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The Art of a Slugslayer: An Exploration of the Process of Extracting Radulae from Lehmannia valentiana for the Purpose of Viewing in the Scanning Electron Microscope

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THE ART OF A SLUGSLAYER: AN EXPLORATION OF THE PROCESS OF EXTRACTING RADULAE FROM *LEHMANNIA VALENTIANA* FOR THE PURPOSE OF VIEWING IN THE SCANNING ELECTRON MICROSCOPE

A Capstone Experience/Thesis Project Presented in Partial Fulfillment

of the Requirements for the Degree Bachelor of Science

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at Western Kentucky University

By

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ABSTRACT

A radula is an anatomical structure found exclusively in mollusks that acts as both tongue and teeth in feeding. Its structure has been studied for more than a century, yet published methods for identifying, dissecting, and cleaning radulae for microscopy are almost unintelligible to anyone not already familiar with molluscan anatomy. The purpose of this project was to identify the best method of garden slug radula isolation and present it in a manner that anyone could understand and successfully use.

After identifying the location of the radula in the slug, the easiest means of removal proved to be simply regurgitating the radula by applying pressure to the slug's body. This pressure was applied using a probe or needle positioned horizontally and firmly pressing from tail to head with a swiping motion. The exserted radula was then cut from the slug with a scalpel and repeatedly rinsed with deionized water. Each excised radula was cleaned of tissue by incubating in a protease solution, then rinsed with DI water and stored in 25% ethanol. For mounting, each radula was placed onto a glass slide to dry before affixing to a scanning electron microscopy (SEM) stub with mounting tape. Finally, the stub was sputter coated and viewed in the SEM. A short web video was created to help anyone with interest in learning this procedure.

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INTRODUCTION

The radula is a remarkable structure found in all molluscan taxa except for Polyplacophora and Bivalvia and plays a vital role in a slug's survival and ecological success (Vortsepneva et al., 2013, 2014). This unique structure, resembling a rasp or a tongue-like ribbon, is covered in rows of tiny, rearward-facing teeth. While inconspicuous, the slug radula possesses an extraordinary array of functions such as the “rasp[ing] of food particles” and “cut[ting] leaves into pieces and in the retention of the food material inside the buccal cavity” (Prakash et al., 2015). This means of food uptake allow for mechanical food processing and gathering, which enables the omnivorous slugs to feed on “a wide range of food” including a diverse range of plant and fungal matter (Dirzo, 1980). However, the significance of the slug radula extends beyond mere feeding. It also plays a role in defense and locomotion. When threatened, slugs can extend and retract their radula rapidly, using it as a defensive weapon to deter predators (South, 2012). The radula's rough surface and sharp teeth can cause discomfort or injury to potential attackers, giving the slug a chance to escape.

By understanding the significance of the slug radula, one may gain insight into the fascinating adaptations and feeding strategies of these intriguing creatures. But while radulae certainly have many significant features necessitating study, published methods for dissecting and preparing radulae are unintelligible to those without prior experience with slugs.

The structure of the slug radula is a marvel of biological engineering. It consists of a flexible, ribbon-like membrane with multiple rows of teeth arranged in a specific pattern, somewhat resembling the appearance of fish scales (South, 2012). The teeth themselves are made of chitin, a tough and durable substance commonly found in the exoskeletons of arthropods (Curtis and Cowden, 1977). The arrangement and shape of the teeth can vary across different slug species, reflecting their adaptations to specific feeding habits and food sources. The odontophore, a u-shaped piece of cartilage which lies beneath and supports the radula, and the connective tissue-filled portion of the radular sac called the collostyle are components of the radular sac in a slug (Curtis and Cowden, 1977). The odontophore can be observed in Figure 1.

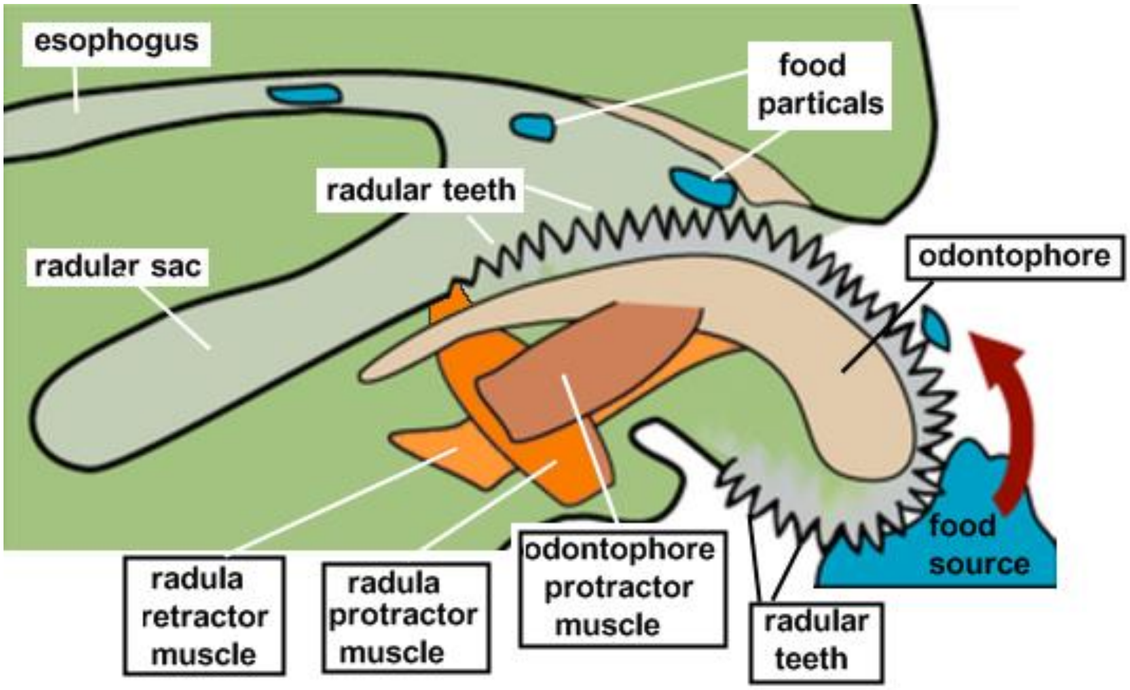

Figure 1: Diagrammatic transverse view of the buccal cavity of a gastropod, indicating the odontophore (Radula in Marine Snails, 2019).

The primary function of the slug radula is feeding. Slugs being omnivores allow them to consume a wide range of plant material such as leaves, stems, and fruits as well as a variety of other foods such as “fungi, mosses and liverworts,” although not as frequently (Dirzo, 1980). The radula acts as a scraping and cutting tool, allowing slugs to extract nutrients from plant tissues (Kawahara et al., 1994). By moving the radula back and forth, slugs can create a rhythmic motion that helps them break down plant matter into smaller, more manageable pieces. The rearward-facing teeth aid in gripping and tearing the food, effectively shredding it into digestible fragments.

Evolutionarily, the slug radula is a remarkable adaptation that has contributed to the success of slugs as a group. It represents a specialized modification of the ancestral molluscan radula, enabling slugs to exploit diverse food resources (South, 2012). The ability to feed on plant material has allowed slugs to colonize a wide range of habitats, from forests and gardens to agricultural fields to even a neighborhood backyard. The evolutionary flexibility of the radula has also led to variations in shape and tooth arrangement, reflecting the diverse ecological niches occupied by different slug species (South, 2012). Slugs can have varying radular tooth sizes and shapes dependent on the environment in which they live.

Furthermore, the slug radula's significance extends beyond its own group. It has served as a source of inspiration for biomimetic designs and engineering. The “chiton tooth, squid beak, and byssal threads of bivalves have inspired the development of new technologies” (Montroni et al., 2019). Researchers have studied the structure and function of the radula to develop innovative materials and tools. The unique self-sharpening properties of the teeth, for example, have inspired the development of more durable cutting instruments. Understanding the significance of the slug radula provides valuable insights into the intricacies of nature's adaptations and serves as a source of inspiration for human technological advancements.

While the significance of the radula is easily apparent, for the common scientist interested in studying these unique structures, the means by which one extracts a radula is difficult to fully comprehend from published research. The aim of this study was to develop a means to remove a radula quickly and effectively for the purpose of viewing in a scanning electron microscope (SEM), and to document that method so that it could be utilized by others.

A scanning electron microscope (SEM) is a powerful imaging instrument used to examine the surface of solid materials at high magnification. It is a type of electron microscope that produces detailed, three-dimensional images of the sample by scanning it with a focused beam of electrons. In the SEM, a beam of electrons is emitted from an electron source, typically a heated filament or a field emission gun. This beam is then accelerated and focused onto the sample using electromagnetic lenses. When the electron beam interacts with the sample's surface, several types of signals are generated, including secondary electrons, backscattered electrons, and characteristic X-rays (McMullan, 2006). The secondary electrons, which are low-energy electrons emitted from the surface, are collected by a detector and used to create an image of the sample's topography. These electrons provide information about the surface morphology, texture, and features of the sample. Backscattered electrons, on the other hand, are higher-energy electrons that are deflected back toward the detector based on the atomic composition and density of the sample. These electrons generate contrast in the image, revealing compositional variations in the material. The characteristic X-rays emitted from the sample can be used for elemental analysis, allowing researchers to identify the chemical composition of different regions on the sample's surface (McMullan, 2006).

In a SEM, the sample is typically coated with a thin layer of conductive material, such as gold or carbon, to enhance image quality and prevent charging effects. The sample is placed in a vacuum chamber to enable the movement of electrons without interference from air molecules. The SEM is then able to produce highly detailed images with resolution down to nanometer-scale, providing valuable information about the surface features, structure, and composition of a wide range of materials, including metals, ceramics, polymers, biological samples, and geological specimens (McMullan, 2006).

Overall, scanning electron microscopes are widely used in various scientific and industrial fields, including materials science, nanotechnology, biology, geology, forensic analysis, and quality control, among others, due to their ability to visualize surface structures at high magnification and resolution.

Through the preparation of slug radula for the SEM, one can better understand their unique structure. The SEM provides detailed images of the sample's surface topography and morphology. In the case of the slug radula, researchers can study the arrangement and shape of the teeth, the surface texture, and the overall architecture of the radula membrane. This information is essential in understanding how the radula functions as a rasp or cutting tool during feeding and how it contributes to the slug's ability to exploit a wide range of food resources. Additionally, understanding the elemental composition of a slug radula observed by the SEM can provide clues about the radula's durability, flexibility, and adaptability to different feeding habits.

Overall while the central aim of this project was to explore and evaluate various methods for isolating the garden slug radula, with the ultimate goal of pinpointing the most effective and efficient approach for the preparing and viewing of the radula with the scanning electron microscope. Beyond mere identification, the project sought to make this technique accessible to all researchers despite their varying background knowledge on the radula. Presenting the method in a clear and comprehensible manner ensures that anyone can grasp and successfully employ it for their scientific endeavors.

METHODS

Fifteen *Lehmannia valentiana* (three band garden) slugs were gathered from a suburban, western Kentucky environment. They were placed in a small terrarium while awaiting the procedure. A dissecting microscope was used to analyze the slugs more easily. Two different approaches were employed to prepare the slugs for dissection as adapted from biologist Dr. Wallace E. Holznagel's article titled *Research Note: a Nondestructive Method for Cleaning Gastropod Radulae from Frozen, Alcohol fixed, Or Dried Material* (Hoznagel, 1998). Five slugs were frozen until euthanasia, the remaining slugs were humanely euthanized with 7% magnesium chloride. In the procedure of removing the radula, all slugs were placed onto small squares of paper towels to prevent the slug mucopolysaccharide (slime) from displacing the slug. Originally, an incision was made from head to tail following right above the foot, and the internal anatomy of the slug was exposed in the search for the radular complex. After referencing many models, and some exploratory means, the radula was identified with help of the z stacking dissecting microscope, a Leica MZ16 using Automontage software (Figure 2).

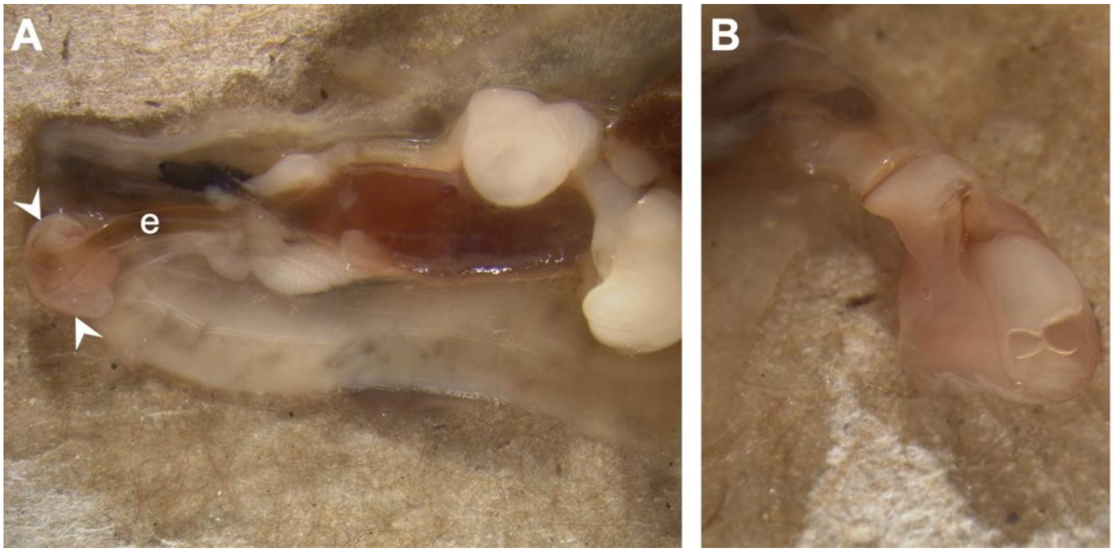

Figure 2. A dissected slug identifying the radula using a light microscope. A) The radular complex is identified with arrows. The radula is connected to the esophagus (e). B) The radula observed from a different angle with emphasis on the connective area between the radula and esophagus.

Slugs retreat their heads into their body as a means of protection when threatened. This means that even though the slugs were humanely euthanized, it was difficult to view the mouth complex. With the frozen slugs, the entire upper portion of the body needed to be removed to access the radula. Overall, this was determined to be an ineffective means of preparation. The radula could be observed more easily in slugs prepared with 7% magnesium chloride and water, allowing the radula to be removed without removing the entire upper portion of the slug body. A small dish was prepared, and the 7% magnesium chloride was added to just coat the bottom. The slugs were individually placed in the dish and allowed to remain until their euthanasia. After determining the location of the radula, an easy means of removal was identified by simply regurgitating the radula from the slug. The radula could be more easily removed if it was first regurgitated from the slug using gentle pressure. This was accomplished by firmly pressing a horizontal probe or needle against the tail and swiping it towards the head. This caused the radula to exsert itself out of the oral cavity so that it could be removed with a scalpel. This means allowed for quick and easy removal without damaging the radular complex. See attached video in the results section for a detailed viewing of this process.

After separating the radula from the rest of the slug, it was rinsed with deionized (DI) water to remove any foreign material or excess mucopolysaccharide. Each radula was then placed in separate 1.5 ml microcentrifuge tubes with 500 μl NET buffer and 10 μl Proteinase K. This protein digested any excess tissue around the radula. The NET buffer was comprised of 1 ml Tris pH 8.0, 2 ml 0.5 M ethylene diamine tetraacetic acid (EDTA), 1ml 5 M NaCI, 20 ml 10% sodium dodecyl sulfate (SDS), and 76 ml deionized water. The tubes were closed and were taped to a mixing platform. The apparatus was then set in the incubator at 37°C for approximately 24 hours.

At the completion of 24 hours, the microcentrifuge tubes were removed from the incubator and placed into a tube holder. All tubes were inspected to confirm all excess tissue had been dissolved. The radulae were then carefully removed from the tubes and rinsed in several changes of DI water to remove all traces of the NET buffer. The radulae were then stored in 25% ethanol until ready for mounting.

Clean radulae were removed from the microcentrifuge tubes and carefully spread onto a glass slide. They were allowed to dry until they could be removed while still retaining their shape, which took approximately one hour. However, it was important to not allow the radula to become too dry, or else they would be too brittle and could crack or rip when being removed from the slide.

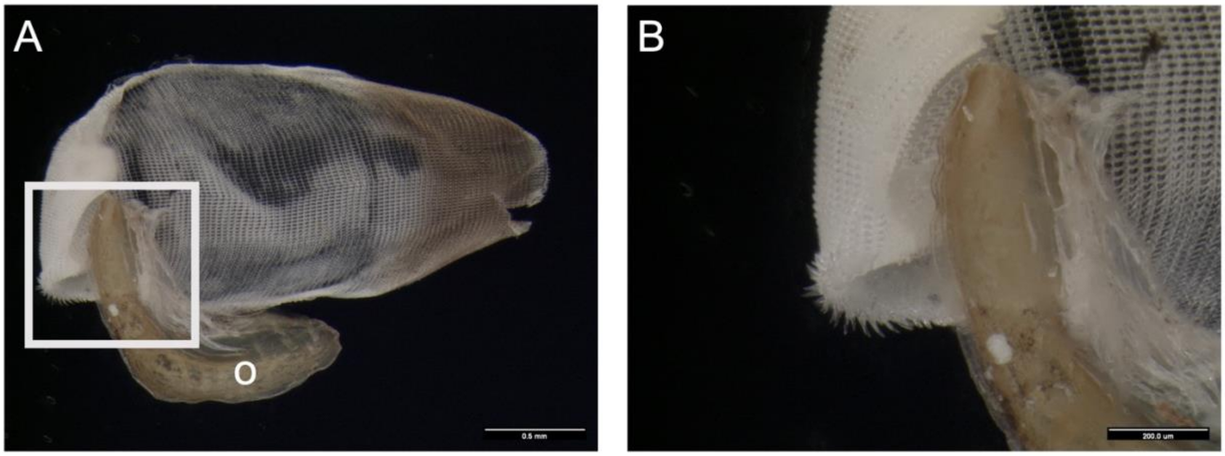

Figure 3. Dried radula viewed under a light microscope with odontophore. A) A large intact radula with part of the U-shaped odontophore (o). The box indicates the enlarged area in B). B) The teeth and odontophore are observed at greater magnification.

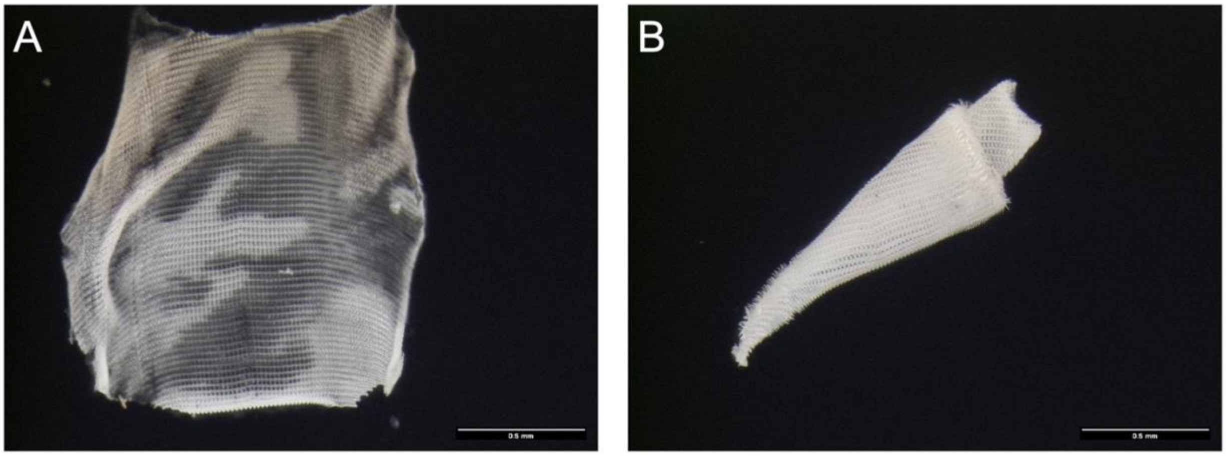

Figure 4. Flat dried radulae and curled dried radulae. A) A radula spread out on double sided carbon tape displaying the interlocking teeth. B) A radula that was allowed to curl during drying allowing for better individual viewing of teeth.

The radula was then carefully manipulated with forceps for mounting using double-sided carbon tape to a JEOL SEM mounting stub. Due to the small size of the radula, approximately three radulae could fit onto one stub. Some radula curled when exposed to air, however this allowed the interlocking teeth to be observed. Figure 5 featured in the results section further explains this phenomenon and significance. In order to enhance image quality and prevent charging effects, the mounting stub was sputter coated with gold. The JEOL SEM mounting stub was then viewed in a JEOL 6510LV SEM at 20kV accelerating voltage.

RESULTS

The optimized radula excision procedure presented in the Methods section was successfully used to prepare garden slug radula for viewing in the SEM as shown in the micrographs in Figures 5. In addition, the preparation procedure was documented in a video that is now available for anyone to view on the web at the following link or QR code.

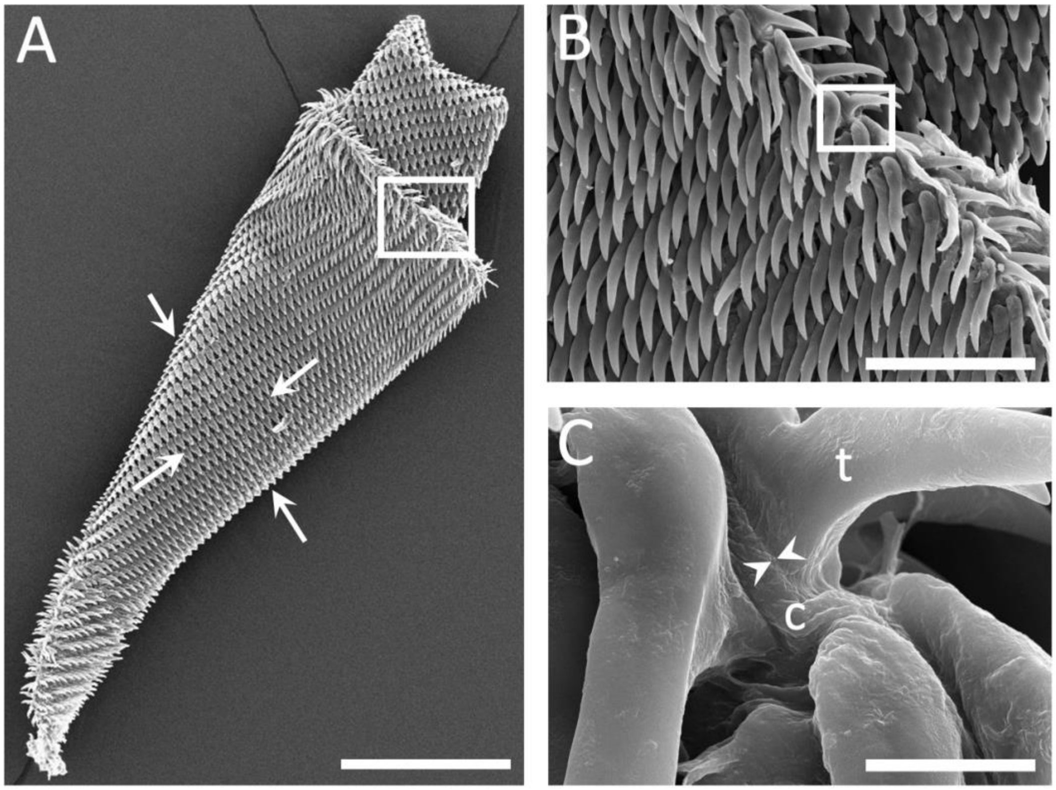

Figure 5: The structures of *Lehmannia valentiana* radulae used for the uptake of food. A single radula sample was isolated using a detergent and proteinase K enzyme, allowed to dry, and treated with 25% ethanol before viewing in the SEM. The radula

shown is curled due to the drying process to expose the interlockings of the teeth. A) The radula comprises several bilaterally symmetrical nearly identical rows of teeth. The teeth are in both horizonal, and vertical rows represented by arrows. The box indicates the enlarged area in B). B) The teeth are observed in greater magnification from a side view to be sigmoidal with a tapering of width from base to tip. When observing the teeth from the top the shoulders are observed. The box indicates the enlarged area in C). C) The teeth are rooted in the cartilaginous remains of the odontophore. The double arrows indicate the boundary between a tooth (t) and the cartilage (c). Scale markers represent 1mm, 50um and 10um in A, B, and C, respectively.

VIDEO PRESENTATION

The following link and QR code leads to a short video presentation I created for the purpose of helping others remove and prepare slug radulae.

https://youtu.be/vNvYB6UEDww

DISCUSSION

The effective means of removal and preparation of the slug radula allowed for the examination of its morphological features with clarity. The curled radula, despite being an accidental occurrence, turned out to be the most revealing. This observation suggests that the shape and arrangement of the radula might have implications for its function and effectiveness in feeding, scraping, and cutting plant material. The curled radula could potentially unveil interesting structural adaptations that contribute to its functionality.

While the study provided valuable insights into the radula of one species of slug, further study is necessary to see if this means of preparation would work on all species of slugs. Slugs are a diverse group, occupying various habitats and niches, and different species may have evolved unique adaptations in their radulae to suit their dietary preferences and ecological roles (South, 2012). Further analysis involving multiple species of slugs is necessary to fully explore this means of radula removal. But one thing is apparent, ecological factors influence the radula's structure and function in relation to the diverse food resources they exploit. Understanding these ecological features can shed light on how and why the radula evolved to meet the dietary requirements of different slug species. From rasping and cutting, to predation and defense, the radula serves as a vital physical attribute of a slug (Prakash et al., 2015).

The video presentation showcasing the effective method for radula removal and preparation serves as a valuable resource for future researchers interested in studying slug radulae. By sharing this knowledge, the study aims to foster the advancement of scientific understanding in this area and encourage further exploration of the radula's morphological and ecological intricacies by a broader community of researchers, both those familiar and unfamiliar with slug radulae.

In conclusion, while the study revealed an effective method for preparing slug radulae and offered insights into its morphological features, there is still a need for further exploration of multiple slug species and different radula shapes to gain a comprehensive understanding of their ecological consequences and evolutionary significance. Sharing this knowledge with future researchers through video presentation can propel the advancement of knowledge in this field and inspire more comprehensive investigations into the fascinating adaptations and functions of the slug radula.

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