Assessing muscle function in the hagfish feeding apparatus of *Eptatretus stoutii*

Jasmine Fuerte-Stone[1,2], Andrew Clark[1,3], Theodore Uyeno[1,4]

Blinks-NSF REU-BEACON 2015

Summer 2015

[1]Friday Harbor Laboratories, University of Washington, Friday Harbor, WA 98250
[2]Department of Bioengineering, University of Washington, Seattle, WA 98195
[3] Department of Biology, College of Charleston, Charleston, SC 29424
[4]Valdosta State University, Valdosta, GA 31698

Contact Information:
Jasmine Fuerte-Stone
UW Department of Bioengineering
William H. Foege Building
3720 15th Ave NE Seattle, WA 98195-5061
jjfuerte@uw.edu

Keywords: Pacific hagfish, *Eptatretus stoutii,* feeding apparatus, muscle strain

Abstract

Hagfish are marine chordates that, despite lacking jaws, are able to feed on large, tough carcasses with the protraction and forceful retraction of toothplates. Using muscle stimulation experiments, we studied the function of the feeding apparatus muscles in the Pacific hagfish, *Eptatretus stoutii*, and compared our results with muscle activity patterns recorded from previously studied Atlantic hagfish *Myxine glutinosa*. Most of our efforts addressed function in the four major feeding apparatus muscles (the deep protractor, sphincter, perpendicular, and retractor muscles), and our stimulations of superficial musculature (superficial protractor muscles and rectus muscles) were assessed qualitatively. Stimulation experiments (tetanic contractions stimulated at 80 V, 60 Hz, 1 ms duration) were performed on *in situ* and excised feeding apparatuses from five animals positioned supine, while toothplate movements and muscle shape changes (strain) were analyzed from video recordings at 120 Hz. Our results confirm previous observations of *M. glutinosa*; in *E. stoutii*, the deep protractor muscles induce toothplate protraction, while activity in the retractor, sphincter, and perpendicular muscles power retraction of the toothplates. In both species, toothplate protraction causes the cylindrical feeding apparatus to decrease in length and increase in diameter, and vice versa during retraction. Upon excision from the body, the feeding apparatus in *E. stoutii* undergoes greater proportional length changes but smaller changes in width, suggesting that the soft tissue connections to the body wall impose constraints on muscle displacement. Because it comprises a large part of the head region (anterior 20% of total length), constraining the cylindrical

shape of the feeding apparatus should work in retaining the cylindrical shape of the head while impeding unwanted head deformations in situations where the head must fit and maneuver in tight spaces (e.g. knotting and burrowing behaviors).

Introduction

Pacific hagfish *Eptatretus stoutii* (Lockington, 1878) are elongate, jawless fishes that can be found in benthic marine environments ranging from 100 m to 500 m deep (Martini, 1998). Hagfish are largely recognized as opportunistic scavengers (Martini, 1998), however there are reports of some hagfishes actively seeking live free-swimming prey (Zintzen et al., 2011). Hagfishes do not benefit from the pliers-like opposing leverage in gnathostome biting systems, yet they are capable of forcefully biting into prey with a complex battery of keratinous teeth and small flexible supportive cartilages collectively called a toothplate (Clark and Summers, 2007). Jawless biting in hagfishes involves cyclic three-dimensionally complex protraction and retraction of the toothplate plate (Figure 1). The protraction movements are dynamic and when maximally protracted provides the hagfish with proportionally immense gapes, and during retraction, the toothplate is capable of exerting forces onto its food with comparable magnitudes to jawed vertebrates of similar size (Clark and Summers, 2007). When struggling with exceedingly large or tough prey items, the toothplate mechanism is often integrated with whole body knotting behaviors. Here, a figure 8 knot is formed at the tail and is slid towards the head (W.A. Haney, pers. comm.). As it passes, the advancing, anterior-most loop of the knot is pressed against the prey and antagonizes the toothplate retraction.(Uyeno and Clark, 2015).

Toothplate movements are controlled by a cylindrical feeding apparatus situated in the anterior 20% of the body, composed of a few unmineralized cartilages and complex arrangements of softer muscle and connective tissues. The hagfish feeding apparatus (HFA) possesses a hard region and a soft region (Clark *et al*., 2010). The toothplate is located in the hard region where it is positioned above a series of supportive cartilages called a basal plate. In addition to supporting the toothplate, the basal plate provides the origin attachment for some of the muscles that control toothplate and body movements (Figure 2). Muscles within the hard component include the four heads of the deep protractor muscles and two heads of the overlying superficial protractor muscles (Cole, 1907). Posterior to the hard component, is the soft part of the feeding apparatus, which includes a cylindrical muscular hydrostat comprised of three muscles: the tubulatus muscle (here, we refer to this muscle as the sphincter muscle), clavatus muscle (here, referred to the retractor muscle), and perpendicularis muscle (here, referred to as the perpendicular muscle) (Ayers and Jackson, 1901) (Figure 3).

The deep protractor muscle is located in the hard component of the feeding apparatus. It originates from the ventral surface of the basal plate and inserts onto the anterior margin of the toothplate. It consists of two medial and two lateral heads with longitudinally arranged muscle fibers. The deep protractor muscle powers protraction of the toothplate (Clark et al., 2010). The retractor muscle bears a strong resemblance to a human gastrocnemius muscle; it is a bipennate muscle with a long, narrow tendon that inserts onto the posterior margin of the toothplate. Posterior to the tendon, the muscle tissue of the retractor originates from the soft component of the feeding apparatus, where it

interconnects with the perpendicular and sphincter muscles. Semicircular sphincter muscle fibers envelope the anterior two thirds of the retractor muscle. Vertically oriented transverse fibers of the perpendicular muscle divide the heads of the posterior third of the retractor muscle (Figure 2)

In a previous study, Clark and his coworkers (2010) described the muscle activity patterns in the protraction of the feeding apparatus in the Atlantic hagfish *Myxine glutinosa*. They determined that simultaneous contraction of all musculature in the soft region (the retractor, perpendicular, and sphincter muscles) caused the area to become turgid and form the origin for the retractor muscle. In that study, it was also hypothesized that the soft region undergoes minimal deformations, or strains, and that it resembles the hydrostats controlling cephalopod beaks, which are known as muscle articulations (Uyeno and Kier, 2005). If this were the case, it would be the first of its kind to be described in a chordate (Uyeno and Clark, 2015)

In this study, I sought to address whether *Eptatretus stoutii* feeding apparatuses function in the same manner as *Myxine glutinosa* feeding apparatuses using electrophysiological stimulation experiments and kinematic analyses of toothplate movements. I observed the effect of direct muscle stimulations to study the muscular function of the *E. stoutii* feeding apparatus. I focused on the roles of specific muscles (deep protractor, sphincter, retractor, and perpendicular muscle) as they pertain to dental plate protrusion. I hypothesized that stimulation of the deep and superficial protractor muscles located near the basal plate are what cause the protrusion of the dental plate while feeding (as seen previously in Atlantic hagfish, *Myxine glutinosa,* Clark et. al. 2010).

Materials and Methods

Animal Care

Twenty specimens of *Eptatretus stoutii* were donated by commercial fishermen, Mr. Rodney and Mr. Bong Jo Kim of the Port Angeles Fish Co. Inc. in Port Angeles, WA. Officer Donna Downs (WA DFW), Mr. Andy Inscore and Dr. Adam Summers (University of Washington) helped us transport the animals back to Friday Harbor Laboratories, WA. The animals were housed in five-gallon buckets that were made to float in a large tank within the flow through seawater system. Holes in the sides of the buckets allowed for circulation. The water was maintained at approximately 10^{o}C and each bucket contained four animals. For acclimation to the testing environment, animals were then transferred to solitary housing in five-gallon buckets that were fitted with perforated lids to allow us to circulating seawater within. The buckets also contained approximately 8 cm of fine to medium grain sand. Buckets were covered with black plastic bags to decrease light; this was found to be a valuable practice, as the animals were less likely to release slime under these conditions.

Stimulation experiments

Five animals (*TL* = 45.2 cm – 54.0 cm) were euthanized with an overdose of tricaine methanesulfonate (MS222). While tissues were still viable, a ventral incision was made to reveal the feeding apparatus and to perform the stimulation experiments. Individuals were positioned supine on a dissection table. An initial incision made approximately three cm posterior to the end of the feeding apparatus on the ventral surface and extended anteriorly to the mouth. The skin and extrinsic musculature were disconnected from the feeding apparatus before applying field electrodes. Stimulations were applied with a pair of 1.20 mm

diameter field electrodes soldered to 0.5 mm diameter piano wire attached to Grass SD9 stimulator (Grass technology, Warwick, RI). Maximal tetanic stimulation was found to occur using trains of stimulations at 60 Hz, 80V, and of 1.0 ms duration. Electrodes were placed on the medial heads of the deep protractor muscles and the anterolateral parts of sphincter muscle while videotaping the protraction and retraction movements of the toothplate. Stimulations were filmed with a Casio Ex-FH25 camera recording at 120 Hz.

Kinematic time variables (protraction and retraction durations) and linear measurements (longitudinal and transverse strains) from the feeding apparatuses were extracted from frames of the video. Protraction duration was measured from the first frame that protrusion could be detected by eye and ended when gape was at its fullest. Retraction time referred to the elapsed time between the frame in which the toothplate began moving back in and full toothplate retraction. Frame counts of these durations were divided by the frames per second of the recording to obtain time. The first and last frames of the protraction duration were used to measure initial and final length of entire feeding apparatus, as well as the initial and final length and width of the soft component. Measurements were made using ImageJ (FIJI; http://Fiji.sc). From these equations longitudinal and transverse strains were calculated using the following equation

$$\text{Strain} = \frac{\Delta L}{L_o}$$

where ΔL was the change in length or width and L_o was the initial length or width. This was done in order to normalize the data and account for variances in

size and shape of feeding apparatuses. Width measurements are thus converted to transverse strain and length measurements to longitudinal strain.

Observational experiments were also performed on two of the animals *in vivo*. Each muscle of the feeding apparatus was stimulated with the specification previously mentioned and the effects of the stimulations were observed. The superficial protractor muscle and each head of the deep protractor muscle were stimulated individually for protraction. The sphincter muscle, retractor muscle, and perpendicular muscle were stimulated individually

Electromyography

Fine wire electrodes were made from approximately 60 cm of 0.06mm diameter bipolar stainless steel fine wire with 1mm of insulation removed from the ends. Electrodes were inserted into target muscles using the tip of a hypodermic needle (Basmajian & Stecko, 1962). Electrodes were connected to A-M Systems Model 1700 differential AC amplifier (A-M Systems, Sequim, WA). The amplifier was set at a gain of 1000x and had a notch filter, as well as a high pass filter with a cut off of 1.0 Hz and a low pass filter with a cut off of 10,000 Hz. Animals were anesthetized for approximately 15 minutes (100mg/L MS222 and 200mg sodium bicarbonate/L in salt water). Electrodes were inserted into muscle wall on either side of body approximately 1cm posterior to the last ventilation hole. Animals were then placed in a glass aquarium and filmed using two Phantom Miro 210 cameras (Vision Research Phantom, Wayne, NJ) filming the lateral and bottom aspects of the aquarium. EMG data were collected as the animal swam. Data were then rectified and a Butterworth filter of 0.0001 Hz was

applied to decrease the presence of noise using Matlab code modified from Uyeno 2007.

Results

Function of Feeding Muscles

Stimulating specific muscles always resulted in specific reactions. I found the superficial protractor muscles and lateral heads of the deep protractor muscles are not required for protraction. The medial heads of the deep protractor muscle were the only locations that would induce protraction of the tooth plate when stimulated. Stimulation of the lateral heads of the deep protractor muscles would not cause protraction, nor did stimulation of the superficial protractor muscles. Retraction of the tooth plate occurred when any of the sphincter, perpendicular, and retractor muscles were stimulated individually. I also observed that stimulating the rectus muscle causes retraction of the toothplate by pulling back on the basal plate. This shows that the basal plate, though not as dynamic as the toothplate, is motile and its motion resulting from the rectus muscle suffices for inducing retraction in the absence of activity from the muscles in the feeding apparatus. It was also noticed that toothplates passively retracted fully and/or partially after maximum protraction.

Timing of Muscle Activity Onset/Offset during Protraction and Retraction Cycles

Average times of active protractions and retractions were measured from stimulation experiments conducted on both *in situ* and excised feeding apparatuses (Figure 4). On average, *in situ* feeding apparatuses induced maximum toothplate protraction in 395 ms and retracted actively in 126 ms. Protractions and

retractions in the excised feeding apparatuses were slower: the apparatus protracted the toothplate in 740 ms on average and retracted it in 118 ms. Protraction and retraction times were not significantly different between in situ and excised muscles. (T test, single tailed, parametric: Protraction; $p=0.164>0.05$. Retraction; $p=0.673>0.05$). A passive elastic recoil was often seen between when the deep protractor muscle was no longer stimulated and when retraction force was being generated by the sphincter muscle. During this period, the tooth plate would partially fold back into the mouth. This occurred during every active retraction and on average took 278 ms with *in situ* feeding apparatuses and 716 ms with excised feeding apparatuses.

Deformation of the Feeding Apparatus

As the roughly cylindrical feeding apparatus was constant in volume (no fluids were gained or lost from this solid block of muscle), increases in length resulted in decreases in width, and vice versa. These length changes were observed and measured in both *in situ* and excised feeding apparatuses. On average, the width of the *in situ* feeding apparatuses was 1.77 cm while retracted and 1.88 cm while protracted. For excised apparatuses, the values were significantly greater ($p=0.045<0.05$): average retracted width was 1.97 cm and the average protracted width was 2.02 cm. There was also a statistically significant difference ($p=0.033<0.05$) in retracted width of excised feeding apparatuses and *in situ* feeding apparatuses. Likewise, the difference in width of protracted feeding apparatuses was also significant ($p=0.033<0.05$). The average length of the *in situ* feeding apparatuses was 10.77 cm while retracted and 9.84 cm while

protracted. Interestingly, in the excised apparatus, the values are no different; the average retracted length was 11.16 cm and the average protracted length was 9.86 cm. There was no significant difference in retracted lengths ($p= 0.745>0.05$), nor was the difference in protracted length ($p=0.393>0.05$). These measurements are displayed in Figure 5. Transverse strain was found to be .07 for *in situ* apparatuses and .02 for excised apparatuses. Longitudinal strain was found to be .08 for *in situ* apparatuses and .11 for excised apparatuses. These values are found in Figure 5.

Electromyography

Results from the electromyography experiments showed muscle activity (spikes in voltage) on the concave side of the animal. By synchronizing high speed video with EMG records, I was able to correlate a right side bend of the body with muscle activation spikes recorded at 5.1s in the right side EMG trace (Figure 6). Similarly, Figure 6 also shows left side muscle activity between 6 and 6.5s that correspond to left side body bends.

Discussion

Function of Feeding Muscles

The qualitative results of the feeding apparatus muscle stimulation experiments are compatible with conclusions made using EMGs recorded during live feeding of *M. glutinosa* (Clark et al., 2010). Specifically, EMGs determined that only the deep protractor muscles were activated during protraction and all three muscles in the soft component were activated during retraction (Clark *et al.* 2010). The observations that only the medial deep protractor muscles induced protraction with stimulation and that all three muscles in the soft component

produced retraction in *E. stoutii* indicate that the two species have similar muscle functions within the feeding apparatus.

While lateral heads of the deep protractor muscle are robust, they lack the ability to protract the toothplate alone. This may imply that they provide another function, such as powering the unfolding of the teeth to a greater extent than protrusion alone. It has been hypothesized that the superficial protractor muscles function in revealing the teeth from overlying soft tissues during protraction, though it is not forceful enough to power toothplate protraction by itself without the aid of the deep protractor muscles (*sensu* Cole, 1907; Dawson, 1963).

Timing of Muscle Activity Onset/Offset during Protraction and Retraction Cycles

The durations of bite cycle components from our protraction/retraction stimulation experiments in *E. stoutii* and those recorded during EMG and kinematic studies on *M. glutinosa* during live feeding (Clark and Summers, 2007; Clark et al., 2010) are comparable. Figure 7 displays the time components of the bite cycle found in the living feeding and stimulation experiments. Though protraction is similar in *M. glutinosa* live feeding and *E. stoutii* stimulation at 300 ms, retraction in live feedings is slower than the average time found in stimulation; approximately 600 ms and 150 ms respectively. Adding in the passive elastic recoil found between stimulating protraction and stimulating retraction gave a comparable time for total retraction.

Deformation of the Feeding Apparatus

In situ and excised feeding apparatuses showed differences in strain patterns and kinematics. While *in situ* feeding apparatuses showed greater transverse strain, excised feeding apparatuses showed grater longitudinal strain

(Figure 5). This could indicate constraints are placed on the feeding apparatus by soft connective tissue that holds the feeding apparatus in place within the body while overall muscle deformation, and perhaps protraction and retraction, remain relatively unaffected. These constraints may also help minimize the time of protraction, as excised feeding apparatuses had a considerably longer protraction time.

Feeding apparatuses in both treatment groups were observed to increase in width and decrease in length when the toothplate was protracted. This pattern is indicative of a pattern proposed for strain in *M. glutinosa*. The pattern proposed a cylindrical shape to the feeding apparatus which would decrease in length and increase in diameter when the toothplate is protracted than retracted as seen in Figure 8 (Clark *et al.* 2010). This pattern appears to hold true for *E. stoutii*, indicating that strain in the muscles of both species are similar despite differences in size and shape (Figure 9).

Electromyography

As seen in Figure 6 and 7, body bends corresponded to EMG muscle activation signatures recorded on the concave side. Hagfish have the ability to make very tight lateral bends in their bodies and this ability is under neural control. These tight lateral bends may support the formation and manipulation of body knots, which in turn are used in a coordinated fashion with toothplate protraction and retraction to make forceful bites.

Future Directions

Further studies are needed to confirm results seen in this study. Because of the inexact nature of muscle stimulation using large field electrodes, EMG data should be collected from the muscles in the feeding apparatus as *E. stoutii* feeds in order to obtain an understanding of the muscle function in a living animal. Another alternative method of identifying muscle function is the use of *Botulinum* toxin injections to chemically denervate muscles in an actively feeding hagfish (O'Niell & Gibb, 2007). A comparison of these data with *in vivo* feeding data from *M. glutinosa* by Clark and colleagues (2010) may confirm that the feeding apparatuses in these species function in the same manner.

Acknowledgements

We would like to thank Mr. Rodney and Mr. Bong Jo Kim of the Port Angeles Fish Co. Inc. in Port Angeles for donating animals, WA as well as Officer Donna Downs (WA DFW), Mr. Andy Inscore (Port Angeles Fish Co.)and Dr. Adam Summers (University of Washington) who helped with transporting the animals. We would also like to thank Dr. Adam Summers for providing animal housing. Crystina Perez and Austin Haney helped in animal husbandry, filming, dissection, stimulation experiments, and collection of electromyogram data as well as providing valuable input in experimental design. Thank you to Dr. Vikram Iyengar for program advising. This projected was funded by the Blinks-NSF REU-BEACON Program and by an NSF grant to TAU and AJC (NSF IOS-1354788).

References

Ayers, H., Jackson, C.M., 1901. Morphology of the myxinoidei: skeleton and musculature. J. Morphol. 15:185–225.

Basmajian, J. V. and Stecko, G. (1962). A new bipolar electrode for electromyography. *J. Appl. Physiol.* **17**, 849.

Clark, A., Maravilla, E. & Summers, A. A soft origin for a forceful bite: motor patterns of the feeding musculature in Atlantic hagfish, Myxine glutinosa. *Zoology* 113, 259–68 (2010).

Clark, A., Summers, A. Morphology and kinematics of feeding in hagfish: possible functional advantages of jaws. *The Journal of experimental biology* 210, 3897– 909 (2007).

Cole, F.J., 1907. A monograph of the general morphology of the myxinoid fishes,based on a study of Myxine. Part 2. The anatomy of the muscles. Trans. R. Soc.Edinb. 45, 683–757.

Martini, F., 1998. The ecology of hagfishes. In: Jørgensen, J.M., Weber, R.E., Malte, H.(Eds.), The Biology of Hagfishes. Chapman & Hall, London, pp. 57–77.

O'Neill, M. & Gibb, A. Botulinum toxin injections as a method for chemically denervating skeletal muscle to test functional hypotheses: a pilot study in

Lepomis cyanellus.*Physiological and biochemical zoology : PBZ* 80, 241–9 (2006).

Uyeno, TA. 2007 The morphology and biomechanics of the muscle articulation: A new class of soft tissue joint (Doctoral dissertation). Retrieved from ProQuest (#3272521)

Uyeno, T., Clark, A. Muscle Articulations: Flexible Jaw Joints Made of Soft Tissues. *Integrative and comparative biology* **55**, 193–204 (2015).

Uyeno TA, Kier WM. 2005. Functional morphology of the cephalopod buccal mass: a novel joint type. J. Morphol. 264:211–22

Zintzen, V., Roberts, C. D., Anderson, M. J., Stewart, A. L., Struthers, C. D. & Harvey, E. S. (2011). Hagfish predatory behaviour and slime defence mechanism. *Scientific Reports* 1, 131.

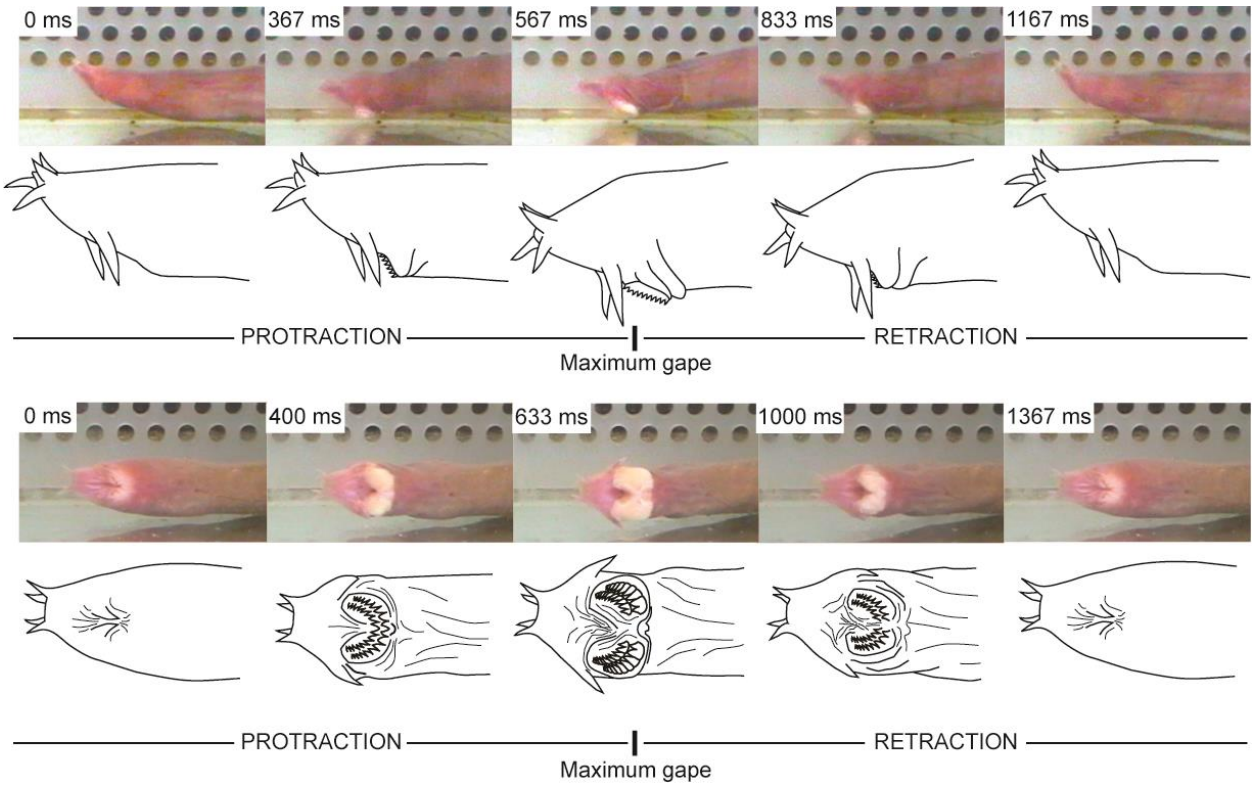


Figure 1. The toothplate is protruded with a cyclic protraction and retraction mechanism, as seen *with Myxine glutinosa*. Top: left lateral view of toothplate protraction and retraction. Bottom: ventral view of toothplate protraction and retraction.(Clark *et. al*., 2010)

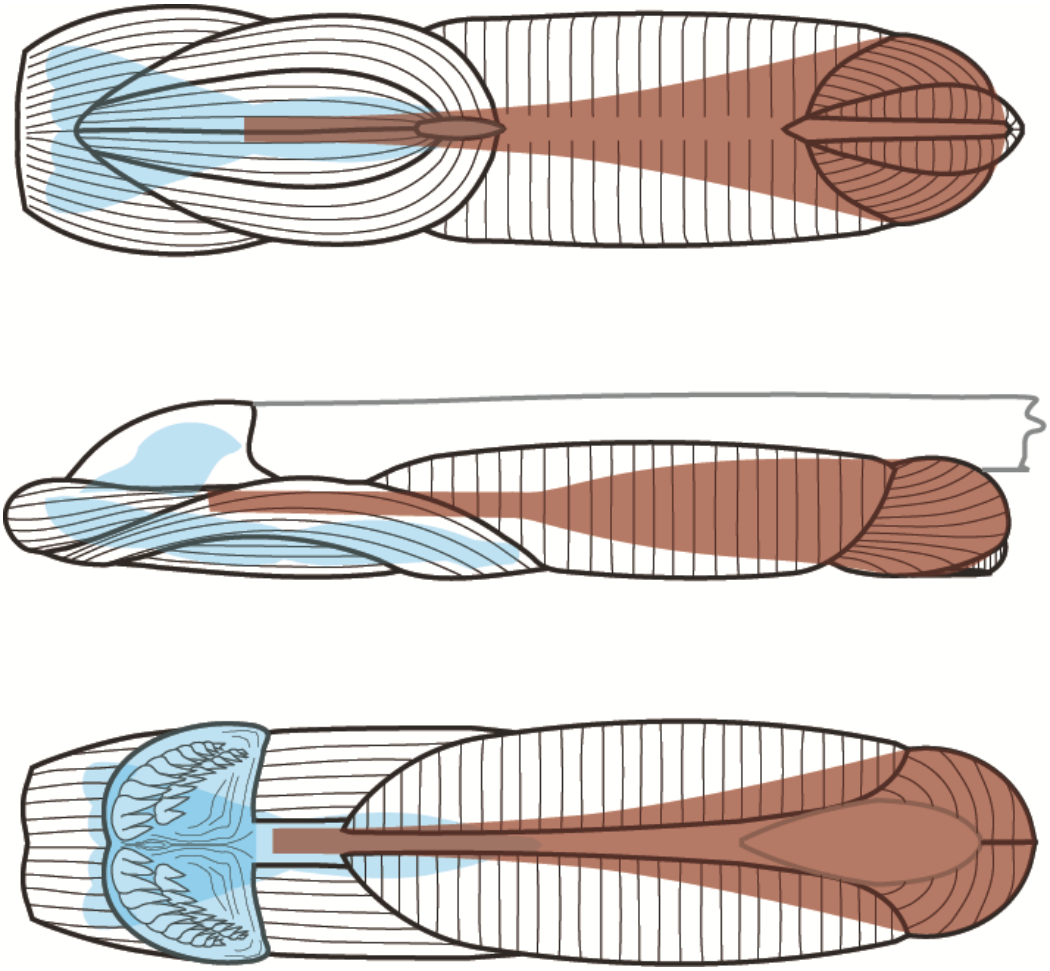


Figure 2. The basal plate (shaded in blue) provides attatchment for the retractor muscle (shaded in red). Top: dorsal view. Middle: lateral view. Bottom: ventral view.

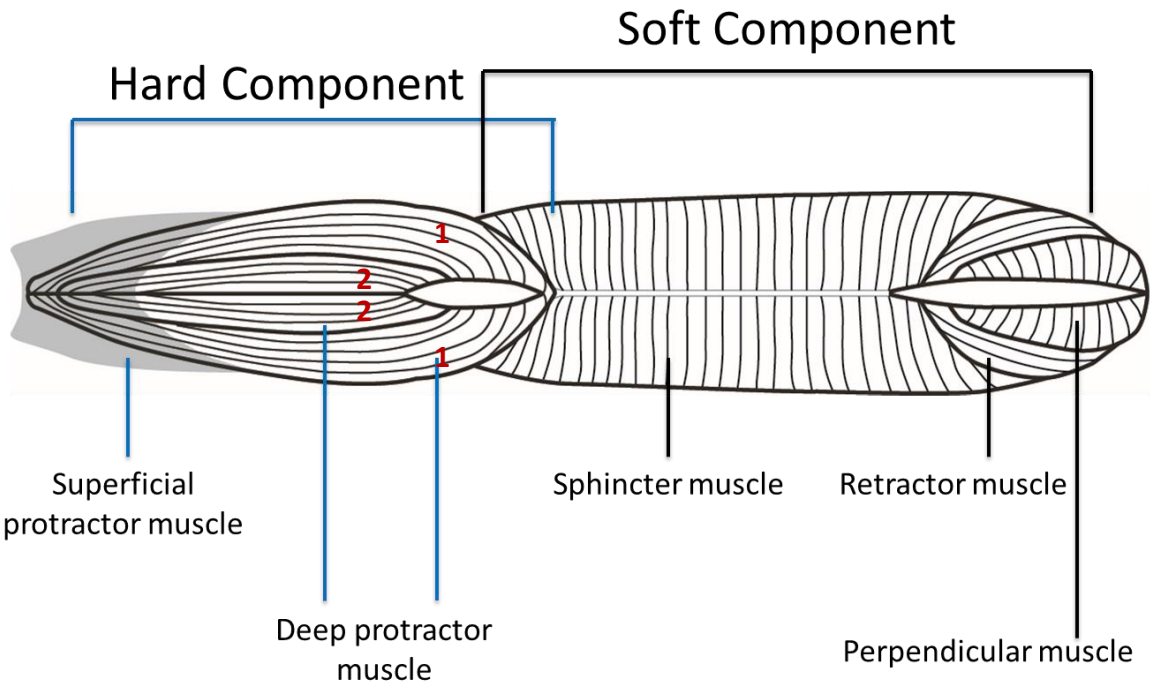


Figure 3. The ventral view of an E. Stoutii feeding apparatus. Labeled are the superficial protractor muscle (SPM), deep protractor muscle (DPM), sphincter muscle (SM), retractor muscle (RM), and perpendicular muscle (PM). Also labeled are the (1) lateral heads (LH) and and (2) medial heads (MH).

Timing of Muscle Activity Onset/Offset during Protraction and Retraction Cycles

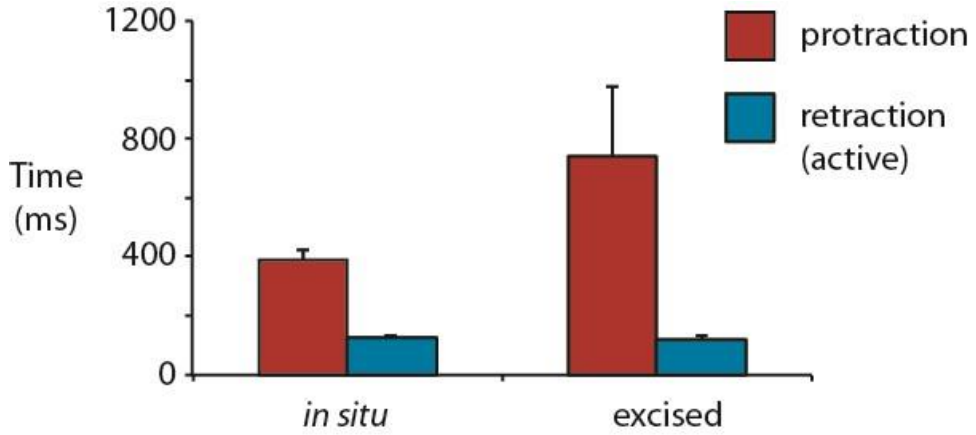


Figure 4. Time of biting cycle components of E. stoutii in in situ and excised feeding apparatuses are comparable for active retraction. Protraction is twice as long in excised apparatuses as in situ.

Longitudinal and Transverse Strain in *E. Stoutii* Feeding Apparatuses during Protraction

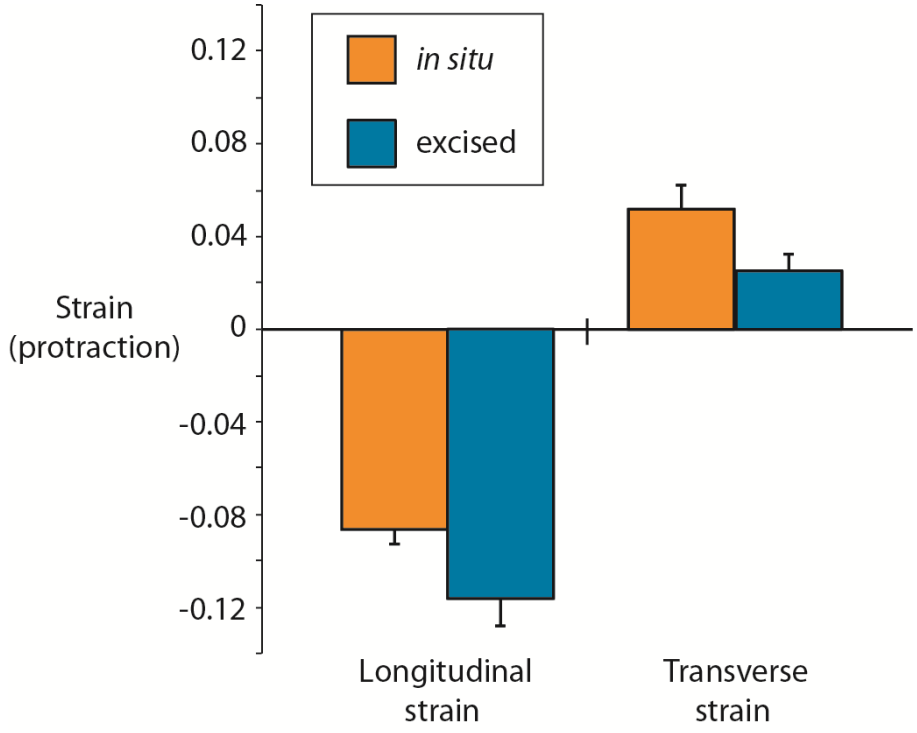
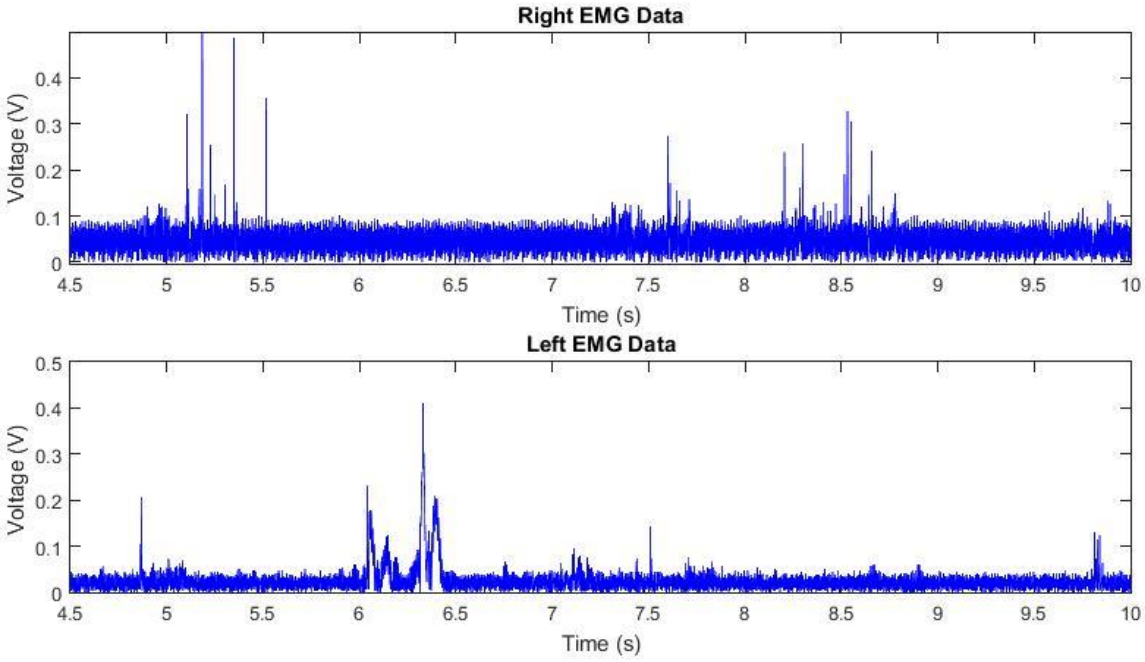


Figure 5. *In situ* feeding apparatus showed greater longitudinal strain while excised feeding apparatuses showed greater transverse strain.



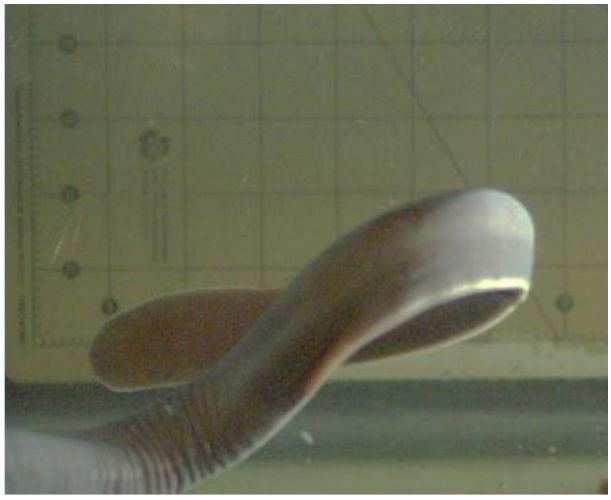
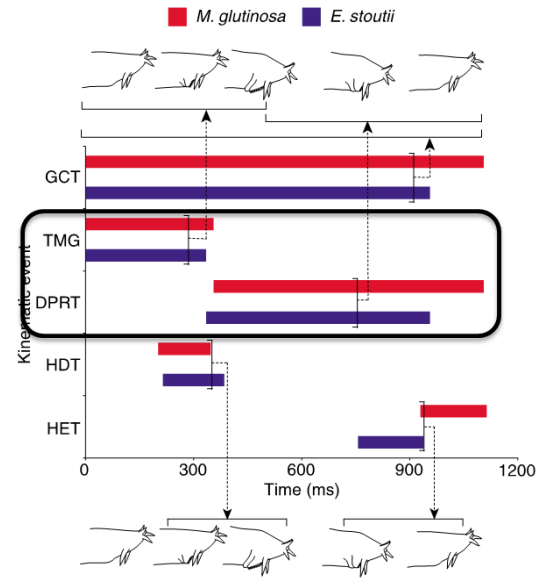


Figure 6. EMG data from the right and left side of the swimming *E. stoutii* (top) show spikes in voltage at 5.1 and 9.8 seconds, respectively. At 5.1 seconds, the animal was observed to be bent towards the right as seen from ventral view (bottom left). At 9.8 seconds, the animal was observed to be bent towards the left, as seen from the lateral view (bottom left).



Clark & Summers, 2007

Pacific Hagfish Biting Cycle Time (Present Study)

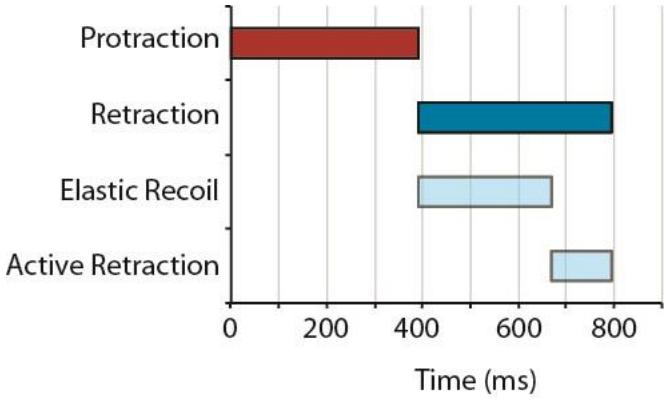


Figure 7. (Left) Similar kinematic times have been seen in *M. glutinosa* and *E. stoutii* protraction and retraction times while using live feedings (Clark and Summers, 2007). These times are similar to data collected from stimulations in *E. stoutii* when measuring active retraction as well as elastic recoil retraction (Right).

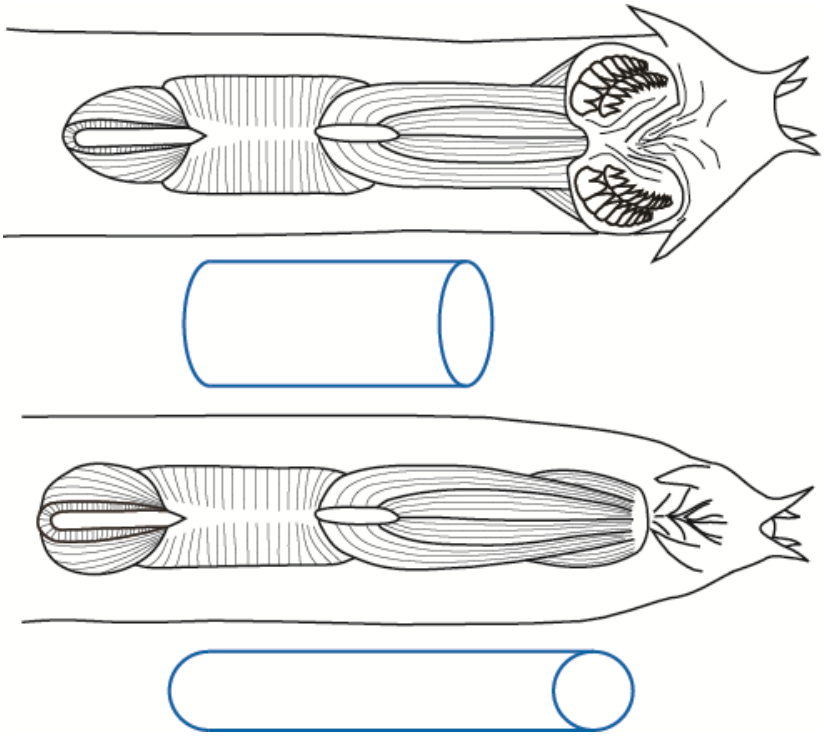


Figure 8. The cyclindrical strain pattern proposed for *M. glutinosa* is applicable to *E. stoutii*.
(Clark *et al.* 2010.)

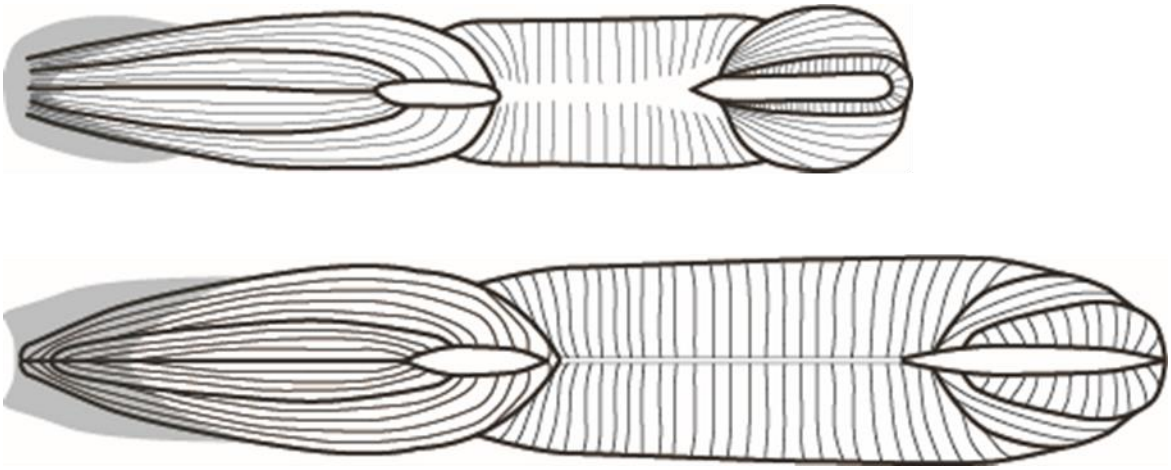


Figure 9. *M. glutinosa* (top) feeding apparatus is smaller and has a slightly different shape than *the E. stoutii* feeding apparatus (bottom).

Appendix: EMG Filter Matlab code

```
% EMG/Movement Monitor Data Analysis Program (from text data
file)
% written by R. Cole & T. Uyeno
% Variables
% A - the matrix
% A(:,1) - Channel 1 data
% A(:,2) - Channel 2 data
% A(:,3) - Channel 3 data
% timearr - the timeline
% meancol1 - average value for channel 1 data
% meancol2 - average value for channel 2 data
% A1aver - channel 1 data rezeroed
% A2aver - channel 2 data rezeroed
% A1abs - rectified channel 1 data
% A2abs - rectified channel 2 data
% fftA1aver - frequency data for channel 1
% fftA2aver - frequency data for channel 2
% A1filt - Butterworth filtered channel 1 data
% A2filt - Butterworth filtered channel 2 data
clear all;
A = xlsread('HF14EMG', 'B2:C29003');
[Rows,Columns]=size(A);
timearr = xlsread('HF14EMG', 'A2:A29003');
% Plotting the basic raw data
figure(1)
subplot(311)
plot(timearr,A(:,1));
subplot(312)
plot(timearr,A(:,2));
subplot(313)
%plot(timearr,A(:,3));
% Calculating means and rezeroing data
 % Column 1
meancol1=mean(A(:,1));
if meancol1>0
 A1aver=A(:,1)-abs(meancol1);
else
 A1aver=abs(meancol1)+A(:,1);
end
 % Column 2
meancol2=mean(A(:,2));
if meancol2>0
 A2aver=A(:,2)-abs(meancol2);
else
 A2aver=abs(meancol2)+A(:,2);
end
% Rectifying data
A1abs=abs(A1aver);
A2abs=abs(A2aver);
% Filtering data (Dr. D. Altshuler's suggestion Butterworth
filter)
[b,a] = butter(2, .0001); %.5 is 1/4 the cutoff frequency.
Altshuler has it between .001 and .01
A1filt = filtfilt(b,a,A1abs);
A2filt = filtfilt(b,a,A2abs);
```

```
% Analyzing filtered data (to make sure that the Butterworth
filter is notcutting off in range data)
meanA1=mean(A1filt);
A1filt=A1filt-mean(A1filt);
rr1=fft(A1filt,4096);
meanA2=mean(A2filt);
A2filt=A2filt-mean(A2filt);
rr2=fft(A2filt,4096);
figure(2)
subplot(211)
plot(fftshift(abs(rr1)))
subplot(212)
plot(fftshift(abs(rr2)))
% building frequency analysis diagram (using a fast fourier
transform using a frequency range of 4K)
fftA1aver=fft(A1aver,4096); % analyzing column 1
fftA2aver=fft(A2aver,4096); % analyzing column 2
% Plotting raw EMG data and frequency spectrum
figure(3)
subplot(221)
plot(timearr,A(:,1)) % plotting column 1 raw data
subplot(222)
plot(timearr,A(:,2)) % plotting column 2 raw data
subplot(223)
plot(fftshift(abs(fftA1aver))) % plotting column 1 frequency data
subplot(224)
plot(fftshift(abs(fftA2aver))) % plotting column 2 frequency data
% Plotting rezeroed, rectified, filtered EMG and movement data
figure(4)
subplot(421)
plot(timearr,A1aver) % plotting column 1 rezeroed data
subplot(422)
plot(timearr,A2aver) % plotting column 2 rezeroed data
subplot(423)
plot(timearr,A1abs) % plotting column 1 rectified data
subplot(424)
plot(timearr,A2abs) % plotting column 2 rectified data
subplot(425)
plot(timearr,A1filt) % plotting column 1 filtered data
subplot(426)
plot(timearr,A2filt) % plotting column 2 filtered data
%subplot(427)
%plot(timearr,A(:,3)) % plotting column 3 data
%subplot(428)
%plot(timearr,A(:,3)) % plotting column 3 data
% Plotting offset
n=1000; %n is the number of points (or msec) for baseline
reference
DURATION=1;
%DURATION is the time that onset has to stay above the threshold
FOLLOWTIME=1;
num_std=2.5;
%num_std is the number of standard deviation(std) for onset
threshold

%A1abs is the EMG data vector for a given muscle
P1 = A1abs(1:n);
```

```
 %P is the vector from the first n points of the emg1 vector
P2 = A2abs(1:n);
threshold1 = mean(P1) + std(P1)*num_std;
%threshold is the point beyond num_std deviations above the mean
baseline reference
threshold2 = mean(P2) + std(P2)*num_std;
figure (5)
subplot(211)
plot(timearr, A1abs, 'b'); hold on;
 %plot the entire vector in yellow first, then hold it
 %Now,plot the onset part over the orginal graph
for i = 1:length(A1abs)-FOLLOWTIME
 if abs(A1abs(i)) & abs(A1abs(i:i+FOLLOWTIME)) > threshold1
%DURATION is the time that data has to stay above threshold,
10msec for me
 plot(timearr(i:i+FOLLOWTIME),A1abs(i:i+FOLLOWTIME),'b');
%using a blue color
 end %if apoint in the data set is greater than the threshold and
stays above thethreshold for 10 ms,
end %then plot this point and the following 10 ms of data in a
different color from the original plot.
subplot(212)
plot(timearr, A2abs, 'b'); hold on;
for i = 1:length(A2abs)-FOLLOWTIME
 if abs(A2abs(i)) & abs(A2abs(i:i+FOLLOWTIME)) > threshold2
 plot(timearr(i:i+FOLLOWTIME),A2abs(i:i+FOLLOWTIME),'b');
 end
end
```