.703	-	-
32	4	13.862	4	6.597
33	4	7.577	3	7.020
34	4	9.570	-	-
35	5	10.366	-	-
36	**4**	**15.714**	**5**	**7.647**
37	5	11.746	4	6.234
38	6	18.101	4	9.324

Table 3. Bifurcation Number and Length Difference Before and After Regeneration

Six worms were successfully measured to determine whether they returned to their previously observed bifurcation number and length after five weeks of regrowth. In this case, branch is synonymous with the number of bifurcations. BR: before regeneration. DR: during regeneration. None of the studied *S. insignis* survived long enough to fully regenerate. I bolded worm #36 to emphasize that it regenerated one more than its original number of bifurcations.

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