

Characterization of Body Knotting Behavior in Hagfish

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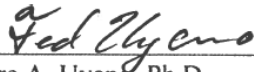
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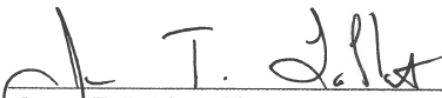


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ABSTRACT

Hagfishes possess a flexibility that allows them to form body knots and then slide the knots along their body. This behavior enhances the hagfish's ability to clean mucus off their body, escape tight spaces, pull prey from burrows and possibly replace the leverage commonly generated by an opposing jaw. Despite the importance of this knotting behavior to the survival of hagfishes, very little has been reported in the literature. This is probably due to the difficulty of studying the behavior in the wild. Using a novel hagfish restraint device, consistent and reliable knotting events were captured with high-speed bi-planar video. I used these recordings to characterize the type and kinematics of knots made by three species belonging to the two families of hagfishes: *Eptatretus stoutii*, *Eptatretus springeri*, and *Myxine glutinosa*. I found that hagfishes statistically preferred simple knots despite the higher internal stresses that these knots theoretically induce. Also, despite the behavioral stiffness (does not coil) of *E. springeri* and *M. glutinosa* when compared to *E. stoutii* (coils) there was no statistical difference in looseness of knots tied when comparing radii of loops between species. However, decreased stiffness may be beneficial: *E. stoutii* was able to tie more complex knots than the other two species. The hagfish body represents an extremely flexible hyper-redundant system that may require a high level of neural input for control. However, kinematic video analysis reveals a potential elegant solution: hagfishes seem to employ only three body movements (crossover the body, tail-wrap, and tail insertion into a loop). These three motions can be re-ordered to create the entire diversity of observed knots as well as more complex theoretical knots. Furthermore, statistical analysis suggests that these motions were performed in the same manner across all

species. This study suggests that knotting may be efficiently controlled by motor primitives and sets the stage for neurophysiological investigations.

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Chapter I

INTRODUCTION

Body Knotting in Aquatic Craniates

There are only three groups of aquatic craniates that utilize body knots: hagfish, water snakes and eels. Aside from hagfish, knotting behavior has been observed in four species of water snakes: the black-banded sea krait, *Laticauda semifasciata* (Reinwardt in Schlegel, 1837), the yellow-bellied sea snake, *Pelamis platurus* (Stoliczka, 1872), the beaked sea snake, *Enhydrina schistose* (Daudin, 1803), and the marine file snake, *Arochordus granulatus* (Schneider, 1799). Mays and Nickerson (1968) suggested the knotting behavior in *L. semifasciata* could have evolved due to a lack of hard substrate to against during ecdysis (skin shedding) since these snakes evolved to live in open water. Pickwell (1971) observed that aquatic knotting behavior in *P. platurus* involved complex loops and coils that may have also facilitated ecdysis. During this process, the shed was usually removed in an inverted and intact state. Interestingly, in their shed skins one or more tight overhand knots were observed. It was also noted that immediately post ecdysis, the snake knotted frequently, perhaps to stretch out the new skin. Pickwell (1971) also observed snakes knotting with no sign of shedding, and hypothesized a function in ectoparasite removal. Another function may be escape; when picked up with forceps *P. platurus* will use a figure-eight knot to wriggle free. Similar to *L. semifasciata*, *P. platurus* may have evolved knotting to live and hunt within the water column. Thus, the lack of substrate against which to rub when shedding may have induced knotting behavior. Voris et al. (1978) saw *E. schistose* using knots for more than shedding. These

snakes will knot frequently after ingestion of a food item in order to aid digestive movements once the food item is approximately halfway to the stomach. The last species of water snake that was observed to utilize body knotting is *A. granulatus* (Lillywhite, 1989). Instead of peeling the skin and inverting it like the other snakes mentioned, they loosen the skin through a series of adduction and abductions of the ribs that serve to expand the circumference of the body and stretch the skin. They then crawl out of the skin which does not get inverted. Shedding through crawling rarely works perfectly as the skin will get bunched up around the fattest parts of the animal. When this happens, knotting is employed to move the skin down the body.

All of these snakes have been described as using relatively simple knots: overhand and figure-eight knots. The observed uses of these knots are aid in shedding, clearing of ectoparasites, stretching during growth periods following shedding, and potentially escape from predators.

Eel knotting behavior was first mentioned as a personal comment by Helfman and Clark (1986) in the painted moray, *Gymnothorax pictus* (Ahl, 1789). They described a behavior in which the eels would swim up to salmon heads at the edge of the water, secure and biting hold and use a knot to tear a chunk of flesh off. As eels possess a very narrow bite width, limitations in gape may be offset by behavioral adaptations, including knotting, that permit manipulation and feeding on parts of prey that are too large to swallow whole. Miller (1987) published the first extensive study on knotting behavior in muraenid eels. Six species were observed in this study, the snowflake moray, *Echidna nebulosi* (Ahl, 1789), the chain moray, *Echidna catenata* (Bloch, 1795), the barred moray, *Echidna polyzona* (Richardson, 1845), the blackedge moray, *Gymnothorax*

nigromarginatus (Girard, 1858), the purplemouth moray, *Gymnothorax vicinus* (Castelnau, 1855) and the leopard moray *Enchelycore pardalis* (Temminck & Schlegel, 1846). Video recordings of knotting behavior in each of these six species showed that they tied overhand knots with the tail draped anterior to the knot to create an incomplete second loop. Miller (1989) further characterized the diversity of feeding behaviors in moray eels. The zebra moray, *Gymnomuaena zebra* (Shaw, 1797) did not employ knotting like the other species in this study, they instead used rotational feeding. This study suggested that an overhand knot is used during feeding and that there is variation in the knots execution; the tail may or may not form a second incomplete loop anterior to the knot by wrapping around the body. There are three different potential uses Miller (1989) observed for knotting in eels. First, the eel repeatedly pulls the prey through a knot to flatten it and facilitate it being eaten whole. Second, knotting is used to remove a piece of the prey that is too large to consume whole. Third, a constrictive knot is used to hold onto the prey for an extended amount of time. Santos and Castro (2003) successfully studied knotting behavior in the white spotted moray, *Gymnothorax ocellatus* (Agassiz, 1831) even though they live on the flat muddy bottom where observation is difficult. They noted that *G. ocellatus* only knotted when trying to eat a food item more than approximately 25% of its total body length. Barley et al. (2015) observed knotting in the fimbriated moray, *Gymnothorax fimbriatus* (Bennett, 1832) through the use of a baited remote underwater video system. *G. fimbriatus* was seen to use a knot to either rip open the stiff plastic mesh bait bag or pull smaller chunks through the mesh holes of the bag. This implies that knotting may be useful for more than just breaking up large food items.

It can also be used for extracting prey items from the interstices of the reefs where morays are known to commonly hunt.

In summary, there are eight confirmed species of muraenid eels that have been shown to employ knotting behavior. Knotting is mainly used in feeding for breaking up large chunks, compressing the food to be eaten whole, or constricting the prey. There are mentions of other potential uses in removing prey from reef crevices and guarding food items from other swarming eels.

Knotting Ecology/Anatomy in Hagfishes

The earliest reference to body knotting in hagfishes is Adams (1960). He noted that knots were used by *Myxine glutinosa* (Linnaeus, 1758) to clean slime off their bodies. Since then, knotting behavior has been documented as a predator escape behavior, to extricate from tight spaces, to pull fish from burrows during active predation, and during macrophagous feeding events (Strahan, 1963; Martini, 1998; Zintzen et al., 2011; Clark & Summers, 2012).

Hagfishes are one of two extant members of the superclass agnatha (“no jaws”; (Cope, 1889). Instead of opposable jaws, they have a feeding apparatus that utilizes a single sided cartilaginous dental plate armed with recurved keratinous teeth (Clark & Summers, 2007). The cyclical protraction and retraction of this dental plate allows for “rasping” of soft and small food items into the oral cavity (Figure 1).

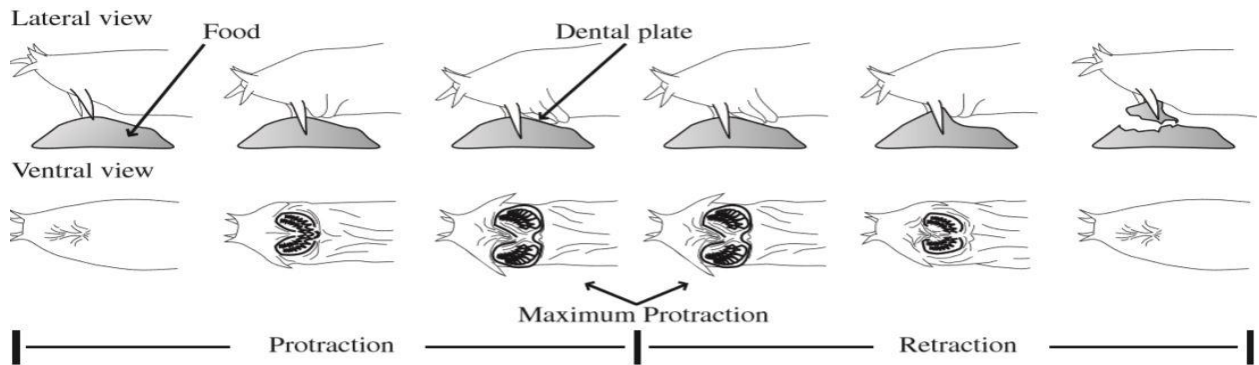


Figure 1. Cyclical Protraction and Retraction of Hagfish Feeding Apparatus (From Clark & Summers, 2012).

During dental plate protraction, the teeth are raked across the food item in a motion that shears off chunks of food. Subsequently, the dental plate is folded and it, along with the attached food are retracted into the oral cavity.

Macrophagic feeding differs from rasping in the following manner: once the food item is impaled on the teeth of the dental plate, and swimming or wiggling does not provide enough retractable force, leverage from a body knot pressed against the surface can be used to tear the chunk away (Clark & Summers, 2012). This knot is formed at the caudal end and slid along the body cranially. Once the knot reaches the head, a loop of the knot is pressed against the surface to create a stable platform (Uyeno & Clark, 2015). Using this stable platform for leverage to oppose the coordinated movements of the head and feeding apparatus is thought to generate an antagonistic “bite” force. This knotting behavior allows for more tearing force to be applied and therefore lets hagfishes exploit larger food items that might otherwise not be available to them.

In order to facilitate knotting, hagfishes have evolved several adaptive features. First, their bodies are relatively flexible due to the absence of vertebrae. Ota et al. (2011) note that embryological data suggests that vertebrae have been secondarily lost. Second, hagfishes also possess loosely attached skin with a large blood-filled sinus and loose

septum allowing for a larger range of motions (Clark et al., 2016). Third the body bears no pectoral or dorsal fins that may get in the way of sliding a knot along the length of the body. Fourth, hagfishes lack a *stratum corneum* that in other fish bears scales (Andrew & Hickman, 1974). Instead, the outer layer of skin is equivalent to the *stratum germinativum* which is entirely smooth, an ideal surface to reduce knot friction and binding. Fifth, knotting friction may be reduced by slime that lubricates the knotting surfaces of the hagfishes (personal communication, summer, 2015).

The Knotting Control Problem

Hagfishes' bodies are extremely flexible; the body can be modeled as a high number of body segments connected by flexible joints each with multiple degrees of freedom. Such models are described as having a large degree of kinematic redundancy. While this "hyper-redundant" body and their flexibility is beneficial for forming knots, neural input parameters of such bodies can be enormous. This is because neural input requirements increase rapidly with increase in the number of joints and their degrees of freedom (Figure 2).

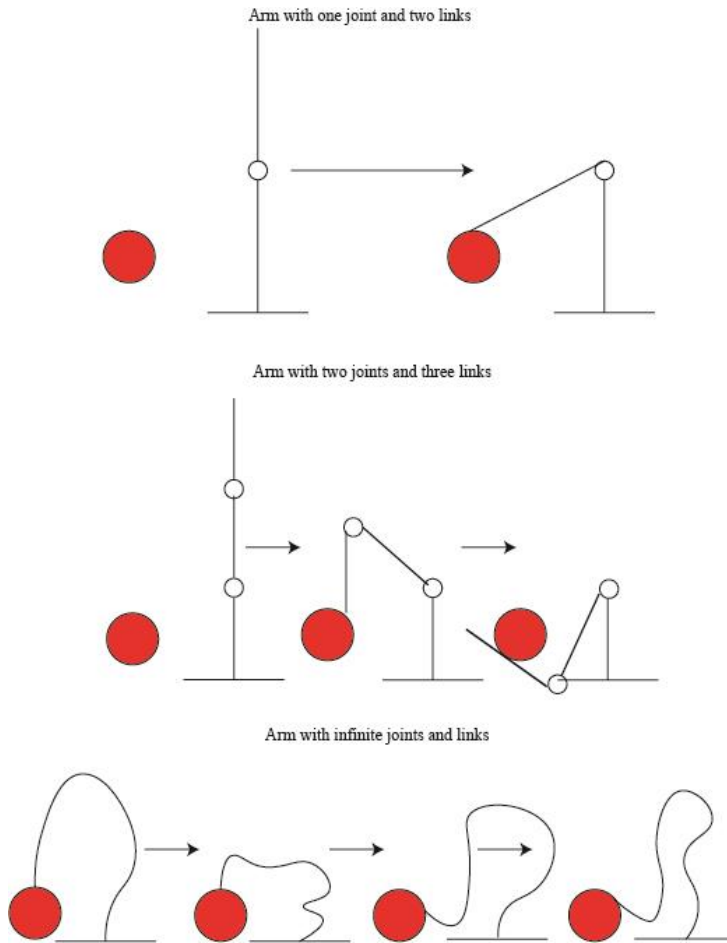


Figure 2. Illustration of a Robotic Arm that Needs Increased Control to Touch a Red Ball

As an example, consider the system at the top of Figure 2 with one joint and two links. To control this system to touch the ball, the translational and rotational positions in the orthogonal x , y , and z axes for one joint are needed. This amounts to six input parameters for every joint. The amount of control needed increases with the number of joints and links. A system that bends at any point along its length represents the most extreme scenario. Such “hyper-redundant” systems have been best studied in the octopus arm. Sumbre et al. (2006) showed that when an octopus reaches for a target, the arm bends at the same three points each time. While the arm could bend at any point, the localization of a “joint” at a fixed position greatly reduces the amount of neural input needed to

control the arm with less “joints” to control. This can be used as building blocks and rearranged in different ways to create a vast array of complex behaviors and are referred to as motor primitives (Mussa-Ivaldi et al., 1994; Thoroughman & Shadmehr, 2000; Flash & Hochner, 2005). These motor primitives are also adaptable to different situations (Yekutieli et al., 2005). For example, when an octopus encounters constraints such as reaching through a hole, the motor primitives adapt to the constraint and are successful in creating reaching motion. Thus, neural control of the highly flexible hagfish body may be reduced through the use of motor primitives.

Knot Theory

The knots that hagfishes employ are well-described by the mathematical field of knot topology. Knot topologists often visualize knots by first tying the given knot into a piece of rope, and then connecting the two ends together. Thus, a rope with no knot in it would look like the letter “O” (often referred to as an “unknot”). A knot that is more complex than an unknot has loops and crossovers in it that cannot be untied without cutting the rope. The pattern of loops and crossovers in a knot therefore determines the type of knot (Adams, 2004). Distinct knots (known as knot “primitives”) are organized from the simplest to increasingly complex. The naming system they employ indicates the number of crossovers used in each knot with a subscript denoting different knots that are tied with the same amount of crossovers (Figure 3).

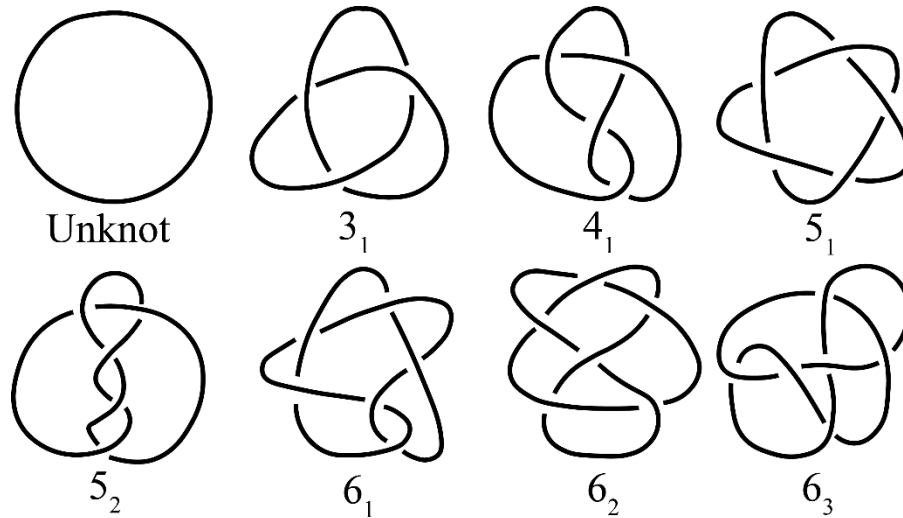


Figure 3. Organization of Knots using Number Classification System.

Once above four crossovers, there can be several fundamentally different knots tied using the same number of cross overs. For an excellent primer on knot topology, see Adams (2004). Ashley (1944) presents a complete listing of the knots depicted here and their common uses.

Hagfishes are not theoretical knots connected at both ends, instead they have a beginning cranial end and a caudal tail end. Functional simulations exist for this more realistic scenario. Using finite element models, Pieranski et al. (2001) predict how forces are distributed throughout knots. Within any given tightened knot, loops create pinch points with increased internal stresses. The authors compared the simple (3_1) overhand knot to the more complex (4_1) figure-eight knot, and found the more complex knot had reduced internal stresses at these pinch points (Figure 4).

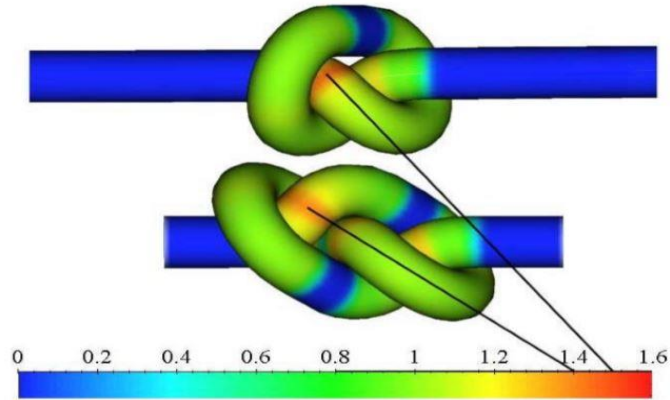


Figure 4. The Reduced Internal Stresses at Pinch Points within a Knot. From Pieranski et al., 2001.

Hypotheses

Body knotting is important to the critical functions of feeding and escape behaviors in hagfishes, and yet it has not been rigorously studied. Thus, my goal is to characterize the hagfish body knotting process through high-speed video kinematics, and to document the diversity of knots between the two major groups of hagfish. This will be accomplished through four hypotheses.

Goal 1

Hagfishes are often seen knotting when they encounter a food item that is difficult to process or in order to escape from tight spaces. I will investigate the effectiveness of these two stimuli as a method of generating consistent and controlled knotting behavior needed for kinematic videography and subsequent analysis.

H₀: There is no way to stimulate consistent controlled knotting behavior.

H₁: Consistent controlled knotting is achieved through trapping the animal and forcing it to escape.

H₂: Consistent controlled knotting is achieved through macrophagous feeding events with the animal.

Goal 2

Based on the average length to width ratio of *Eptatretus stoutii* (Lockington, 1878) (23.82) there are knots that are too complex for hagfishes to tie. This is because more complex knots require longer lengths of material. Thus I predict that the most complex knot they will be able to tie is a 4_1 knot. This is based on models of knots I created with rope scaled to the average length to width ratio of *E. stoutii*. To compensate for potential dimensional changes that may occur in live animals I will investigate knots that include up to a 6_3 knot. An additional consideration for predicting knot selection is the reduction of internal stress. Pieranski et al. (2001) showed that the internal stresses in the more complex 4_1 knot were less than in the simpler 3_1 knot. Within the simple knot set considered here, the more complex knots reduce loop radius and therefore internal stress, I predict that hagfishes prefer more complex knots.

H₀: Hagfishes show no preference for a certain knot type.

H₁: Hagfishes prefer more complex knots.

H₂: Hagfishes prefer simpler knots.

Goal 3

According to current phylogenies there are two distinct and robust families of hagfishes (Fernholm et al., 2013; Cortez et al., 2015; Schumacher et al., IN PRESS). An interesting behavioral distinction between Myxinae and Eptatretinae seems to be that the Myxines tend to hold their bodies more stiffly than do Eptatretines. Because of this, coiling behavior is more frequent in Eptatretine species and not common in Myxine species. Therefore, I hypothesize that Eptatretines will be able to tie more complex/tighter knots than Myxines.

H₀: Behavioral stiffness does not affect complexity/tightness of knots tied between species.

H₁: Behavioral stiffness does affect complexity/tightness of knots tied between species.

Goal 4

Given the trend towards neural control simplification using motor primitives in other hyper redundant structures (e.g., octopus arms, Sumbre et al., 2006), hagfishes may not neurally control all portions of their body. Thus, I hypothesize that complex behaviors such as knots in hagfish may be composed of distinguishable simpler movements organized as motor primitives.

H₀: Hagfishes can control every aspect of their body in all degrees of freedom.

H₁: Complex hagfish movements are composed of a sequence of simple stereotyped movements.

Chapter II

METHODS AND MATERIALS

Overview

I obtained 27 individuals across three species of hagfishes. There were 20 *E. stoutii* (Average: length = 493mm, Width = 22mm, Weight = 162g) from Olympia seafood company in Port Angeles, WA. Three *Eptatretus springeri* (Average: length = 536mm, Width = NA, Weight = 253g) (Bigelow & Schroeder, 1952) from Dr. Dean Grubbs at Florida State University off the shore of Panama City, FL. Four *M. glutinosa* (Average: length = 452mm, Width = 16mm, Weight = 67g) from Dr. Andrew Clark at the College of Charleston. Hagfish were maintained in a temperature and light controlled room in a flow through tank system. The water temperature was maintained at 13° C and salinity was maintained at standard seawater levels. For the first 3 months, the hagfishes were fed chunks of mullet weekly until feeding behavior in the animals ceased, then they were presented mullet chunks once a month.

I obtained consistent and controlled knotting behavior in hagfishes using a custom hagfish restraint device (Figure 5).

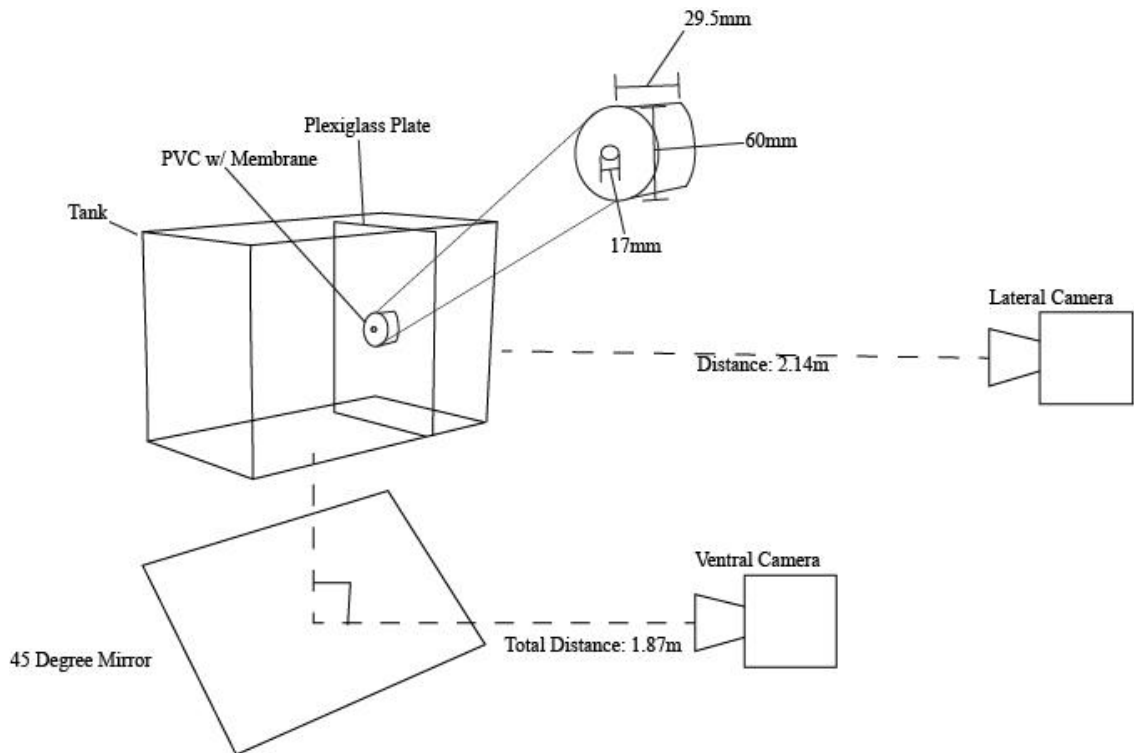


Figure 5. Illustration of Hagfish Restraint Device used.

The device consisted of a tank with a vertical plexiglass plate inside holding a short piece of PVC pipe with a membrane stretched across it and held on with an O-ring (45mm, nitrile). The membrane had a hole in the center that held a sedated hagfish's head securely in place (4mL of 20mg sodium benzoate in 400mL of ethanol dissolved in 2L of salt water until hagfish was unresponsive to tail pinch, approximately 10-15 minutes). Sodium benzoate was found to be preferable to MS-222 (200mg MS-222 with 400mg sodium bicarbonate in 2L of salt water) due to prior success in juvenile lamprey (Christiansen et al., 2013). Sodium benzoate seemed to have a faster recovery time for a more realistic representation of natural knotting events by limiting the effects of anesthesia on knotting behavior (see Appendix B - IUCUC approval). Underneath the tank was a 45° angled mirror so that one high-speed camera (Phantom Miro 320) could obtain a ventral view while the other was aimed at the side of the

tank for a lateral view. This resulted in .CINE file formatted high-speed bi-planar video of knotting events which was converted to .mp4 format for kinematic analysis using Phantom Camera Controller software for PC. Most trials were shot at 200 fps but ranged from 100-300 fps. The lens used for all trials was a Zeiss MAKRO-PLANAR T* 2/100mm ZF.2. A Nikon ED AF NIKKOR 80-200mm 1:2.8 D lens was tried as well as a Bower 1:1.4 35mm. The Zeiss was easier to focus in on the target area and captured crisper more defined images with less aberrations around the edge.

The PC video analysis program Tracker (ver. 4.96) was used to measure five geometric knot parameters to describe knot formation movements (Brown, 2017). First, the angle of crossover (XO angle) is defined as the angle between two parts of the body that overlap but do not wrap around (Figure 6). Once a crossover occurs, a loop is formed. Second, the normalized loop radius (NLR) is the radius of a circle fit to the tightest curve of the loop created by a body crossover added to half the width of the body, resulting in the radius from the center of the loop to the midline of the hagfish (Figure 6). This is then divided by the body width to standardize for body size between species and individuals. Third, the crossover overhang (XO overhang) is defined as the amount of the body that crosses over the other side of the body before it begins to wrap around the body. Fourth the tail wrap angle (TW angle) is the angle the tail wraps around a portion of the body (Figure 7). Fifth, the tail insertion point (TI point) is the distance that the tail inserts into the loop from the opposite side of the loop (Figure 8). The angular measurements were then standardized. If the angle of the body/tail was angled cranially and the measurement was greater than 90° , it was subtracted from 180° , if the measurement was less than 90° , it was left alone. If the

angle of the body/tail was angled caudally and the measurement was greater than 90° it was left alone, if the measurement was less than 90° then it was subtracted from 180° . This was done so that for all angular measurements, the same angle was being compared. All angles were measured by running a tangent line along the body maximizing contact with the body.

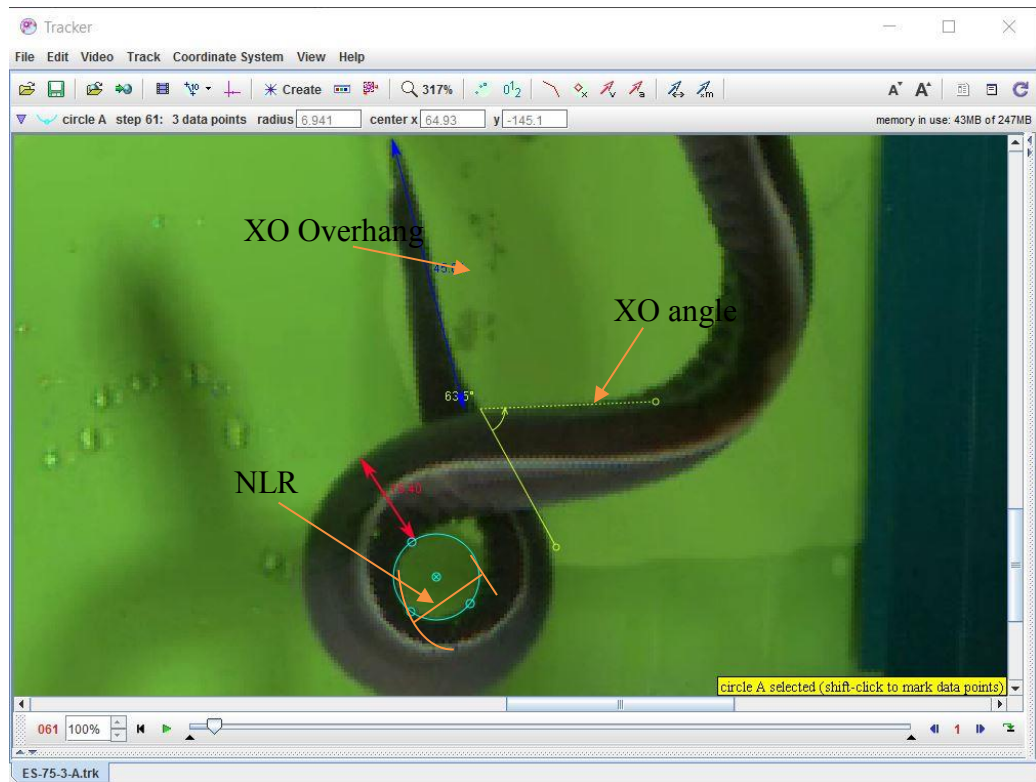


Figure 6. XO Angle, RF and XO Overhang Measurements in Tracker.

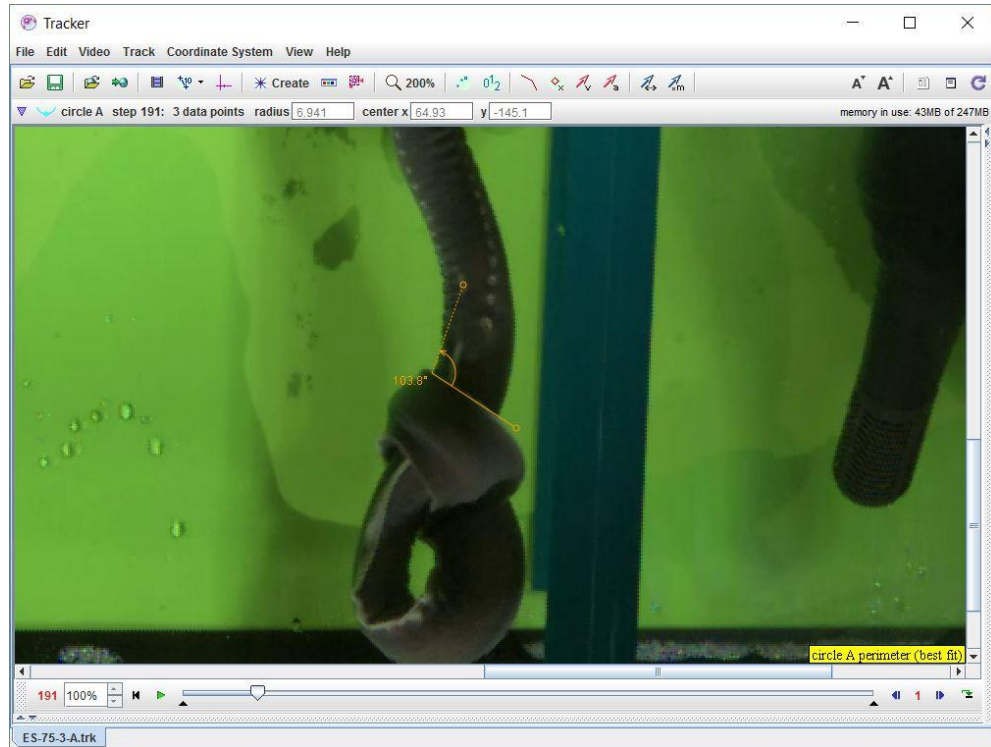


Figure 7. TW Angle Measurement in Tracker.

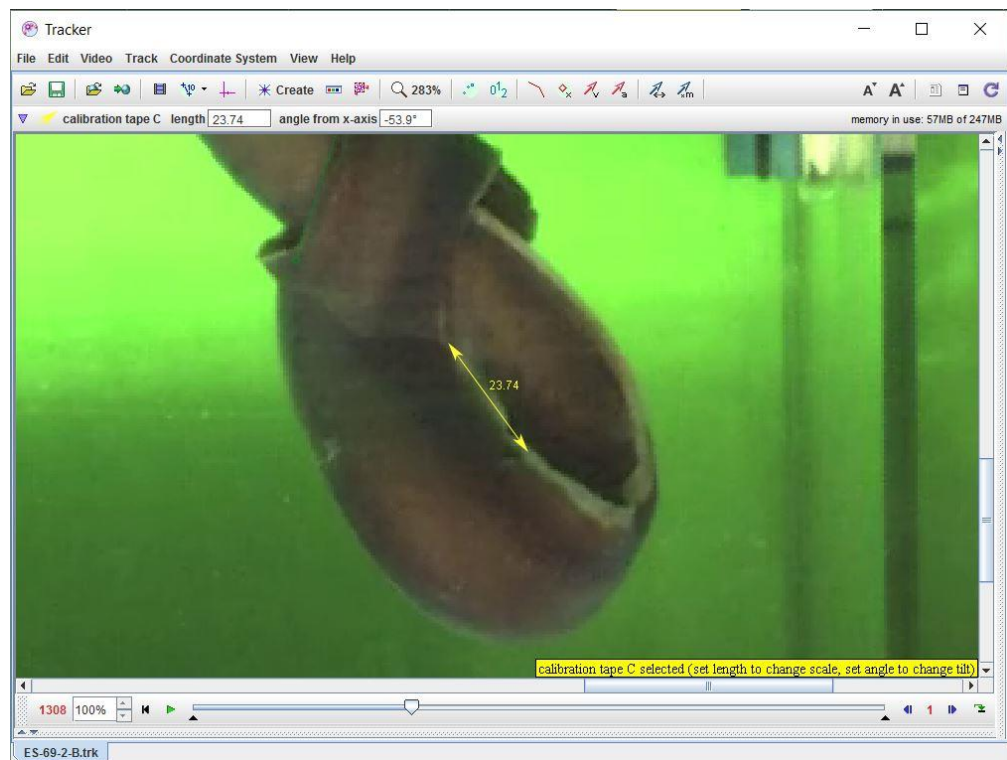


Figure 8. TI Point Measurement in Tracker.

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) statistics software (IBM, Armonk, NY). First, each measurement was tested for normality using the Shapiro-Wilk test of normality. If the data was normally distributed, then univariate analysis was done between species and knot types. For each univariate comparison, if the variance was assumed to be equal based on Levine's test of equality of error variance then Tukey *post-hoc* analysis was performed, if equal variance was not assumed then Games-Howell *post-hoc* analysis was performed. If the data was not normally distributed then the non-parametric Kruskal-Wallis test was performed to account for lack of normality.

Chapter III

RESULTS

Types of Knots

I recorded and analyzed a total of 100 knotting events using high-speed bi-planar video. 84 events were captured from *E. stoutii*, 6 from *E. springeri*, and 10 from *M. glutinosa*. Hagfishes performed five types of knots to escape the restraint device. The first knot, a 0 knot or unknot, which I refer to as a “loop-leverage,” represents a movement where a loop of an unformed knot is pressed against the surface of the membrane and used as leverage to extricate the head. Additionally, there were three complete knots used, the 3_1 knot (or overhand knot), the 4_1 knot (or figure-eight) knot and the 5_2 knot (or Miller-Institute knot). The “other” category includes an assemblage of escape behaviors that did not involve pressing a loop against the restraining membrane to generate leverage. These movements included shaking out of the membrane (lateral uncoordinated thrashing), swimming backwards (lateral coordinated movement), failure to escape (rarely some animals just gave up), and swimming forward through the membrane. Table 1 shows the breakdown of the types of knots used between each species.

Table 1. Types of Knots for Each Species

	Total	<i>E. stoutii</i>	<i>E. springeri</i>	<i>M. glutinosa</i>
Loop-Leverage	11	10	1	0
Overhand	45	37	3	5
Figure-8	33	31	2	0
Miller-Institute	4	4	0	0
Other	7	2	0	5

Movements and Rules of Knotting

Video analysis of knotting events allowed me to describe the diversity of knots and escape behaviors. One significant discovery was that each of these diverse motions were comprised of three distinct motions and two underlying rules.

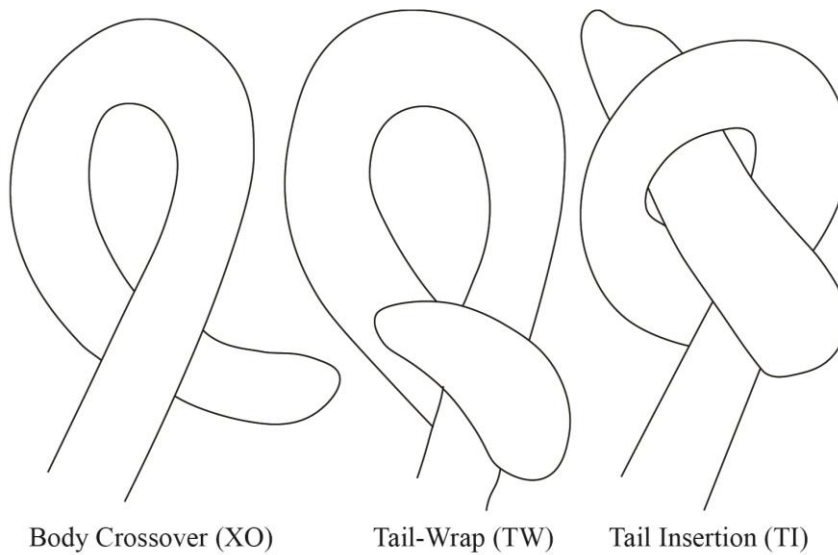


Figure 9. Illustrations of the Three Distinct Motions.

The first distinct motion is a crossover (Figure 9, XO), when the body of the animal crosses over itself. The second motion is a tail wrap (Figure 9, TW), when the tail of the

hagfish wraps around the body. The third motion is a tail insertion (Figure 9, TI), when the tail is inserted into a loop of the knot, usually completing a knot. While these three motions are the only ones used to tie knots, there are some general rules that govern their use. First, throughout the body knot formation process; the knot is formed in the caudal region and propagation travels cranially until failure. Movement of failed knot loops is how most of the loop-leverages were formed; a crossover and tail-wrap that was not followed by a tail-insertion that propagates cranially. Second, each movement must alternate in its handedness. This means that the next crossover, tail-wrap, or tail insertion in the sequence must change direction and side of the body. If the first crossover is left and under the body the next must be right and over the body. Failure to conform to this rule results in an incomplete knot and restarting of the knotting process.

Movements for Each Knot Type.

Different knots are created using a specific sequence and combination of the three cardinal movements. The order of the three motions can be rearranged to create different types of knots. Table 2 shows the theoretical motions needed to create the eight simplest knots.

Table 2. Motions for Each Knot Type
(XO = crossover, TW = tail-wrap, TI = tail insertion)

Type of Knot	Motions	Used by Hagfish?
0	XO, TW	Y
3 ₁	XO, TW, TI	Y
4 ₁	XO, TW, TW, TI	Y
5 ₁	XO, TW, TI, TW, TI	N
5 ₂	XO, TW, TW, TI, TW, TI	Y
6 ₁	XO, TW, TW, XO, TI	N
6 ₂	XO, TW, TI, XO, TW, TI	N
6 ₃	XO, TW, TI, XO, XO, XO, TI	N

The constituent motions of this model were identified by tying these knots into rope and noting the steps and sequence each knot required (Figure 10).

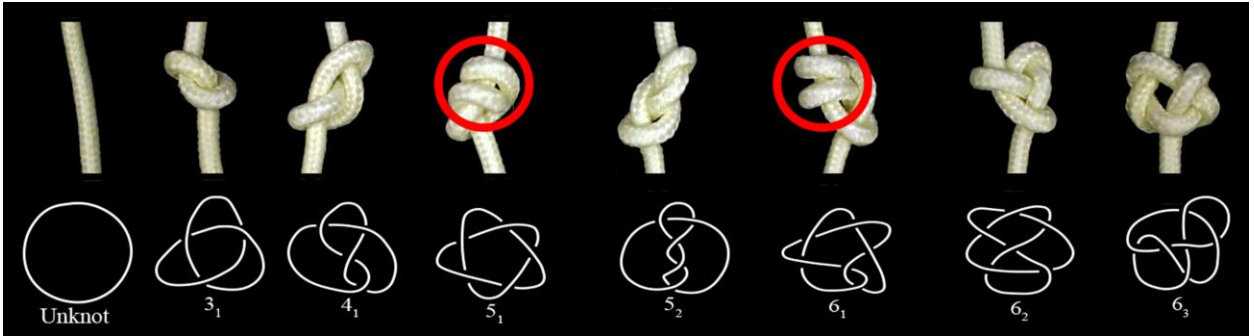


Figure 10. Rope Representation of Knots 0 to 6_3 with their Respective Line Drawings Underneath. The circles represent areas of knots that require two consecutive motions in the same direction.

Hagfishes are capable of tying only 0, 3_1 , 4_1 , and 5_2 knots (see Table 2). In attempting to tie these knots, other motions were sometimes attempted before and after the formation of the knot. However, the order of motions followed those listed in Table 2 during a successful attempt 100% of the time. No deviations from these orders were ever observed.

Knot Geometry

Between knot types, crossover angle, normalized loop radius, and crossover overhang were statistically similar. Tail-wrap angle between all knot types were statistically different ($P < .0001$). Tukey *post-hoc* analysis resolved which knots were different from each other (Figure 11); overhand knots were statistically different from both figure-eight knots and miller-institute knots ($P < .0001$), while figure-eight and Miller-Institute knots were not significantly different ($P = .168$). Since there were not enough tail-insertion point measurements for Miller-Institute knots, only overhand and figure-eight knots were compared and it was found to be statistically different ($P <$

.0001). Since variance of tail insertion point between knot types could not be assumed to be equal, an Independent-Samples Kruskal-Wallis test was performed (Figure 12).

Between species all measurements were statistically the same at the $\alpha = 0.05$ level. For all non-significant output from SPSS statistical analysis, see Appendix A.

Multiple Comparisons

Dependent Variable: TW_Angle
Tukey HSD

(I) Knot_Type	(J) Knot_Type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Overhand	Fig-8	26.9583*	4.37372	.000	16.4884	37.4283
	Miller-Institute	44.4690*	9.38059	.000	22.0135	66.9246
Fig-8	Overhand	-26.9583*	4.37372	.000	-37.4283	-16.4884
	Miller-Institute	17.5107	9.58235	.168	-5.4278	40.4492
Miller-Institute	Overhand	-44.4690*	9.38059	.000	-66.9246	-22.0135
	Fig-8	-17.5107	9.58235	.168	-40.4492	5.4278

Based on observed means.
The error term is Mean Square(Error) = 321.375.
*. The mean difference is significant at the .05 level.

Figure 11. SPSS Output for Tukey Post-hoc Test for TW Angle between the Three Knot Types.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of TI_Point is the same across categories of Knot.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Figure 12. SPSS Output of Independent-Samples Kruskal-Wallis Test for Tail Insertion (TI) Point between 3₁ and 4₁ Knots.

Chapter IV

DISCUSSION

Inducing Knotting Behavior

My first question tested the efficacy of different stimuli on inducing knotting. Initial tests using food items that were large enough to require knotting resulted in inconsistent knots that were difficult to control. The two major difficulties were that the hagfishes would not always feed, and if they did, they did not always knot. Furthermore, it was almost impossible to control the orientation in which the hagfish knotted. However, subsequent experiments that restrained a hagfish using a membrane allowed for reliably induced, consistent escape knots. Thus, I used a hagfish restraint device as opposed to feeding events. With the restraint device, I set the starting orientation of the hagfish and focus the cameras on the region of interest where the knot would be tied. This resulted in clear images of knotting events. Even though there were some instances of escape without knotting, the vast majority of escape events required the use of a body knot. These no-knot escapes were further reduced when I found that the smaller hagfishes performed more reliably when I used a membrane with a smaller opening. Thus, the hagfish restraint system can now be adapted to different sizes of hagfishes and provides a reliable means of successfully inducing consistent and controlled knotting behavior for kinematic analysis. This experimental protocol has the potential to be expanded to other species of knotting aquatic craniates such as water snakes and eels.

Knot Complexity

My second question assessed the types of knots that hagfish prefer. I postulated that more complex knots would be favored due to decreased internal body stress. I found that *E. stoutii* had the largest repertoire of knots of the three species studied. They were able to tie the 0, 3₁, 4₁, and 5₂ knots. *E. springeri* were found to be able to tie 0, 3₁, and 4₁ knots, while *M. glutinosa* had the least diverse range of knots and could only tie the 0 and 3₁ knots. Even though *E. stoutii* was able to tie more complex knots, it still statistically favored the simple 3₁ overhand knot. Thus, my results do not support the hypothesis that *E. stoutii* will tie more complex knots due to reduced internal stress. However, in *E. stoutii*, the species for which I had the most recordings, the number of 3₁ knotting incidences and 4₁ knotting incidences was close. This leads me to further hypothesize that the chosen knot could actually be random and depend on whether the first tail wrap happens to be directed cranially or caudally. In comparison to other aquatic craniates that tie body knots, Pickwell (1971) notes that the long slender *P. platurus* water snake used figure-eight knots when escaping and overhand knots when shedding skin. Presumably, the decreased internal stress at pinch points in the figure-eight knot lends itself to easier manipulation and escape, while higher knot stresses in the overhand knots lends itself to loosening and shedding skin. This gives rise to the interesting hypothesis that different knots, with their different levels of internal stresses and loop configurations may have different sliding, holding, and other properties that may lead to different functions.

Behavioral Stiffness

One described difference between the two major groups of hagfish, the Eptatretines and the Myxines, is that the myxines tend to hold their bodies in a more erect

position. I describe this as “behavioral stiffness” because there are no great morphological differences in body construction between the two groups that would lead me to suspect differences in body flexibility. Thus my third question investigated this difference in behavioral stiffness by measuring the radius of curvature that each hagfish species could form during knotting events. The results showed that being an Eptatretine or a Myxine did not seem to play a predominant role in the looseness of tied knots. The normalized loop radius between species was not statistically different. However, behavioral stiffness did seem to play a role in complexity of knots tied. The less behaviorally stiff *E. stoutii* tied more complex knots than the more behaviorally stiff *E. springeri* and *M. glutinosa*. While there are no documented morphological differences, I cannot currently rule out that this behavioral stiffness may be caused by body stiffness. Interestingly, Miller (1989) noted that several eel species began another loop behind the overhand knot, perhaps in an attempt to create a more complex knot. In this case, the stiffness associated with having tightly attached skin and vertebrae may prevent them from doing so. Further functional and anatomical tests need to be performed in order to describe the range of body-stiffness represented by a larger diversity of hagfish bodies.

Knotting Control

The fourth question investigated the neural control of knotting in hagfish. Because hagfish have so many points of flexibility along their slender, rope-like bodies, the neural control parameters involved could be enormous; a “hyper-redundant” system as described by mathematicians, roboticists, and engineers. While there have not been very many neurobiomechanical considerations of such systems, investigations of the best-understood biological examples (octopuses, e.g., Sumbre et al., 2006; Yekutieli et al., 2005) suggest

a simplification of knotting control via an organized underlying sequence of simple and invariant motions known as “motor primitives.” Thus, I hypothesized that knotting in hagfish may not reflect a massive “brute force” control of every point of flexibility in the body, but may rely on a neural control simplification based on motor primitives. Indeed, my findings refute the “brute force” hypothesis as there is a) statistical similarity to many aspects of knotting regardless of the knot formed and b) all knots were found to be composed of a set of three basic motions that were combined with varying repetition and order depending on which knot was being tied.

Regardless of species, all knot characteristic measurements (crossover angle, normalized loop radius, crossover overhang, tail-wrap angle, and tail-insertion point) were statistically identical. This reinforces the idea that these motions are stereotyped and suggests that their further characterizations as motor primitives is warranted. The few variations between knot types that were detected seem to be related to the physical characteristics of the knot being tied. The angle of the first tail wrap was statistically different between the two most common knots, the 3_1 knot and the 4_1 knot. Because of the physical structure of these two knots, the tail either slides caudally behind the body and inserts into the loop creating a 3_1 knot, or it wraps cranially to the body to continue making the 4_1 knot. The other statistically different measurement between the 3_1 and the 4_1 knot, the tail insertion point, was also dictated by the physical differences between these knots. This makes intuitive sense because the loop into which the tail is inserted is much larger in a 3_1 knot than in a 4_1 knot because of the twist that the loop undergoes giving the 4_1 knot its characteristic figure-eight shape.

I have described three distinct motions that, when strung together in different numbers and order can explain a simplified mechanism by which knots could be tied. My analysis of these sequences also showed that there were two rules that governed their organization: once a sequence is started it moves cranially until failure, and sequential motions always alter handedness. Sequences of these basic motions could even be used to create failed knots and theoretical knots that were not tied. Investigations of these sequences may explain why the 5_1 and the 6_1 knot were not tied. It may be because they require two consecutive tail wraps in the same direction as seen in Figure 12, and this would violate the second rule of alternation of handedness for each motion. Additionally, the 6_1 knot may lay outside the morphospace of what is possible for the hagfish; they may lack the required length to width ratio needed to create these more complex knots.

Future Directions

This study validates the need for further assessment of the basic knotting motions described above as neurobiomechanical motor primitives. To do this, one would need to show that the neural commands and muscle activation patterns are generated in the same stereotyped pattern. Recording these data requires highly flexible electrodes that have yet to be developed. These results will allow us to see if the muscle activation patterns meet the statistical definition of stereotypy. I suggest that hagfishes represent an ideal model organism for this purpose as they are extremely flexible and yet their motions may rely on a surprisingly small set of commands. An automated kinematic analysis program is currently being coded that will allow us to create 3D line representations of the center the cross sections of hagfish bodies throughout the knotting duration. This paired with

electromyographical records, would represent a powerful advance towards a better understanding of the control aspects of hagfish knotting behavior.

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APPENDIX A:

IBM Statistical Package for the Social Sciences (SPSS) Output for Statistical Analyses

Tests of Normality

	Kolmogorov-Smirnov ^a			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Normalized_Loop_Radiu s	.112	26	.200 [*]	.947	26	.200
XO_Overhang	.089	26	.200 [*]	.956	26	.311
TI_Point	.118	26	.200 [*]	.954	26	.280

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Tests of Normality

	Kolmogorov-Smirnov ^a			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
XO_Angle	.123	69	.011	.972	69	.120
TW_Angle	.072	69	.200 [*]	.976	69	.216

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Between Species

Levene's Test of Equality of Error Variances^a

Dependent Variable: XO_Overhang

F	df1	df2	Sig.
2.192	2	59	.121

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Species

Multiple Comparisons

Dependent Variable: XO_Overhang

Tukey HSD

(I) Species	(J) Species	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
E. stoutii	E. springeri	18.8633	9.31333	.115	-3.5282	41.2549
	M. glutinosa	9.0825	8.13481	.508	-10.4756	28.6406
E. springeri	E. stoutii	-18.8633	9.31333	.115	-41.2549	3.5282
	M. glutinosa	-9.7808	11.99751	.695	-38.6258	19.0641
M. glutinosa	E. stoutii	-9.0825	8.13481	.508	-28.6406	10.4756
	E. springeri	9.7808	11.99751	.695	-19.0641	38.6258

Based on observed means.

The error term is Mean Square(Error) = 246.755.

Levene's Test of Equality of Error Variances^a

Dependent Variable: Radius_Factor

F	df1	df2	Sig.
6.256	2	57	.003

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Species

Multiple Comparisons

Dependent Variable: Radius_Factor

Games-Howell

(I) Species	(J) Species	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
E. stoutii	E. springeri	.0740	.03252	.103	-.0141	.1620
	M. glutinosa	-.3397	.16568	.243	-1.0155	.3361
E. springeri	E. stoutii	-.0740	.03252	.103	-.1620	.0141
	M. glutinosa	-.4137	.16577	.164	-1.0891	.2617
M. glutinosa	E. stoutii	.3397	.16568	.243	-.3361	1.0155
	E. springeri	.4137	.16577	.164	-.2617	1.0891

Based on observed means.

The error term is Mean Square(Error) = .030.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of TI_Point is the same across categories of Species.	Independent-Samples Kruskal-Wallis Test	.062	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Levene's Test of Equality of Error Variances^a

Dependent Variable: TW_Angle

F	df1	df2	Sig.
2.000	2	71	.143

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Species

Multiple Comparisons

Dependent Variable: TW_Angle

Tukey HSD

(I) Species	(J) Species	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
E. stoutii	E. springeri	-3.8754	10.69054	.930	-29.4668	21.7160
	M. glutinosa	-20.6904	11.86671	.196	-49.0973	7.7165
E. springeri	E. stoutii	3.8754	10.69054	.930	-21.7160	29.4668
	M. glutinosa	-16.8150	15.45250	.524	-53.8057	20.1757
M. glutinosa	E. stoutii	20.6904	11.86671	.196	-7.7165	49.0973
	E. springeri	16.8150	15.45250	.524	-20.1757	53.8057

Based on observed means.

The error term is Mean Square(Error) = 530.621.

Levene's Test of Equality of Error Variances^a

Dependent Variable: XO_Angle

F	df1	df2	Sig.
2.914	2	66	.061

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Species

Multiple Comparisons

Dependent Variable: XO_Angle

Tukey HSD

(I) Species	(J) Species	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
E. stoutii	E. springeri	13.0201	10.06179	.404	-11.1051	37.1453
	M. glutinosa	-12.6549	10.06179	.424	-36.7801	11.4703
E. springeri	E. stoutii	-13.0201	10.06179	.404	-37.1453	11.1051
	M. glutinosa	-25.6750	13.78473	.158	-58.7267	7.3767
M. glutinosa	E. stoutii	12.6549	10.06179	.424	-11.4703	36.7801
	E. springeri	25.6750	13.78473	.158	-7.3767	58.7267

Based on observed means.

The error term is Mean Square(Error) = 380.038.

Between Knot Types

Levene's Test of Equality of Error Variances^a

Dependent Variable: XO_Angle

F	df1	df2	Sig.
.076	2	66	.927

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Knot_Type

Multiple Comparisons

Dependent Variable: XO_Angle

Tukey HSD

(I) Knot_Type	(J) Knot_Type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Overhand	Fig-8	.9359	4.86429	.980	-10.7272	12.5990
	Miller-Institute	-19.5284	10.22124	.144	-44.0359	4.9791
Fig-8	Overhand	-.9359	4.86429	.980	-12.5990	10.7272
	Miller-Institute	-20.4643	10.38026	.127	-45.3531	4.4245
Miller-Institute	Overhand	19.5284	10.22124	.144	-4.9791	44.0359
	Fig-8	20.4643	10.38026	.127	-4.4245	45.3531

Based on observed means.

The error term is Mean Square(Error) = 377.125.

Levene's Test of Equality of Error Variances^a

Dependent Variable: Radius_Factor

F	df1	df2	Sig.
.312	3	56	.817

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Knot

Multiple Comparisons

Dependent Variable: Radius_Factor

Tukey HSD

(I) Knot	(J) Knot	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Overhand	Figure-Eight	.0363	.05295	.902	-.1039	.1765
	Loop-Leverage	.0731	.11812	.926	-.2397	.3858
	Miller-Institute	-.0487	.11812	.976	-.3614	.2641
Figure-Eight	Overhand	-.0363	.05295	.902	-.1765	.1039
	Loop-Leverage	.0368	.11855	.990	-.2771	.3507
	Miller-Institute	-.0850	.11855	.890	-.3989	.2289
Loop-Leverage	Overhand	-.0731	.11812	.926	-.3858	.2397
	Figure-Eight	-.0368	.11855	.990	-.3507	.2771
	Miller-Institute	-.1218	.15875	.869	-.5421	.2986
Miller-Institute	Overhand	.0487	.11812	.976	-.2641	.3614
	Figure-Eight	.0850	.11855	.890	-.2289	.3989
	Loop-Leverage	.1218	.15875	.869	-.2986	.5421

Based on observed means.

The error term is Mean Square(Error) = .038.

Levene's Test of Equality of Error Variances^a

Dependent Variable: XO_Overhang

F	df1	df2	Sig.
.094	2	58	.911

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Knot

Multiple Comparisons

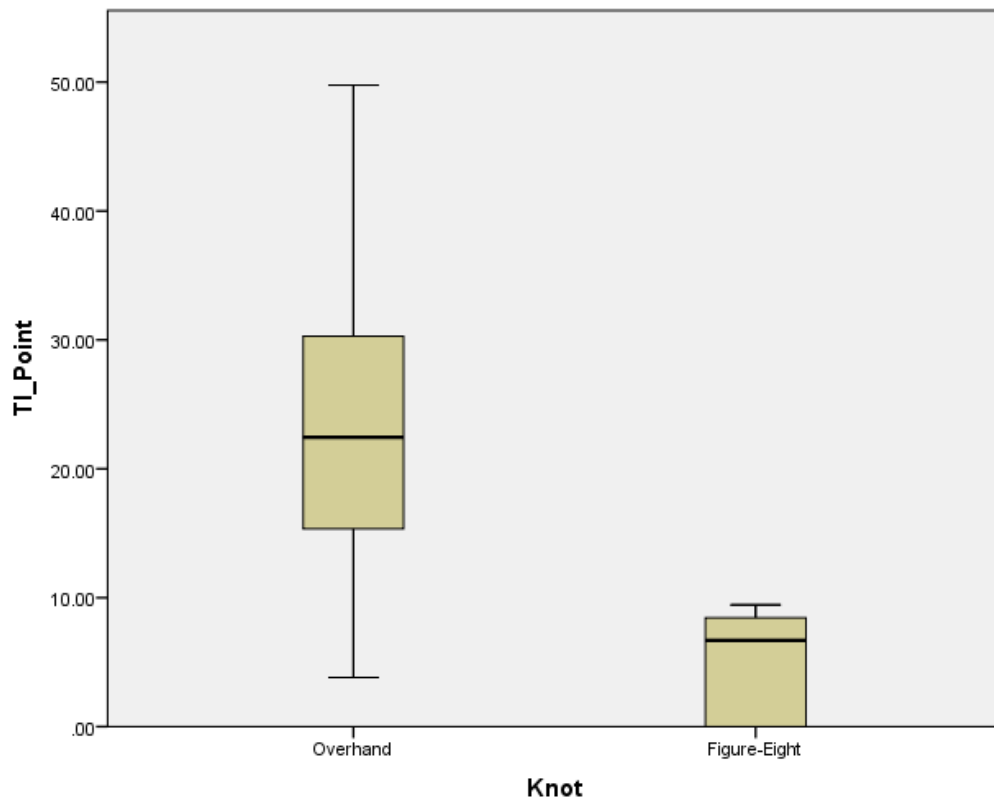
Dependent Variable: XO_Overhang

Tukey HSD

(I) Knot	(J) Knot	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Overhand	Figure-Eight	-.2534	4.37762	.998	-10.7830	10.2761
	Miller-Institute	1.5718	8.78400	.983	-19.5564	22.7001
Figure-Eight	Overhand	.2534	4.37762	.998	-10.2761	10.7830
	Miller-Institute	1.8253	8.84122	.977	-19.4406	23.0912
Miller-Institute	Overhand	-1.5718	8.78400	.983	-22.7001	19.5564
	Figure-Eight	-1.8253	8.84122	.977	-23.0912	19.4406

Based on observed means.

The error term is Mean Square(Error) = 272.324.



APPENDIX B:

Institutional Animal Care and Use Committee Approval



Institutional Animal Care and Use Committee (IACUC)

ANIMAL USE PROTOCOL APPROVAL

February 13, 2017

Dr. Theodore Uyeno
Department of Biology
Valdosta State University

Dear Dr. Uyeno;

Animal Use Protocol (AUP) "*RU: Collaborative Proposal: Biting Hard with soft Feeding Apparatuses*" (AUP-00070-2017) has been approved by the Institutional Animal Care and Use Committee (IACUC). This approval is from 02/13/2017 – 02/13/2020. Each year, an animal report must be submitted to the IACUC to keep your protocol active. You will be contacted by the Office of Sponsored Programs and Research Administration approximately one month before the annual report is due.

Please remember that you must obtain IACUC approval before amending, or altering the scope, or procedures of the protocol. You are also required to report to attending Veterinarian, the IACUC Chair, and the IACUC Administrator any unanticipated problems with the animals that become apparent during the course, or as a result of the research, or teaching activity.

Should you have questions concerning your approved research, please contact Tina Wright, Research Compliance Specialist, at 229.253.2947, or email IACUC at iacuc@valdosta.edu.

Sincerely,

Elizabeth Olphie

Elizabeth "Ann" Olphie
IACUC Administrator

cc: Dr. James LaPlant, Institutional Official
Dr. Teresa Doscher, Attending Veterinarian
Dr. Robert L. Gannon, Department Head