How water flow interacts with the rhinophores in *Tritonia tetraquetra*

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Abstract

We were interested in learning how olfactory cues that guide navigation in the gastropod nudibranch *Tritonia tetraquetra*, formerly known as *Tritonia diomedea* are received by the rhinophores in turbulent flow. We measured boundary layer thickness at various speeds, and characterized water flow turbulence caused by their rhinophore. This was done using Particle Image Velocimetry (PIV) around a fixed (dead) rhinophore on a clay model slug. We predicted with increasing flow speed, boundary layer thickness would become thinner. Results from this experiment were deemed inconclusive. We also characterized cilia-generated flow on the clavus of three species of nudibranch (*Tritonia diomedea*, *Tritonia festiva*, *Armina californica*). Dye flow patterns show cilia-driven currents from near the distal region of the rhinophore to the proximal region of the rhinophore. In *Tritonia*, the current originated at the distal tip and flowed proximal into the folds of the clavus, then spread outward towards the circumference of the base of the clavus. In *Armina*, the flow was unidirectionally proximal along the infolds of the rhinophore. The cilia on the rhinophore may help the animal respond faster to odor changes by removing the boundary layer (i.e. sniffing). Scanning electron micrographs showed that the tuft at the distal end of the rhinophore (a.k.a. clavus) had large patches of dense cilia on its vertical inner folds, but lacked dense cilia on the parts of clavus that are more exposed around the circumference.

Introduction

Many marine animals use cues such as water flow and odor during navigation. Several studies have shown that aquatic gastropods are primarily dependent on odor-based navigation (Croll, 1983; Cummins and Wyeth, 2014). The nudibranch mollusk *Tritonia tetraquetra* (Pallas, 1788) (formerly known as *Tritonia diomedea) (*Bergh, 1984) primarily uses odor plumes to navigate through its turbulent environment. An odor plume is defined as a series of fine filaments of odor in the water. Turbulent water flow creates spreading odor plumes with odorless gaps between filaments of concentrated odor as the odor disperses, apparently randomly, from its source. The turbulent nature of an odor plume creates an intermittent temporal chemical cue that the animal will follow as water carries the chemical. While navigating within a turbulent odor plume, *Tritonia tetraquetra* will navigate upstream in the presence of attractive odors (i.e. prey and conspecifics) and downstream in the presence of predators (Wyeth and Willows, 2006a). This navigational strategy where animals travel upstream in the presence of attractive odors is called odor-gated rheotaxis (OGR), and typically, animals that live in a high Reynolds number environment use this strategy. A high Reynolds environment is chracterized by turbulent water flow due to dominant hydrodynamic forces being inertial as opposed to viscous forces. Thus at high Reynolds numbers, parts of the fluid are moving past other parts due to relative velocities, but in low Reynolds numbers the fluid moves in a more laminar flow due to viscous interactions between layers of fluid flow.

T. tetraquerta has two sensory structures used during odor based navigation. The first organ is the oral veil, a flap overhanging dorsal to the mouth, and is thought to be involved in flow direction detection (Murray and Willows, 1996) as well as touch and taste (Willows, 1978). The rhinophores, which are a pair of rod shaped structures located on the head in marine gastropod opisthobranch mollusks, is another second structure used during navigation (Figure 1). It has been shown that the rhinophores are specialized chemosensory structures necessary for navigating within turbulent odor plumes (Wyeth and Willows, 2006b). For the purpose of this paper, we will focus strictly on the rhinophores.

Figure 1. Photograph of *Tritonia tetraquetra* with rhinophores and oral veil present.

In this project, we were interested in studying boundary layer thickness around the rhinophores in *T. tetraquerta* because boundary layer thickness will dictate how fast an animal can respond to odor changes in the water. By definition, a boundary layer is a layer of fluid that sticks to and is carried along the surface of an object or body. When water interacts with an object, the water will slow down and form a boundary layer around the surface of the object. Boundary layer thickness will dictate how fast odor can diffuse across and reach the slug's rhinophore, thus limiting the latency of the slug's response to odor changes. It is expected that molecular diffusion across a thick boundary layer will slow response time to changes in odors, whereas molecular diffusion across a thinner boundary layer would allow for faster response time to changes in odor.

Although a thin boundary layer would allow for a faster response time, it is possible the slug might have evolved to delay responses and instead average their odor input over time to increase signal to noise. Turbulent water flow creates odor plumes as the odor disperses, apparently randomly, from its source. The nature of an odor plume creates a patchiness of scent that comes and goes, thus providing a noisy "on/off" signal. With the animal's olfactory targets being slow moving (i.e. sessile prey, slow slugs, fairly slow predator), *T. tetraquerta* may only need to update their olfactory information every few seconds or longer.

T. tetraquetra is a good model for examining the neural basis underlying navigation. Unlike other animals that navigate over long distances, *T. tetraquerta* have shorter navigational distances that allow for behavioral observations in the laboratory and the field to be feasible. With this project we were interested in studying how water flow interacts with the rhinophores in *T. tetraquerta* to gain a greater understanding of how olfactory cues that guide navigation are detected. Our objectives were to measure boundary layer thickness around a fixed *T. tetraquetra* rhinophore clavus by measuring velocity fields using Particle Image Velocimetry (PIV). The second objective was characterize the nature of water flow turbulence around the rhinophore. We predict that the boundary layer becomes thinner with increasing speed. With these objectives in place, we hope to help address very important questions: how often does the slug update sensory information from the rhinophore, and how is odor transduced by the rhinophore.

Material and Methods

Animals

Tritonia tetraquetra and *Ptilosarcus gurneyi* were collected by SCUBA diving and maintained in the sea tables at Friday Harbor Laboratories, Washington, USA. Slugs were fed sea pens, *Ptilosarcus gurneyi ad libitum.*

Procedure for making model of rhinophore

A slug was relaxed in a phenoxypropanol solution for a total of two hours. Once the slug was fully relaxed, with the rhinophores fully extended, a hemostat was clamed at the base of a rhinophore. While holding the hemostat in place, a cut was made proximal to the rhinophore and the still-inflated rhinophore was placed in a small petri dish. In order to fix both rhinophores, a glutaraldehyde solution was poured into each petri dish and left to soak in the refrigerator overnight. The next day, the glutaraldehyde solution was removed from both petri dishes and replaced with a 0.2M Millonigs phosphate-buffered saline (PBS) solution. Both rhinophores were soaked in the PBS solution three times: 2 minutes (1x) and 10 minutes (2x). Immediately after this step, the rhinophores were dehydrated in series of ethanol solutions: distilled water, 30%, 70, 80%, 90%, 95% and 100%. Each rhinophore was allowed to soak once in the solution for a total of 10 minutes, however, with the exception of the 100% ethanol solution being repeated three times while the rhinophores were placed on an orbital shaker. The rhinophores were then dried in the a critical point dying (CPD) machine, a Sandri-790 Critical Point Drier in the Lab 8 Microscopy room. This machine allowed for the tissue to dry without leaving artificial markings on the rhinophore. To properly use this machine,

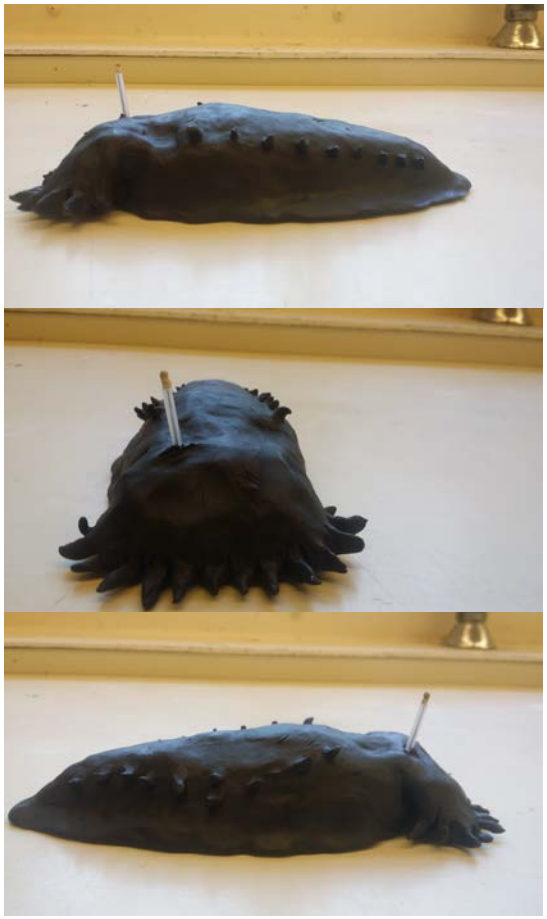

Figure 2. Clay model of *Tritonia tetraquetra* with rhinophore clavus attached.

locate the Sandri-790 Critical Point Drier in the Lab 8 Microscopy room. Pictures of both pre-dehydrated and dehydrated rhinophores were taken under a dissecting microscope. Lengths and widths were taken in ImageJ to calculate differences between rhinophore sizes.

A model of the basic body shape of *T. tetraquetra* was created by molding Sculpey III Polymer Clay. A model rhinophore was attached to the top of the model's head, consisting a needle wrapped in clear plastic tubing with a dehydrated rhinophore clavus at the top of the needle (Figure 2).

Video recording experiments

The clay *T. tetraquetra* model was placed into a recirculating water flow flume (124 X 17 X 20 centimeters). A 37 cm working area tank showed a mostly laminar flow. Three flow straighteners were pressed together (from upstream to downstream: 37 mm thick with 3mm holes, 53 mm thick with 3mm holes, and 62 mm thick with 5 mm holes) were placed upstream of the working section to help generate more laminar flow. Flow straighteners were tall enough to prevent foam and overflow. Video recordings of water movement around the slug's rhinophores were captured using a video camera mounted

above the tank (Sony Model TRV-75). Video was digitized at 30 frames a second using a Canopus model ADVC-300 and Security Spy software v.3.4 (Bensoftware). A laser light (low power setting) (Wicked Lasers S3 Spyder III Krypton**)** equipped with a lens (Krypton extended laser kitt) that turn the beam into a thin sheet (~ 1mm; 30° lateral spread), was placed on side of flume tank to illuminate the flow of water, particles and rhinophore of animal. Neutrally buoyant old (~1996) brine shrimp eggs cysts (brand: Premium Sanders Great Salt Lake *Artemia* Cysts) were added to the water circulating the tank to mark the flow of water in video recordings. Five flow speeds were used for each experiment: 0.5, 1, 2, 4, 8 and 10 cm/s per second. Before each new speed, a short video recording of a ruler immersed within the tank was recorded to calibrate the speed of water flow. A video sequence of the ruler in water at the same level of the laser sheet was imported into ImageJ (v.2.0.0) to calibrate the scale bar. Control video experiments involved video recordings of unimpeded flow (i.e. no slug or ruler) to quantify flow speed and to characterize the absence or degree of turbulence. The flow of 100 particles was tracked manually for each flow speed. After each experiment, a short recording was made to ensure flow speed did not change. For each trial, a slug with fully extended rhinophores (no large gap between rhinophore sheath and rhinophore) illuminated from the right side by a laser sheet, was recorded, with recording lasting for 5 seconds or longer. Water temperature was maintained at 12°C throughout experiment using plastic bottles sea ice downstream to the working section before the propeller. Between all trials, the tank was drained and all surfaces were scrubbed with a soft sponge to avoid scratching and rinsed with fresh water. All data was analyzed with ImageJ2 with a PIV (Particle Image Velocimetry) plug-in created by Oingzong Tseng.

Results

Particle Image Velocimetry Results

Water speed in PIV videos is coded according to vector length (arrows) and color. Slower water flow is indicated by small purple and blue arrows, whereas faster water flow is indicated by larger orange and red arrows. A transparent image of the left rhinophore was added to all PIV videos.

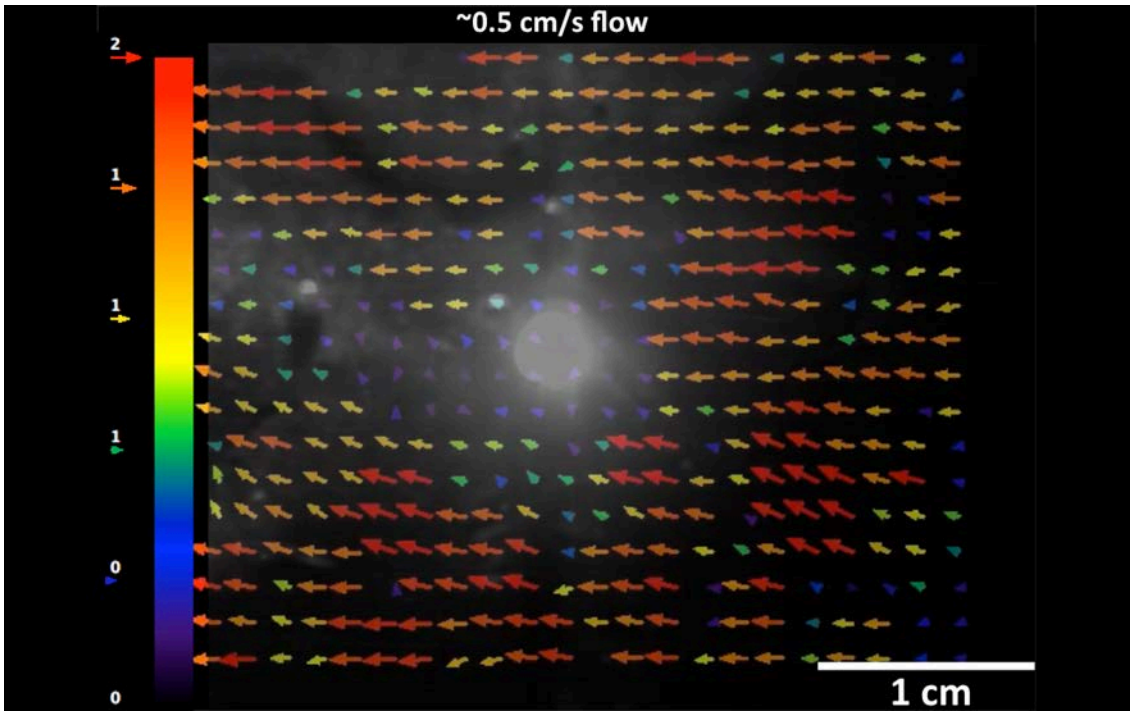

Figure 3. PIV results for 0.5 cm/s water flow around rhinophore clavus.

In 0.5 cm/s laminar flow, there was an approximately 1 mm thick boundary layer upstream and lateral to the rhinophore clavus, as well as turbulence several mm downstream (Figure 3).

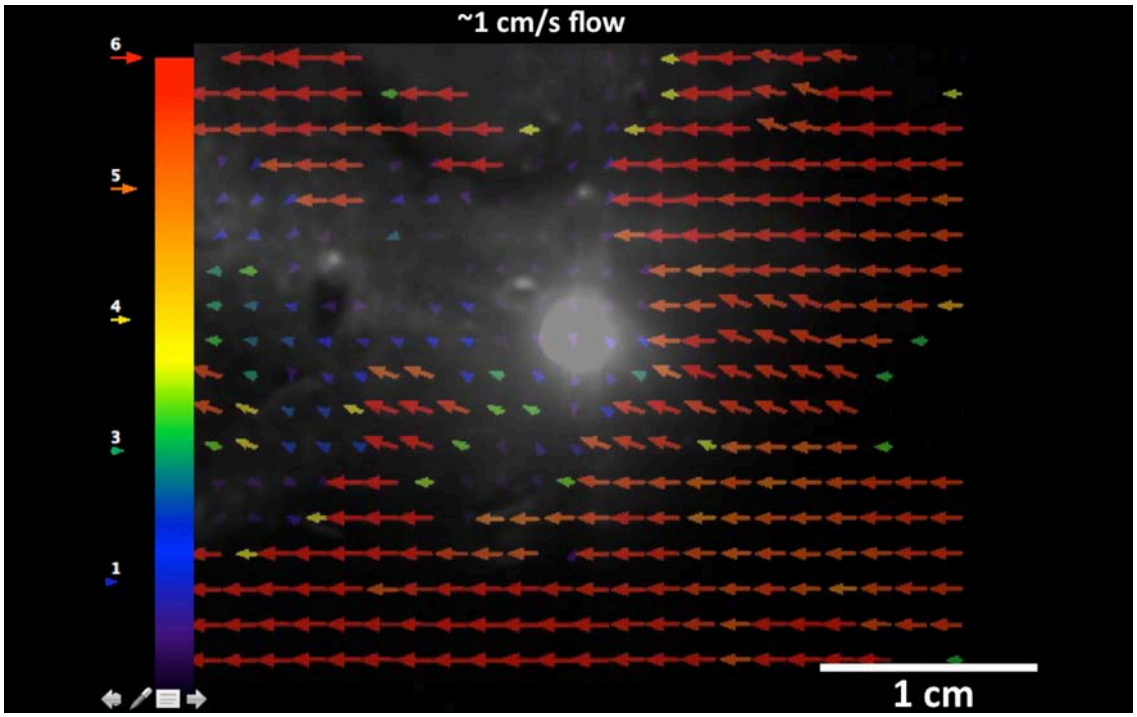

Figure 4. PIV results for 1 cm/s water flow around rhinophore clavus.

In 1 cm/s laminar flow, there was an approximately 2-3 mm thick boundary layer upstream and lateral to the rhinophore clavus, as well as turbulence for several mm downstream (Figure 4).

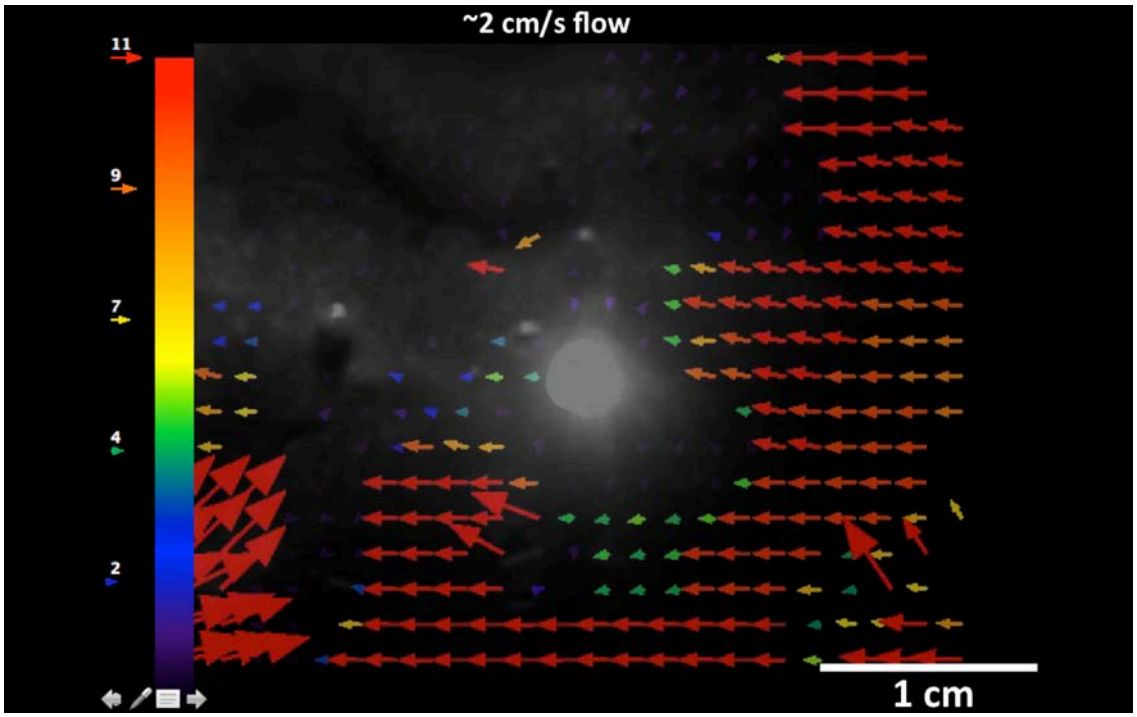

Figure 5. PIV results for 2 cm/s water flow around rhinophore clavus.

In 2 cm/s laminar flow, there was an 3 mm boundary layer upstream and lateral to the rhinophore clavus, as well as and evidence of turbulence for several mm downstream. In this video, there were areas of highly accelerated water flow (large red arrows near bottom corners and center of photo) (Figure 5).

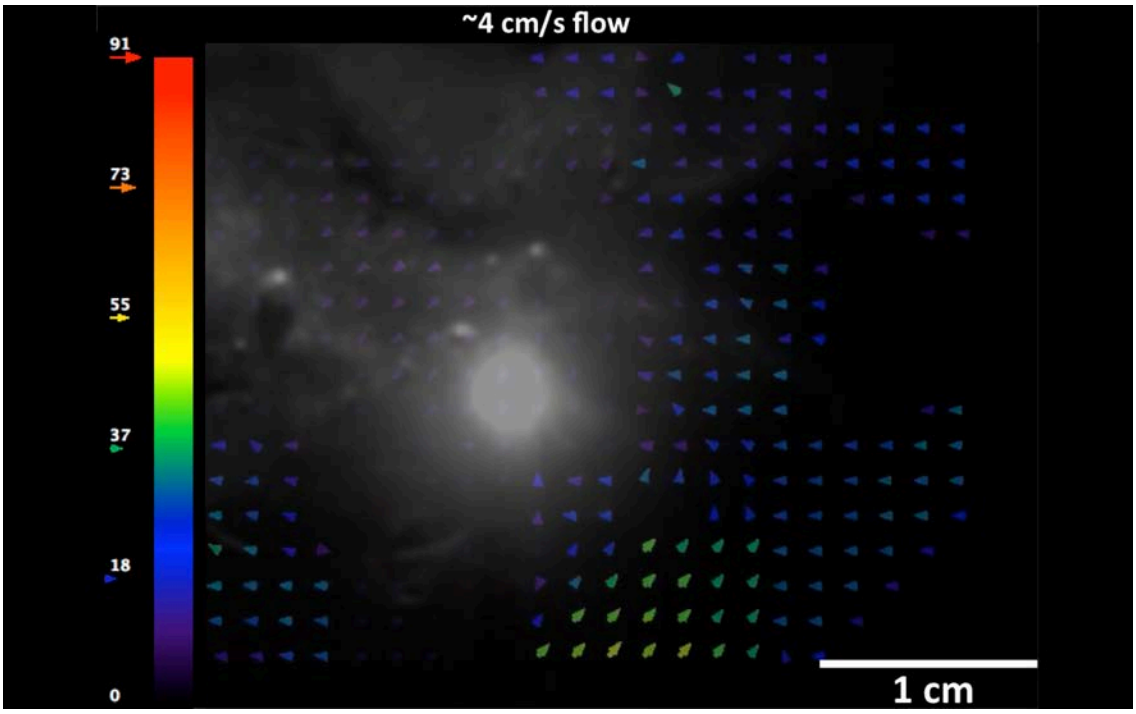

Figure 6. PIV results for 4 cm/s water flow around rhinophore clavus.

In the 4 cm/s laminar flow, the presence of a boundary layer and turbulence was left undetermined (Figure 6).

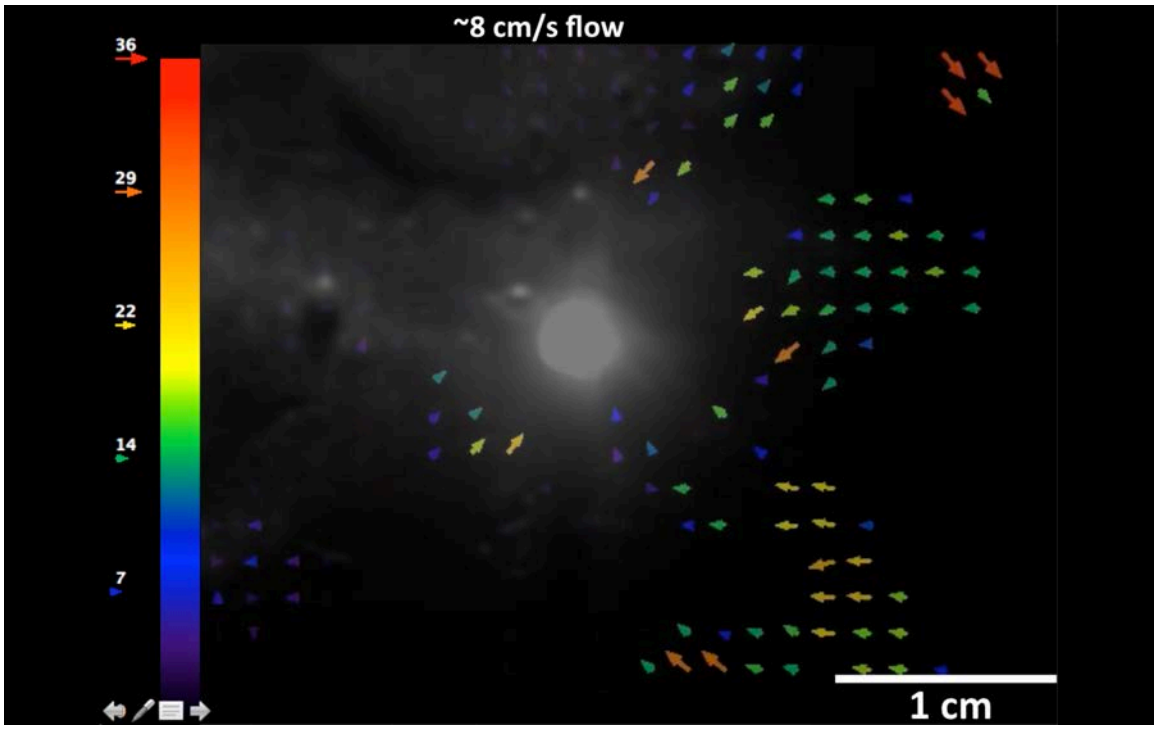

Figure 7. PIV results for 8 cm/s water flow around rhinophore clavus.

In the 8 cm/s laminar flow, the presence of a boundary layer and turbulence was left undetermined (Figure 7).

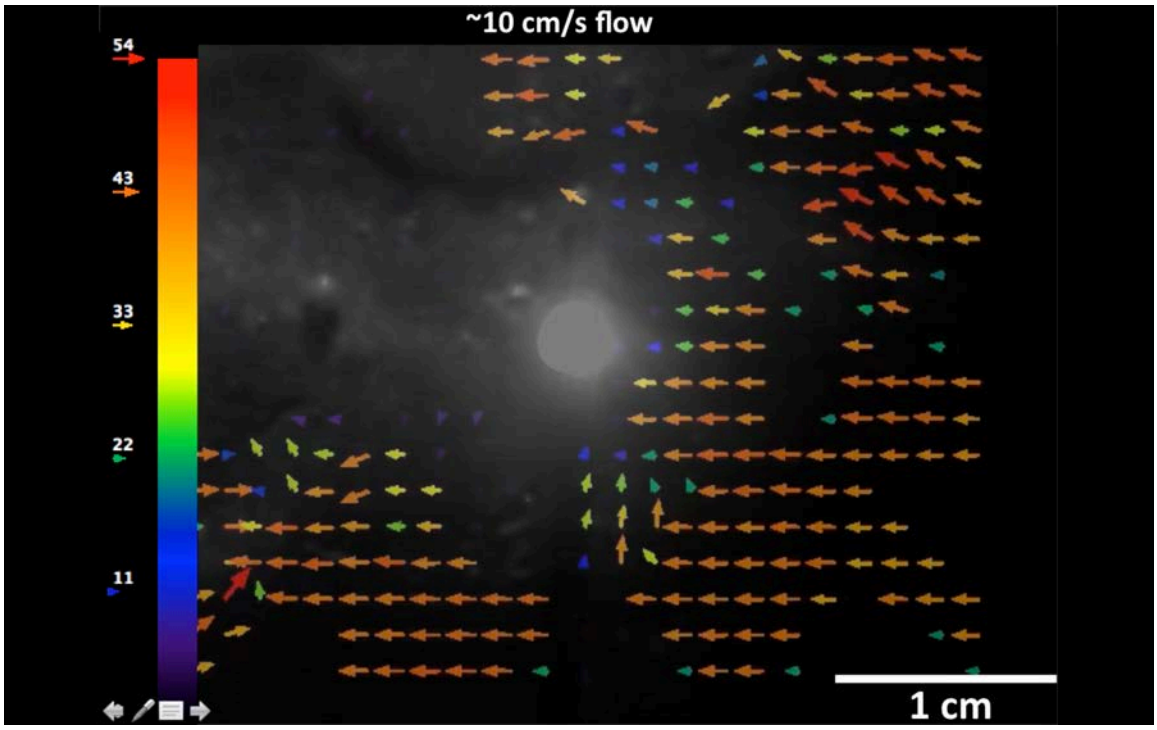

Figure 8. PIV results of 10 cm/s water flow around rhinophore clavus.

In 10 cm/s laminar flow, there was an approximately 1 mm thick boundary layer upstream and lateral to the rhinophore clavus, as well as turbulence several mm downstream (Figure 8).

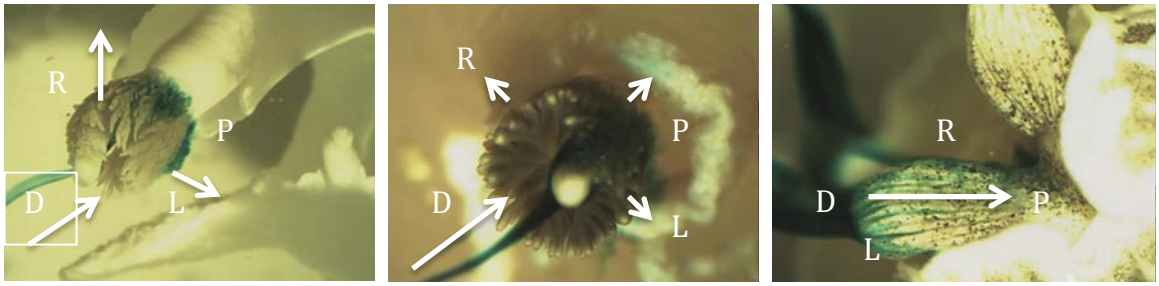

Figure 9. Magnified image of cilia located on the interior vertical folds of a *Tritonia tetraquetra* clavus. "L" indicates left. "R" indicates right. "D" indicates distal. "P" indicates proximal. Arrows indicate flow of dye.

Dye flow experiments were performed on anesthetized *Tritonia festiva*, *Tritonia tetraquerta* and *Armina californica*. The same flow pattern was seen in both *Tritionia* species. The cilia generated current originated at the distal tip of the rhinophore and flowed proximal into the folds of the clavus, then spread outward towards the circumference of the base of the clavus (Figure 9). A scanned electron miscopy (SEM) image of the interior vertical folds of the distal end of a T. tetraquetra clavus had large patches of dense cilia (Figure 10). This observation was not seen on the outer exposed regions of the clavus. For *Armina californica*, dye moved in a unidirectional pattern with dye moving from distal to proximal all along the infolds

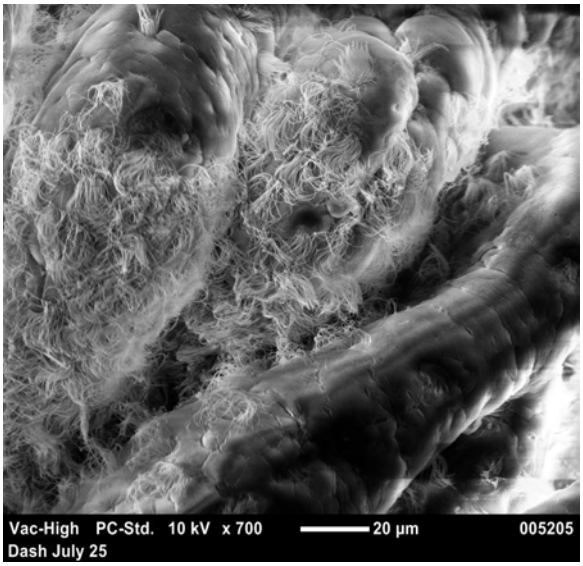

Figure 10. SEM photograph of cilia located on the interior vertical folds of a *Tritonia tetraquerta* rhinophore clavus.

of the rhinophore (Figure 9).

Discussion

We were interested in measuring boundary layer thickness around the rhinophore at various speeds. We predicted that with increasing speed, boundary layer thickness would become thinner. Results from this experiment were not clear enough to support or reject our prediction. The boundary layers in the 0.5, 1, 2 and 10 cm/s PIV videos ranged from 1-3 mm thick. Additionally, the 4 and 8 cm/s PIV videos were hard to interpret. Thus the results from this experiment were deemed inconclusive.

One possibility that contributed to the results being inconclusive is the body shape of the clay model. Although the clay slug was modeled after the body shape of *Tritonia tetraquerta,* the body shape of the model was not anatomically correct. The bulky body shape of the slug may have interfered with the laminar water flow and caused the flow to accelerate in certain areas of the video. This would explain the large red arrows seen in figure 5.

It is possible *Tritonia* may have behaviors that are able to manipulate boundary layer thickness. In a study analyzing the behavior of *T. tetraquerta* in prey odor plumes, the animal displayed a behavior of lateral head sweeping while crawling upstream towards an odor source (McCullagh et al., 2014). This behavior is predicted to increase sensitivity to odor detection within odor plumes by tearing of the boundary layer around the rhinophores. Thus this tactic would give the animal a faster response to odor changes within the water.

It is also possible the cilia located on slug's rhinophore is another strategy for the animal to manipulate boundary layer thickness. Results from the dye flow experiment showed a general pattern of cilia pulling the dye from the distal tip to the promixal end of the rhinophore in the slug species used in this study. This is important to note because when the animal projects its rhinophores into the water column, part of its rhinophores lie slightly outside the boundary layer. As the cilia pulls in currents from outside the boundary layer, it is possible chemical cues in the surrounding water will be detected by the rhinophore. We predict this strategy allows the animal to respond faster to odor changes within the water.

Cilia generated current as a possible navigational tool for *A. californica* is interesting because it spends its day buried in the sand with its rhinophores slightly projecting above the surface of the sediment (Marcus, 1961). While buried in the sand, it is possible the animal uses the cilia on its rhinophore to pull odors from the surrounding sediment.

There are two general types of cilia: motile and non-motile cilia. Motile cilia drags currents along the surface of the cell while non-motile cilia act as a sensory antenna for the cell to receive signals from external cues (Shah et al., 2009). We believed the cilia seen in the dye study were motile cilia because these types of cilia are usually present on a cell's surface in large numbers and beats in coordinated waves. In the SEM photographs there were large patches of dense cilia on the interior folds of the clavus in both *Tritonia* species. Addtionaly, the rhythmic beating of the cilia is most likely responsible for driving the dye across the surface of the rhinophores. Thus, this further

supports the likely presence of motile cilia on the rhinophore's in the three slug species used in this study.

Conclusions and Future Directions

Our results were unable to support or reject our hypothesis. For example, we did not see boundary layer thickness get thinner as water flow increased. Instead we saw boundary layers ranging from 1-3 mm thick despite the speed of water flow in most PIV videos. Additionally, two videos (4 and 8 cm/s) were hard to determine the presence of a boundary layer and turbulence. Thus the results from this part of the experiment were deemed inconclusive. It is possible the anatomically incorrect slug model was cause of weak results. For future experimentation, a slug that is anatomically correct would help in getting conclusive results.

Dye flow patterns show cilia-driven currents from near the distal region of the rhinophore to the proximal region of the rhinophore. In *Tritonia*, the current originated at the distal tip and flowed proximal into the folds of the clavus, then spread outward towards the circumference of the base of the clavus. In *Armina*, the flow was unidirectionally proximal along the infolds of the rhinophore. We predicted that cilia generated currents would allow for the animal to respond faster to odor changes in the water. For future experimentation, characterizing the physical differences between the two types of cilia should be conducted. This would help gain an understanding on the role cilia play in odor-based navigation.

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