

THE EFFECT OF REGIONAL PHENOTYPIC DIFFERENCES OF *PROCAMBARUS CLARKII*
OPENER MUSCLE ON SARCOMERE LENGTH, FIBER DIAMETER, AND FORCE
DEVELOPMENT

THESIS

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ABSTRACT OF THESIS

THE EFFECT OF REGIONAL PHENOTYPIC DIFFERENCES OF *PROCAMBARUS CLARKII* OPENER MUSCLE ON SARCOMERE LENGTH, FIBER DIAMETER, AND FORCE DEVELOPMENT

The opener muscle in the walking legs of the crayfish (*Procambarus clarkii*) has three distinct phenotypic regions although innervated by only one excitatory motor neuron. These regions (distal, central and proximal) have varied biochemistry and physiology, including synaptic structure, troponin-T levels, EPSP amplitudes and facilitation, fiber diameter, input resistance, sarcomere length, and force generation. This investigation confirmed that excitatory postsynaptic potentials (EPSPs) in the proximal fibers have larger amplitudes and facilitate less than those of the central fibers with distal fibers showing intermediate levels. As a result of a larger facilitation index, the force generated by the central fibers when the excitatory neuron was stimulated at 40Hz was more than the force generated by the other regions. This increase in force was correlated with the central fibers having longer sarcomeres when measured in a relaxed claw. These data support the idea that the central fibers are tonic-like and proximal fibers are phasic-like. The addition of serotonin directly to the fibers was hypothesized to increase the force generated by the central fibers more than in the other regions, but this did not occur at 40Hz stimulation. This study demonstrates how malleable the motor unit is with modulation and frequency of stimulation.

KEY WORDS: invertebrate, neuromodulation, tonic verses phasic fibers, neuromuscular junction

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CHAPTER 1

INTRODUCTION

On the first two crayfish pereopods, or walking legs, are small claws, which crayfish use to feed themselves. The muscle used in this study, the opener muscle, is aptly named because it opens those claws. Located in the dorsal region of the propodite leg segment, contraction of the opener muscle causes the dactylopodite segment to rise. Since crayfish have no internal skeleton, they have invaginations of the cuticle called apodemes that act as tendons. Therefore muscle fibers are attached cuticle-to-cuticle giving the opener muscle a pinnate fiber arrangement with three regions: distal, central, and proximal. These different regions have several known characteristics that show the proximal region has phasic like fibers, the central region has tonic like fibers, and the distal region is mixed.

Generally, skeletal muscle can be classified as either slow twitch (tonic) or fast twitch (phasic) based on the speed of contraction and the myosin isoforms causing different ATPase activity. Tonic fibers in vertebrates can be darker in color because of the increase in myoglobin and mitochondria in the muscle, and the increase in circulation bringing more red blood cells to that type of muscle. These fibers are used for continual motion, like the muscles in a panda's (*Ailuropoda melanoleuca*) abdomen used for standing. Whereas, phasic fibers can be paler in color and used by animals for fast motion, like the muscles in a cheetah's (*Acinonyx jubatus*) hind limbs which allow for high speed running (Goto et al., 2012). More specifically, in a tonic muscle fiber the sarcomeres are usually longer, contract slower, and therefore can generate more force because there are more myosin heads attaching to the actin over this longer distance. It is important that they can generate more force with less fatigue because these fibers are used to maintain posture in the animal or hold a claw open waiting for food to swim by. There are generalities about motor neurons as well. The phasic synapses have more active zones and therefore can have higher synaptic output because there are more readily releasable vesicles in the terminals. This study was designed to see whether

these general properties of tonic and phasic fibers hold when looking at the different regions of the crayfish opener muscle.

There is a lot of data on the difference between the regions of the opener muscle with respect to the gross anatomy, physiology, biochemistry, and nerve terminals, but no information about how the different regions develop force. The goal of this project was to correlate all the regional differences found in the literature with the generation of force, length of sarcomere, and sensitivity to serotonin. Using the data found in the literature since 1888, I hypothesized that the proximal region would be more phasic-like; therefore, generate force quickly, have shortest sarcomeres when passively stretched, and have the least influence by serotonin. The central region would be more tonic-like; generating force more slowly, having the longest sarcomeres, and giving rise to the greatest increase in force when serotonin was added since it is more influenced with respect to mean quantal output. The distal region would have an intermediate amount of force generation and length of sarcomere.

Since force generation of the opener muscle has not been studied before, first this investigation had to determine the appropriate dissection technique to measure force and the proper stimulation paradigm to generate a measurable force; therefore, much of the data was not used in the statistical analysis and results. It was determined that the dorsal dissection of the opener and the muscle stimulated at 40 Hz was the optimum experimental design, hence this limitation on the data was used to make the study more manageable and focus more narrow. Other manipulations of the data might be able to answer other questions, but are beyond the scope of this project.

These questions about force generation in the opener muscle could be answered either by using intact muscles or stripped muscle fibers. "The demembranated (skinned) muscle fiber preparation is widely used to investigate muscle contraction because the intracellular ionic conditions can be precisely controlled. However, plasma membrane removal results in a loss of osmotic regulation, causing abnormal hydration of the myofilament lattice and its proteins (Tanner et al., 2012)." Emanuel Azizi's studies in *in situ* muscles have shown that the physical arrangement is important to determine the

mechanical function (Azizi et al., 2008). By using the intact animal, I hoped to understand the locomotor performance. However, I found that ablating the proximal fibers caused damage to the nerve such that other fibers could not generate force when stimulated. These phasic fibers appear to anchor the apodeme to the cuticle, thus making it impossible to stimulate and record force generation from the distal and central fibers alone. If the effect of serotonin on force generated by different regions in the crayfish opener muscle were to be studied again, using *ex situ* muscle fibers would most likely produce different results, which could perhaps be more in line with the mean quantal output data found in the literature. The limitation of not being able to remove the proximal fibers in the crayfish opener muscle will need to be addressed in the design of future studies with pinnate muscles like the opener.

Chapter 2 of this thesis will explain background information on general muscle structure in both invertebrates and vertebrates. I will then describe more specifically the phenotypic differences of muscle, and end Chapter 2 with a literature review of the currently known differences in the regions of the opener muscle. Since several dissection techniques were attempted, Chapter 3 gives an extensive description of the methodology used to conduct this investigation. This information should be valuable to other researchers studying opener muscles in other decapods. Chapter 4 explains the statistics employed as well as the interpretation of the research, i.e., the amount of force these different regions can generate, the difference in sarcomere length of these regions, and the effect that serotonin has on the force generation. Since the known differences in the regions of the opener muscle never have been correlated with muscle output or force development, Chapter 5 ties together this investigation with already reported data in the literature.

CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

Muscle transforms chemical energy into mechanical energy, and although the molecular mechanism is similar in all multicellular animals, there is astonishing variation in the overall process. In vertebrates and invertebrates alike, muscles are a part of a lever system that creates an inverse relationship between force and velocity, allowing a muscle to be specialized for strength or speed, respectively. Muscle is categorized broadly as striated or smooth with the former deriving its name from the appearance of the muscle cell. There are two types of striated muscle, skeletal and cardiac, and two main phenotypes of skeletal muscle, fast twitch (phasic) and slow twitch (tonic). This review will focus on general skeletal muscle morphology, excitation-contraction coupling, mechanical behavior of muscle, differences between vertebrate and invertebrate muscle physiology, phasic versus tonic fibers, and document specifics of the crustacean opener muscle.

Contractile Apparatus: The Sarcomere

In many animals, each skeletal muscle is composed of many muscle cells called muscle fibers that are packed in parallel. These fibers contain nuclei located on the surface and all the remaining space is occupied by bundles of filaments called myofibrils, which are subdivided longitudinally into the contractile units of a muscle, the sarcomeres. Each sarcomere is composed of a thick filament, myosin, and a thin filament, actin, that interact together powering motility of striated and smooth muscles. These filaments are highly arranged and their interactions in the cross bridge cycle are highly regulated. Within a filament, the Z line demarcates the ends of one sarcomere and on either side of the Z line is the I band, which is composed primarily of actin. The center of the sarcomere is the A band which contains primarily myosin; however, the actin and myosin overlap at the ends of the A band forming a dark region and a lighter region towards the center composed of myosin only called the H band. The dark line

down the absolute center of the sarcomere is the M line, which contains proteins that help align the myosin filaments. At the ends of the myosin is the protein titin that tethers the myosin to the Z line (discussed more in detail later). When the sarcomere shortens during contraction the A zone does not change in length, but the H zone disappears and the I zone becomes shorter as a result of the filaments sliding with respect to each other (Gordon et al., 2000).

The thin filament is formed by the spontaneous aggregation of globular actin into a helical two-stranded actin filament (Koubassova and Tsaturyan, 2011). Also within the thin filament are the proteins nebulin, tropomyosin (Tm), and troponin (Tn). Nebulin regulates the length of the thin filament (Witt et al., 2006), tropomyosin covers the myosin binding sites on the actin filament, and the three types of troponin (TnT, TnC, and TnI) form a complex that influence the position of the tropomyosin and thus the binding of actin to myosin during the cross bridge cycle (Koeppen and Stanton, 2010). Each troponin is named from its first identified property: TnT binds tropomyosin, TnC binds calcium ion, and TnI is an integral protein for the conformation of the tropomyosin/ troponin complex and inhibits binding of myosin heads (Gordon et al., 2000). Calcium ion binding to troponin C enhances the interaction between troponin C and troponin I and thus weakens the troponin I attachment to actin. This calcium binding also exposes the myosin binding sites on the actin by causing tropomyosin movement (Gordon et al., 2000). TnT is a highly asymmetric molecule with many isoforms and acts as the structural glue holding the Tn-Tm-actin complex together through binding Tm, TnI, TnC, and actin (Perry, 1998). This interaction helps determine the position of tropomyosin on the thin filament and the different isoforms of TnT can lead to important functional differences in the regulation of contraction, such as altered calcium sensitivity and enhanced activation of ATPase (Gordon et al., 2000).

The thick filament consists of many myosin motor proteins in a bipolar arrangement formed by the tail-to-tail association of these myosin molecules centered on the M line of the sarcomere that interact with actin to produce sarcomere shortening and force (Koeppen and Stanton, 2010). All myosins contain one or two heavy chains

and several light chains (Koubassova and Tsaturyan, 2011) and often is composed of six different polypeptides, two heavy chains and four light chains. The heavy chains wind together to form the long rod-like region and a portion of the large globular head of the myosin molecule with the light chains necessary for the hydrolysis of ATP by the myosin heads. The bipolar arrangement of the thick filament is critical for shortening of the sarcomere during contraction (Gordon et al., 2000). The thick filament contains other non-motor protein (myosin binding protein C, MyBP-H, and MyBP-X) which appear to play a role in forming the myosin filament by bundling the myosin molecules together during development. The thick filament also contains the large elastic molecule, titin, which links the thick filaments to the Z-discs and plays a major role in contributing to the stability and passive elasticity of the sarcomere (Horowitz and Podolsky, 1987).

Sliding filament theory and cross bridge cycling

Using high-resolution microscopy, the sliding filament theory was independently developed by Hugh Huxley and Andrew F. Huxley in 1954 which provided a molecular basis for muscle contraction (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954). It states that force produced by the shortening of a muscle occurs through interactions of the two motor proteins: actin and myosin (Herzog et al., 2008). During contraction, they observed the A band (containing the thick filaments of myosin) remaining constant in length but the other regions of the sarcomere shortened.

As mentioned above, each myosin protein has the globular heads, called the S1 region, with multiple hinged segments, which can bend and facilitate contraction (Hynes et al., 1987; Spudich, 2001). This S1 region bending and the flexibility of the myosin tail region, or S2, explains how myosin moves along the actin (Krans, 2010). The traditional sliding filament theory says once the myosin binding sites are uncovered, the energized myosin head (which has ADP and inorganic phosphate bound) binds to actin. The ratchet action occurs when hydrolysis of ATP is completed and ADP and inorganic phosphate are released. This ATP hydrolysis results in release of chemical energy that is transformed into mechanical work (Koubassova and Tsaturyan, 2011) and the myosin head relaxes to a low energy conformation (Hill and Olson, 2012).

Specifically, this ATP hydrolysis provides the energy for myosin to go through cross bridge cycling: release actin, change its conformation, contract, and repeat the process again (Krans, 2010). The conformational change in the myosin molecule pulls the actin filament toward the center of the sarcomere in a power stroke and thus shortening the sarcomere and the I band. A new ATP binds to the globular myosin head causing a decrease in the affinity of myosin to actin and therefore a release of the cross bridge (Koubassova and Tsaturyan, 2011). The energy released in a partial hydrolysis of the newly bound ATP is used to recock the myosin head, which is now ready to bind again. If intracellular calcium ion concentration is still elevated and ATP is available the cycle repeats producing further contraction of the muscle, and if it is low, relaxation occurs (Gordon et al., 2000). Each second, the 350 heads of each thick filament form and reform about five cross-bridges (Hill and Olson, 2012). Myosin would remain bound to actin indefinitely — causing the stiffness of rigor mortis — if new ATP molecules were not available (Lorand, 1953).

The mechanism of the swinging lever arm model of cross bridge action has been determined using X-ray (Smith and Rayment, 1996), electron micrographs (Rayment et al., 1993), and chemical mapping (Holmes et al., 1990). These data suggest four sites produce the interaction associated with contraction: two ionic interactions of amino acid residues, one hydrophobic interaction of residues, and one proline interaction (Gordon et al., 2000). First, the myosin binds to actin with the ionic interactions causing a change in the cleft, which allows the release of the phosphate. This cleft change also causes the movement of the lever arm attached to the globular head (Gordon et al., 2000).

Some observations of muscle cells show they can behave in ways that do not match the current model. For example some muscles in mollusks and arthropods can generate force over long periods which is called force hysteresis or catch tension (Krans, 2010; Hoyle, 1969). Other unresolved questions that still have not been answered are depression of force with shortening, high thermodynamic efficiency of actively shortening muscles, low cost of force production during active stretch (Monroy et al.,

2012), and enhancement of force with stretch (Herzog et al., 2008). However efforts over the past 50 years to explain these observations have led to modifications of the original theory and alternative hypotheses (Monroy et al., 2007). Monroy et al. suggest there is evidence that active muscles behave as if they possess an internal spring, which is capable of storing energy, and the protein titin is the likely candidate. They propose an addition to the sliding filament swinging cross-bridge theory: a winding filament model. This theory says the titin is activated by Ca^{+2} influx and then the titin is wound on the actin. (Monroy et al., 2012).

Regulation of sarcomere shortening

ATP and calcium are required for the contraction of muscle cells and act as non-protein components of enzymes called cofactors. As mentioned above, the ATP provides the energy and the calcium regulates the muscle contraction. In a resting sarcomere, tropomyosin blocks the binding of myosin to actin. With the increase in intracellular Ca^{2+} in response to membrane depolarization, Ca^{2+} binds to troponin C causing tropomyosin to move toward the actin cleft and exposing the myosin binding sites on actin. In 1994, William Lehman and his colleagues demonstrated how tropomyosin rotates by studying the shape of actin and myosin in calcium-rich and calcium-depleted solutions (Lehman et al., 1994). By comparing the action of troponin and tropomyosin under these two conditions, they found that the presence of calcium is essential for the contraction mechanism. Once the myosin-binding sites on the actin are exposed, and sufficient ATP is present, myosin binds to actin to begin cross-bridge cycling. Then the sarcomere shortens causing the muscle to contract. If calcium is not present then this binding cannot occur, showing that the presence of calcium is an important regulator of muscle contraction.

Each myofibril is surrounded by the sarcoplasmic reticulum (SR), an intracellular membrane network, and each muscle fiber is surrounded by a cell membrane called the sarcolemma. T tubules, formed through invagination of the sarcolemma, run along the ends of the A band of the sarcomere near the terminal cisternae of the SR. Together

these distinct membrane systems participate in the regulation of intracellular calcium that causes contraction of the sarcomere and therefore the muscle fiber.

In mammals and maybe all vertebrates, an action potential originating in the CNS is transmitted along an α -motor neuron to the neuromuscular junction. Once it reaches the junction, Ca^{2+} enters the end plate and causes acetylcholine to be released into the synapse. Acetylcholine binds and activates nicotinic receptors, which causes an influx of sodium ion and efflux of potassium ion. The muscle fiber depolarizes and the action potential is transmitted along the sarcolemma traveling down the T tubule causing Ca^{2+} to be released from the terminal cisternae of the SR into the myoplasm of the muscle fiber. This increase in intracellular Ca^{2+} concentration promotes actin-myosin interaction and therefore initiates contraction.

This description of the activities at the endplate is not the same in all organisms. Much like a vertebrate cardiac muscle, invertebrate skeletal muscle requires extracellular calcium (Zacharová and Zachar, 1967). If the action potential is being carried on an excitatory motor neuron, the transmitter glutamate is released (Kawagoe et al., 1982) and if an inhibitory motor neuron, γ -aminobutyric acid (GABA) is released (Takeuchi and Takeuchi, 1965). In crayfish and *Drosophila* the sarcolemma has voltage gated Ca channels which allow Ca^{2+} to enter from the extracellular fluid and not only the SR (Erxleben and Rathmayer, 1997). However this influx of Ca^{2+} through the sarcolemma is not sufficient for excitation-contraction (E-C) coupling (Mounier and Goblet, 1987), instead contraction comes from calcium-induced Ca^{2+} release (CICR). Györke and Palade reported that CICR is more closely controlled by a small amount of Ca^{2+} entering through T-tubule membrane than by a larger amount released by the SR. This allows CICR to remain graded rather than an all-or-none (Györke and Palade, 1992). In contrast, excitation-contraction coupling in vertebrate skeletal muscle is not generally believed to involve CICR, but rather relies on the mechanism mediated by charge movement in the T-tubule membrane, explained below.

The molecular mechanism behind this Ca^{2+} release is a physical protein-protein interaction between the L-type voltage gated Ca^{2+} dihydropyridine receptor (DHPR)

embedded in the T tubule and the ryanodine receptor (RyR) embedded on the SR. An action potential causes both an influx of extracellular Ca^{2+} through the DHPR and a conformation change in the DHPR which in turn causes the RyR to open and release Ca^{2+} stored in the SR into the myoplasm. Several proteins work together to increase the concentration of Ca^{2+} at the terminal cisternae allowing for the quick efflux of Ca^{2+} from the SR when the RyR opens. Calsequestrin, a low affinity Ca^{2+} binding protein, is found in the lumen of the SR and facilitates Ca^{2+} storage creating a favorable concentration gradient. Other membrane proteins, triadin and junctin, bind both the RyR receptor and calsequestrin to increase the amount of Ca^{2+} at the terminal cisternae, the site of calcium release. Histidine-rich calcium-binding protein (HRC) is another low affinity calcium binding protein found in the SR lumen that binds triadin in a calcium dependent manner.

Relaxation of vertebrate skeletal muscle requires the resequestration of Ca^{2+} by the SR through the action of the Ca^{2+} pump named SERCA (sarcolemmal endoplasmic reticulum calcium ATPase). This reuptake occurs throughout the longitudinal and terminal SR and the Ca^{2+} is moved back to the terminal SR by the protein sarcalumenin. However in crayfish NCX (basolateral $\text{Na}^+/\text{Ca}^{2+}$ exchanger) and PMCA (plasma membrane Ca^{2+} -ATPase) move intracellular Ca^{2+} across the membrane (Wheatly et al., 2007; Ashley et al., 1974). The crayfish NCX has similar physical and chemical properties to excitable mammalian tissue; except its optimum temperature is 20°C since crayfish are poikilotherms (Ruscák et al., 1987).

Tension- force development

When muscles contract, they decrease in length and generate force which is often measured as tension or stress. The length tension relationship can be studied in one of two ways, either by using isometric contractions, muscle length is held constant, or isotonic contractions, force is held constant. When a muscle at rest is stretched it will resist that stretch by a passive force that will increase slowly at first and then more rapidly as the stretch increases. Herzog and colleagues think this passive component is caused in part by a "calcium-dependent increase in passive force, which is likely

associated with an increase in stiffness of the molecular spring titin.” They go on to speculate that the “rest of the passive force enhancement is related to a calcium or troponin C, or force dependent regulation of titin attachment to actin (Herzog et al., 2008).” In 1954 Huxley and Niedergerke reported that short sarcomeres contract more rapidly than fibers with long sarcomeres because of the larger number of sarcomeres in series with a given length of muscle fiber (Huxley and Niedergerke, 1954). However, Jahromi and Atwood showed in 1969 that if the muscle is stimulated to contract at different lengths, the force generated will increase as the muscle length is increased, up to a point which is designated as the optimum length (L_0). As the muscle is stretched beyond L_0 the force decreases which is consistent with the sliding filament theory because in a very long sarcomere the actin will no longer overlap with the myosin heads (Jahromi and Atwood, 1969). In contrast, a very short sarcomere will have thin filaments colliding in the middle of the sarcomere (Koeppen and Stanton, 2010).

Phasic versus tonic fibers

It has been known since the 1678 that there are different types of muscle fibers when Stefano Lorenzini first described red and white muscles of Elasmobranchii (sharks and rays) (Bone, 1966). Two hundred years later, Ranvier reported that red muscles in rabbits contracted more slowly than white muscles. Recently, scientists have shown the existence of a variety of muscle fiber types with distinct biochemical, physiological and morphological properties (Schiaffino and Reggiani, 2012). I will begin with a description of mammalian skeletal muscle types.

In mammalian skeletal muscles there have been four different fibers classified through histochemical reactions for myosin ATPase- slow fibers or type 1 and fast fibers or type 2A, 2B, and 2X (Schiaffino et al., 1989)- and through SDS-PAGE gels hybrid fibers with mixed composition have also been identified (Termin et al., 1989). Physiological studies correlate an increase in the speed of shortening to the different fiber types, with an increase from type 1 to type 2A, type 2X, and fastest with type 2B fibers (Schiaffino and Reggiani, 2012). Type 1 fibers appear red because they have an abundant amount of myoglobin, mitochondria and oxidative enzymes; whereas type 2B fibers appear

white because they have an opposite metabolic profile with low amounts of myoglobin, high levels of glycolytic enzymes, and larger surface area of sarcoplasmic reticulum (Eisenberg and Kuda, 1976).

Muscle fibers are organized in motor units, and the type of fiber is determined by its innervation. All fibers in a motor unit are innervated by a single α -motor neuron and all fibers within a motor unit are the same type. Kugelberg and Burke demonstrated this homogeneous composition of fiber type and identified three major types of units: slow (type 1), fast fatigue resistant (type 2A) and fast fatigable (type 2B). These properties are directly related to activity of oxidative enzymes (Schiaffino and Reggiani, 2012). Slow twitch are recruited first and as more and more force is needed, fast twitch are recruited. First muscle fibers recruited are those that have high resistance to fatigue.

In mammalian skeletal muscle, action potentials are uniform and lead to a reproducible release of Ca^{+2} from the SR, and a single action potential causes the release of enough Ca^{+2} to cause a twitch (Koeppen and Stanton, 2010). There is great variation in fiber type profiles between species and within species. For example genetic differences led to human sprint runners having predominately type 2 fibers and endurance runners having type 1 (Simoneau and Bouchard, 1995). Table 1 states a few of the properties of muscle fiber types to show that slow fibers are more suitable for low intensity and long lasting activity and fast fibers for short and strong contractile performance.

Properties of Type 1 and Type 2B fibers in vertebrates

Properties	Type 2B (Fast / Phasic)	Type 1 (Slow/ Tonic)
Network of SR	T-tubules and invaginations are more extensively developed	Less extensive
Calcium pumps	DHPR and SERCA are more abundant in the T-tubules	Slower
Calcium release channels	Greater abundance	
Calcium entry	Less from extracellular space	Greater both at rest and during depolarization
Calcium sensitivity	Higher affinity and higher cooperatively	
Twitch		5x longer
Speed of shortening	Higher speed against any given load	
Mitochondria		Many
Myoglobin (O ₂ storing)		Abundant
Resting membrane potential	More negative	
Sarcomere length	Shorter sarcomeres	Longer nebulin and actin filaments would imply longer sarcomeres

Table 1: (Information in the table comes from Schiaffino and Reggiani, 2012).

Whole crustacean muscles are often composed of muscle fibers with a range of contractile properties and can be adapted for slow and fast movement. The most obvious attribute of a muscle fiber is its diameter and there is great variation in crustacean muscle fiber diameter; however, within the same organism the smaller diameter fibers tend to produce slower contractions. With few exceptions, the sarcomere length is also correlated to contraction speed, with short sarcomere fibers producing less force per unit area more quickly (Chapple, 1982). Other features of muscle morphology are also correlated with speed of contraction. Atwood stated sarcomeres of slow fibers are at least twice as long as those of fast fibers and tonic muscle fibers often have wide irregular Z bands, indistinct H band, and the M line may or may not be present (Jahromi and Atwood, 1969). These slow fibers also have thick layers of mitochondria beneath the sarcolemma, high concentrations of glycogen granules, and neuromuscular junctions deeply embedded within fibers (Hoyle and Burrows, 1973).

Differences in vertebrates and invertebrates

There are many major differences in vertebrate and invertebrate muscle physiology and this section will summarize the differences that have been mentioned in the previous sections. The invertebrate thick filament shows great variability and can even vary in a single muscle; however, the thin filament has been shown to be similar to vertebrates. The review by Hooper et al. in 2008 states “describing invertebrate thick filament structure has been an enormous undertaking, and even today detailed understanding of their structure is available for only a few types of thick filament (Hooper et al., 2008).”

Crustaceans do not have tendons, but invaginations of the cuticle called apodemes. At the distal end of these apodemes are tension receptors that can sense the sheering forces and act much like our Golgi tendon organs (Cooper and Hartman, 1994). Invertebrate muscles show considerably more variety in their structural organization than do vertebrate muscles (Caillé et al., 1985). Crustacean muscles can

have huge fibers, and specifically in the crayfish opener muscle the individual fibers are not bundled like in vertebrates and attach directly to the apodeme.

Like vertebrates, crayfish can have phasic neurons innervate phasic muscles, but unlike vertebrate neuromuscular junctions, a motor unit may include more than one muscle (Atwood, 1976), can be dual innervated by both tonic and phasic motor neurons (e.g. leg extensors), or different muscle fiber types can be innervated by the same phasic motor neuron (e.g. claw opener) (LaFramboise et al., 2000). Vertebrates only have excitatory neuromuscular junctions, whereas the opener muscle is innervated by both excitatory and inhibitory axons (Gunzel et al., 1993).

Muscle tension in invertebrates occurs with facilitation of excitatory postsynaptic potentials (EPSP) and those EPSPs are graded and not an all-or-nothing event like with vertebrates (Atwood, 1964). There can also be different synaptic responses depending on the region of the muscle even though it is innervated by the same motor neuron (Iravani, 1965).

Serotonin modulation of muscles

A neuromodulator regulates or modifies neuronal processes and travel in the systemic circulation, regulating a target far from the site of synthesis. Several neuromodulators are present in crayfish hemolymph, including octopamine, 20-hydroxy-ecdysone, and 5-hydroxy-tryptamine (commonly called serotonin). Serotonin is produced in serotonergic neurons through a two-step reaction involving tryptophan and then stored in vesicles until release. The effects of serotonin are numerous and varied in both vertebrates and invertebrates. For example, serotonin regulates social stress and depression-like behavior (Mineur et al., 2013), decrease aggressive and locomotor behaviors in beta fish (Kohlert et al., 2012), and can cause mate rejection in female cabbage butterflies (Obara et al., 2011). The type of response elicited from serotonin is dependent on the type of receptors present on the target tissue.

In crustaceans, serotonin can be released directly by the neuron onto their target, or released into the hemolymph and bathe the muscles and target tissues (Florey and Rathmayer, 1978). Serotonin has been reported to enhance synaptic transmission

at crayfish neuromuscular junctions by increasing mean quantal content (Dudel, 1965). Serotonin can modulate the physiology of the opener muscle of the lobster walking leg (Goy et al., 1984), enhance the release of transmitter from the excitatory and inhibitory axons that innervate the muscle, and act directly on muscle fibers to produce a contraction (Kravitz et al., 1980). Serotonin causes long-lasting increases in both spontaneous and evoked release of transmitter on the excitatory and inhibitory nerve terminals (Dudel, 1965). As evoked EPSPs show depression, serotonin can cause an increase in the amplitudes of the evoked EPSPs. These findings indicate that serotonin could be recruiting reserve pool (RP) into the readily releasable pool (RRP) (Wu and Cooper, 2012b).

Summary

The crustacean muscle shows great diversity in structure and physiology and thus provides opportunity to learn about the relationship between structure and function. For example the muscles in the walking leg of decapods has a pinnate arrangement with attachments at the central apodeme and the exoskeleton that increases the force generated by that muscle (Alexander, 1968). Because of the specialization of these muscle fibers they can produce different amounts of force with the same cross-sectional area (Jahromi and Atwood, 1969). The crustacean model also has little variability in the morphology among preparations, the neurons are large and easily identifiable, and both excitatory and inhibitory motor neurons synapse on the same muscle fiber (Atwood, 1976).

Chapple suggests that mechanical properties of muscles can be illustrated by four general experimental procedures: “(1) the isometric contraction– estimating muscle speed from the time-to-peak contraction or from the relaxation time; (2) the isometric length-tension curve– identifying the relationship between the contractile properties of a muscle and its length; (3) the isotonic force-velocity curve– relating the speed of shortening of a muscle to its load; (4) the muscle stiffness– relating the instantaneous force of a muscle to its length. However, there are difficulties associated with measuring these mechanical properties. For example, crustacean muscle

excitation-contraction coupling is graded which makes it difficult to establish a basal level of activation. Differences in speed or force produced by two muscles may be the result of differences in their degree of activation rather than the properties of the contractile apparatus (Chapple, 1982). Using the opener muscle removes this source of error.

Determinations of maximum tension are different depending on whether potassium contracture, caffeine contracture, direct stimulation of the muscle, or direct stimulation of the nerve is used to produce tension. "Since the force generated by a muscle is a function of its length, measurements of time to peak contraction, force velocity relationships, or muscle stiffness will be ambiguous unless optimum length of the muscle is defined. Length should ideally be expressed in terms of sarcomere length" (Chapple, 1982), which is why the measurement of sarcomeres was completed in this thesis.

To induce contraction in the opener muscle, a motor neuron fires bursts or trains of action potentials that will become increasingly larger based on the phenomenon of facilitation (Dudel and Kuffler, 1961). This is perhaps a truer measure of what is happening in the crayfish opener muscle under physiological conditions than the measure of a single EPSP amplitude. Since the facilitation indices among high and low output terminals is known, this thesis compares that data with the force that can be generated by the different regions of the opener muscle. In addition, this thesis correlates the force increase with the addition of serotonin to the EPSP facilitation increase with the same neuromodulator.

CHAPTER 3

METHODOLOGY

Introduction

Crayfish muscles offer not only easily accessible preparations but also allow for the study of postsynaptic responses from a single identifiable motor neuron. In particular the claw opener muscle is innervated by one excitatory motor neuron, but the excitatory postsynaptic potentials (EPSPs) in different regions can vary over 50 fold in the dorsal superficial fibers (Bittner, 1968) and as much as 8 fold in ventral superficial fibers (Iravani, 1965). Since all the muscle fibers are innervated by the same motor neuron what is causing the phenotypic differences? It is not fully understood. However, there are differences in the synaptic structure of the nerve (Mykles et al., 2002). Over the central fibers the synaptic structure is similar to tonic terminals with long nerve terminals and many varicosities; whereas, synapses over the proximal fibers are similar to a phasic terminals having short nerve terminals and few varicosities (Atwood and Cooper, 1996). The idea of retrograde influences has been investigated in frog skeletal muscle (Nudell and Grinnell, 1983), in the lobster (Katz et al., 1993), and in crayfish (Lnenicka and Mellon, 1983) with reasonable convincing evidence.

The varicosities on the central fibers have low synaptic outputs compared with the high outputs of varicosities in the proximal region (Mykles et al., 2002). There are more active sites per synapse in the proximal's high-output varicosities than in the low-output varicosities which contain many synapses with no active sites (Govind et al., 1994). This indicates that there are more silent synapses in the low-output varicosities (Atwood and Wojtowicz, 1999). The differences in the synaptic structure among the varicosities may in part explain the differences in the Ca²⁺ influx during stimulation at various frequencies (Cooper et al., 1995). This synaptic complexity within the varicosities appears to account for much of the difference in EPSP amplitudes between the proximal and central fibers (Cooper et al., 1996).

There is a measurable difference in EPSP amplitudes in the different regions of the opener muscle when a single stimulus is given to the excitatory motor neuron (Iravani, 1965; Cooper et al., 1995). In the proximal region the EPSP amplitude is very large, in the central region it is very small by comparison, and the distal region has intermediate size EPSP amplitude. These differences are most likely caused by the different fiber diameter in the regions (see figure 1). The central fibers have a large diameter which gives rise to a smaller input resistance (see figure 2). Thin fibers have a higher input resistance. If the number of leak channels assumed to be the same per area of membrane, then the thin fibers have less surface area, fewer leak channels, and therefore a higher input resistance. As seen in figure 4, the central fibers have smaller EPSPs they show a higher facilitation index like tonic fibers (Crider and Cooper, 2000). This data shows that the proximal fibers are not pure phasic fibers because the EPSPs facilitate and do not depress during a train stimulation as would be expected in purely phasic fibers (see figure 3). The stimulation paradigm is important and depending on how facilitation is measured will show different results of which region facilitates more (Crider and Cooper, 1999).

Another known difference is how serotonin affects regional EPSPs. Serotonin is a neurotransmitter that is directly released into the hemolymph of crayfish. It can activate readily releasable pools in the nerve terminal. Since the central fibers are more tonic-like, it is thought that they have more readily releasable vesicles and therefore serotonin would have a greater effect on these fibers than on the distal fibers (Southard et al., 2000; Sparks and Cooper, 2004).

Lastly there are regional differences in the isoforms and amount of troponin T (Mykles et al., 2002). Proximal fibers have the highest levels of TnT1, with lower levels in distal fibers; central fibers lacked TnT1. Although this data does not match the idea that proximal tissue is phasic-like, it is important to note that there is a lot of data on the differences between the regions with respect to the gross anatomy, physiology, biochemistry, and nerve terminals, but no information about how the different regions

develop force. My goal is to correlate all the regional differences I have just mentioned with the generation of force and length of sarcomere.

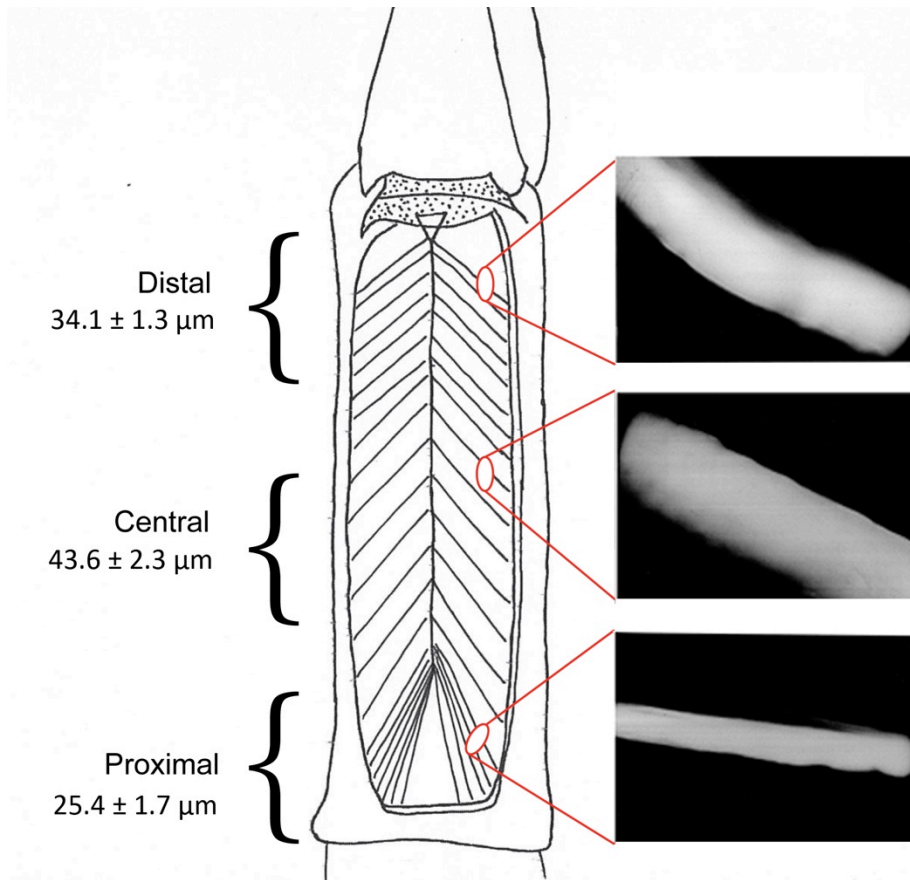


Figure 1: Fiber diameter in different regions of opener muscle.

Fibers were injected with Lucifer Yellow before measuring. Each fiber type was measured 6 times. Diameter of muscle fibers were measured by an eyepiece ocular micrometer. Fibers were injected with Lucifer yellow to obtain more accurate measurements, although the values that were collected before and after the injection were not different. Data shows the proximal fibers were the smallest and the central fibers were the largest. A T-test was used for the fiber diameter analysis ($p=0.003$). Drawing by Rachel Holsinger (unpublished data Cooper, 1993).

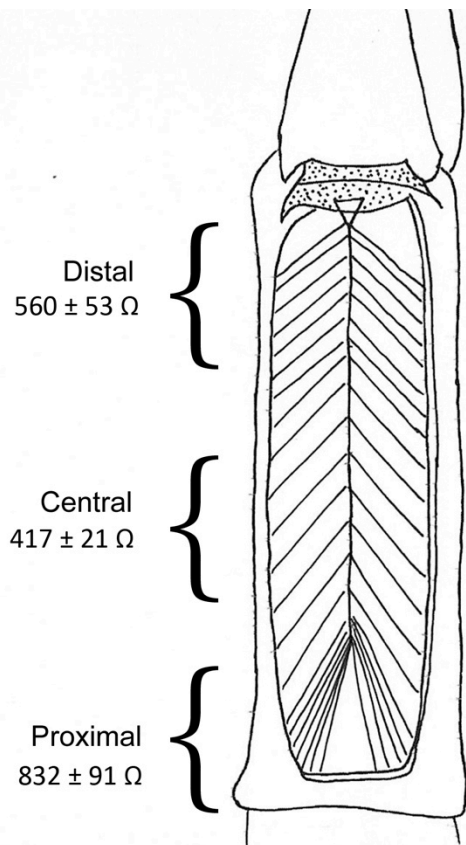


Figure 2: Input resistance in different regions of opener muscle

When calculating input resistance, a single-electrode voltage method was used. Electrodes that were used had resistances in the range of 10-20MΩ. Preparations were always cooled with perfusion of chilled saline (14-15°C). The Mann-Whitney rank sum test was used for the input resistances analysis ($p < 0.045$). Drawing by Rachel Holsinger. (unpublished data Cooper, 1993).

Input resistance and diameter of different regions of opener muscle.

	Input Resistance (k Ω)	Diameter (μ m)
Distal	560 \pm 53 (14)	34.1 \pm 1.3(5)
Central	417 \pm 21 (41)	43.6 \pm 2.3 (6)
Proximal	832 \pm 91 (17)	25.4 \pm 1.7 (6)

Table 2: The numbers in the parenthesis are the number of samples.

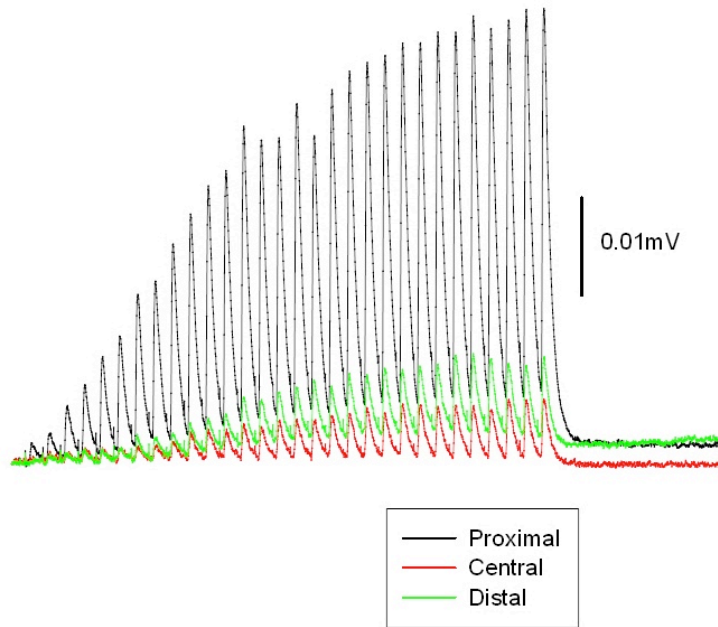


Figure 3: Regional differences of EPSP generation

To determine the facilitation of EPSPs in the different regions, a 40Hz train of 30 pulses in duration was applied to the excitatory nerve and the responses in each region was measured and compared. Proximal has the largest amplitudes and central has the smallest amplitudes. The facilitation index is reported in figure 4.

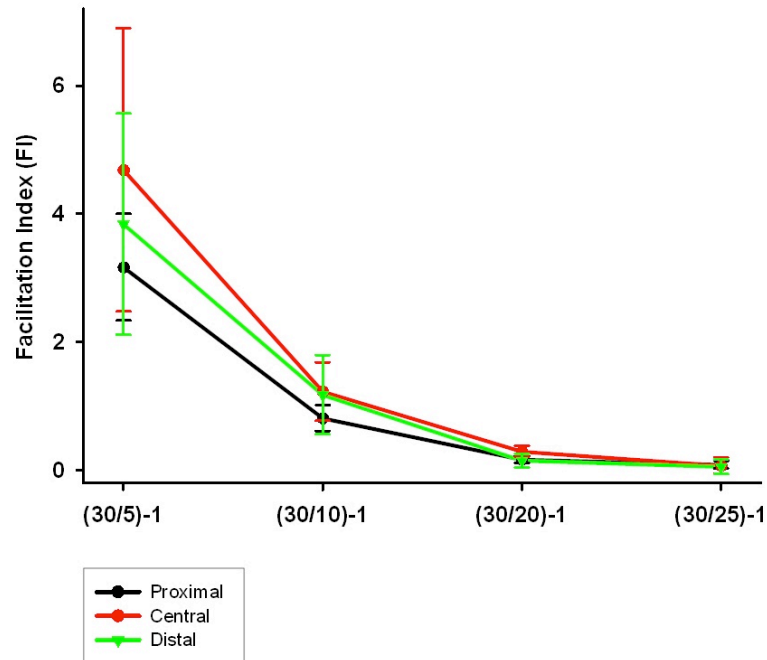


Figure 4: Facilitation Index

The facilitation index was determined by starting with the fifth pulse. The EPSPs start to plateau by the 10th pulse and have no additional facilitation by the 25th. At the beginning of the train, the central fibers facilitated the most (4.69 ± 2.21) and the proximal fibers the least (3.17 ± 0.83).

Again the goal of this project was to correlate all the regional differences found in the literature with the generation of force, length of sarcomere, and sensitivity to serotonin. I hypothesized that the proximal region would be more phasic-like; therefore,

generate force quickly, have shortest sarcomeres when passively stretched, and have the least influence by serotonin. The central region would be more tonic-like; generating force more slowly, having the longest sarcomeres, and giving rise to the greatest increase in force when serotonin was added. The distal region would have an intermediate amount of force generation and length of sarcomere.

Methods and Materials

Animals and Care

Medium sized *P. clarkii* (orbital-thorax length 32 to 44mm) were transferred from the shipping box to individual aquaria 33 x 28 x 23cm with a water depth of 10-15cm. They were fed commercial fish food pellets (Aquadine) and their water was changed weekly. The water was aerated for several days and carbon-based filters removed the chloramines of local water (Cooper and Cooper, 2004; McRae, 1999) before adding to the aquaria. To make sure that the opener muscle was not compromised, crayfish that appeared to be molting or had visual parasites were excluded from this study. To ensure that the animals could continue to eat after the experiment, no organisms that were already missing walking legs were used. No gravid females were used.

Dissection

There are two approaches that could be used to dissect the chelated first walking leg of *P. clarkii* in order to expose the opener muscle in the propodite: the ventral approach and the dorsal approach. These two different dissection techniques permit two different means of force measurement that the intact opener muscle can generate: (1) the stimulated muscle contracting and pulling on the force transducer or (2) the stimulated muscle causing the claw to open and the dactylopodite pushing on the force transducer (see figure 5).

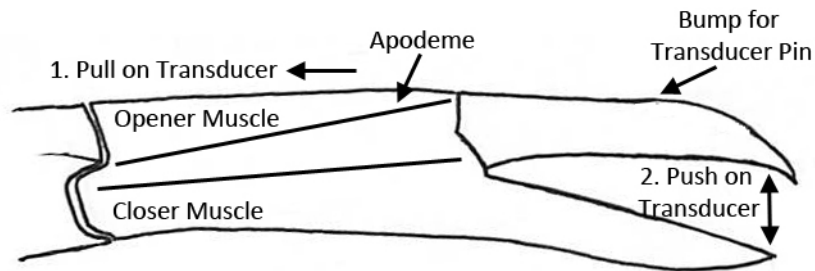


Figure 5: Side view of the chelated first walking leg.

The two different opener muscle dissections, ventral approach with removal of the closer muscle and dorsal approach, give rise to two different ways to measure the force generated by the stimulated opener muscle, (1) pulling on the transducer pin inserted into the dactylopodite or (2) pushing on the transducer pin resting on the top of the dactylopodite. This side view shows the location of the apodemes for opener and closer muscles in the propodite, with the opener muscle apodeme being used for force transducer pin attachment in the ventral approach. In the dorsal approach the force transducer pin rests on the bump on the dactylopodite illustrated in this drawing. Drawing by Rachel Holsinger.

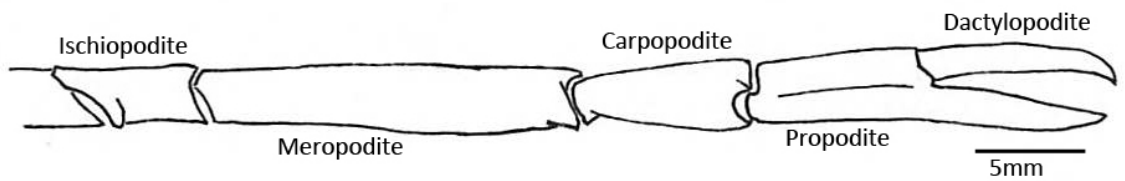
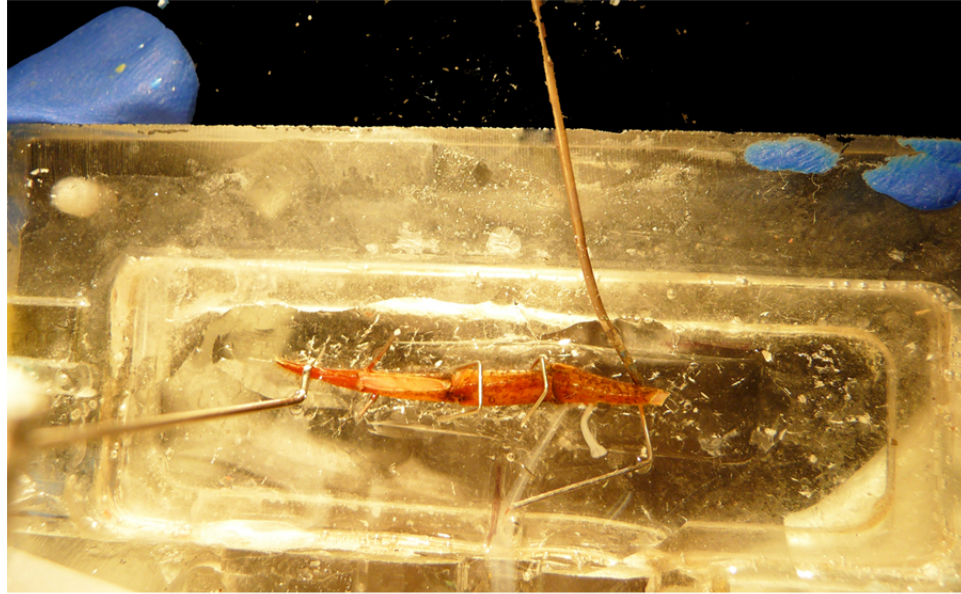


Figure 6. Names of chelated first walking leg segments.

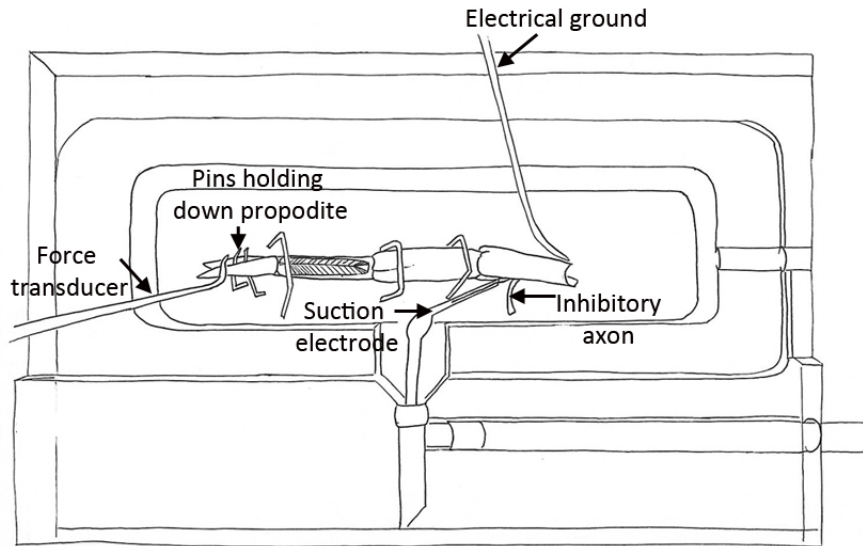
Opener muscle is located on the dorsal side of the propodite, the excitatory opener nerve is dissected out of the meropodite, and the dactylopodite is used for attachment of the force transducer pin in both the ventral and dorsal approaches. Drawing by Rachel Holsinger.

Ventral or dorsal dissection begins with inducing the crayfish to autotomize the walking leg by forcibly pinching the ischiopodite segment. A window was cut in the meropodite cuticle to expose the nerve of the opener muscle, and then a window was cut in the propodite cuticle, either on the dorsal or ventral side depending on the dissection (see figure 6 for segment names).

Once the preparation was covered with modified Van Harreveld's solution (in mM: 205 NaCl; 5.3 KCl; 13.5 CaCl₂·2H₂O; 2.45 MgCl₂·6H₂O; 5 HEPES adjusted to pH 7.4 (Cooper and Cooper, 2009; Van Harreveld, 1936) in a dissecting dish, the cuticle of the meropodite and propodite were removed to expose the nerve bundle and opener muscle, respectively. The nerve bundle was transected to separate the excitatory and inhibitory opener nerves (Dudel and Kuffler, 1961). The excitatory nerve was stimulated using a plastic suction electrode (see figure 7). The preparation must be pinned to the recording chamber in a crisscross fashion to make sure that the entire leg does not vibrate when the opener muscle is stimulated. With the dorsal approach the ventral section of the propodite must be pinned to allow the dactylopodite to press against the force transducer pin when the opener muscle is stimulated.



A



B

Figure 7. Recording chamber showing pinning of preparation and suction electrode.

A. Photograph shows a dorsally dissected preparation pinned in the recording dish with the force transducer pin resting on the dactylopodite. The suction electrode holding the excitatory nerve can be seen in the bottom center of the dish and the inhibitory nerve is floating free. B. Diagram detailing the recording chamber setup specifically showing how the preparation is pinned between the dactylopodite and ventral propodite. Drawing by Rachel Holsinger.

Each leg was weighed, the dorsal propodite length and orbital-thorax length were measured, and the gender was recorded before beginning the dissection of the propodite and meropodite. At the completion of the experiments each leg was stained with methylene blue and the opener muscle was photographed at 100x magnification to determine the number of bundles that were ablated.

Stimulation Paradigm

Several different stimulation paradigms were explored before determining that stimulating the excitatory opener nerve for 15sec and then increasing the frequency was appropriate for addressing the questions posed in this study. When stimulation was applied for 15sec and then turned off before increasing the frequency, the preparation twitched, which appeared to cause vibration in force measurements. However, a continual stimulation produced more stable force measures and therefore was determined to be the best approach. 15sec at 5, 10, 20, 30, 40, 50, 60, 70Hz was used because the force generated leveled off after 10sec at each of the frequencies. When the preparations were stimulated at 80Hz depression occurred after 16sec in some preparations and up to 62sec in others. At 40Hz the preparation can be stimulated for up to an hour without failure of the nerve.

Measurement of Force

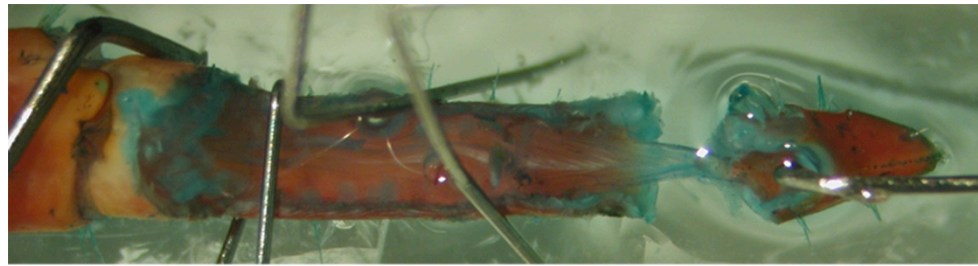
The 2g range ADInstruments force transducer (model MLT0402) was used because it is sensitive enough to detect small forces when the tissue is ablated, but has a large enough range to measure intact opener muscle in walking legs even when serotonin is added. The transducer was connected to an ADInstruments Bridge Amplifier (ML221) and the support rod of the transducer was attached to a micromanipulator to allow for precise positioning. The transducer was calibrated using 1.00 and 2.00g weights hung from the attached pin used in the experiments. The force transducer was zeroed using LabChart before calibration and before each experiment.

First, the ventral dissection of the propodite (with the full removal of the ventral portion of the claw) was used to measure force the opener muscle could generate. The force transducer was outfitted with a hook, which was inserted into the dactylopedite.

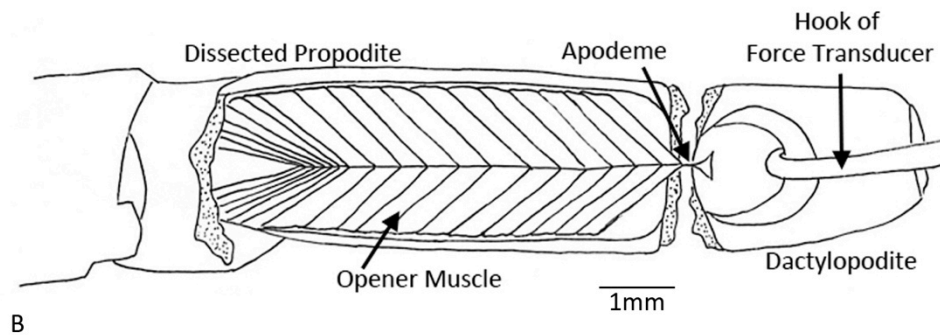
As the opener muscle was stimulated at 5, 10, 20, 30, 40, 50, 60, 70Hz, the apodeme (invagination of the cuticle that acts as a tendon) pulled on the dactylopodite and therefore the force transducer (see figure 8). To ensure that the dactylopodite did not dampen the force when the muscle contracted, it was pulled far enough away from the joint as not to touch it. The problem with this dissection technique was that the dactylopodite moves down into the dish when the opener contracts therefore it does not pull on the transducer in the direction needed for proper measurement. This preparation also was unstable, swaying side to side with stimulation, and the attachment of the force transducer overstretched the muscle into a non-physiological state; therefore a new approach in measuring force was attempted.

To keep from pulling the muscle out of its natural location and reduce the drag on the transducer, the force transducer was attached directly to the apodeme. The apodeme of the cheliped is much larger allowing a hole to be drilled for attachment of the force transducer. The same ventral dissection technique was used; however, a rotary tool instead of a razor was used to cut the cuticle because of its thickness in comparison to the first walking leg (see figure 9). Drilling the hole in the apodeme seemed to cause damage to the lower muscle bundles of the opener muscle, and these damaged muscle fibers were observed when the walking leg was stained with methylene blue.

Next a string was tied around the apodeme and attached to the hook of the force transducer, but the string would not stay attached to the apodeme because of the pyramidal structure (see figure 10). Once it became apparent that this new technique would not affectively measure the force generated by the *in vivo* opener muscle, the dorsal dissection of opener muscle of the cheliped was used.



A



B

Figure 8. Ventral dissection of opener with transducer hook placement.

To measure the force generated by the opener muscle the condyle between the propodite and dactylopodite is carefully cut allowing the apodeme (tendon like tissue) to be freed. A. Photograph of preparation showing the hyperextension of the opener muscle due to the removal of the condyle. B. Diagram detailing the anatomy of the preparation showing the pinnate arrangement of the opener muscle, its attachment to the apodeme, and the hole left in the dactylopodite when the ventral portion of the propodite was removed. Drawing by Rachel Holsinger.

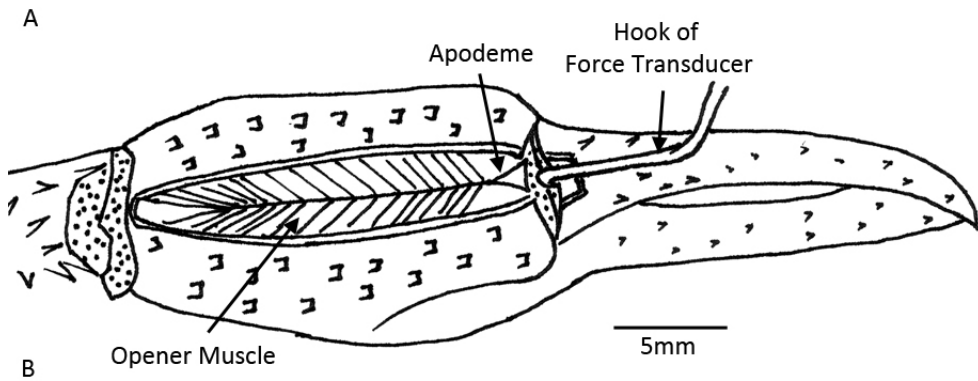
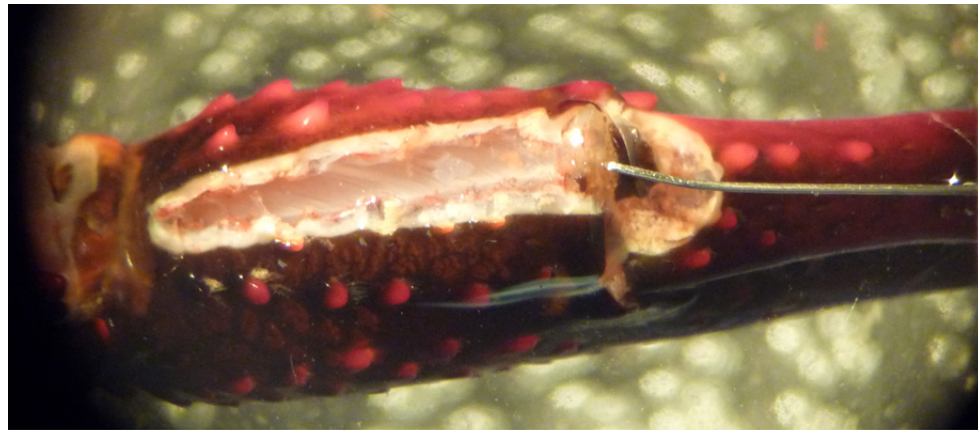


Figure 9. Dorsal dissection of cheliped with force transducer location.

A. Photograph of cheliped showing the location of the force transducer hook attached to the apodeme. This photograph shows the increased thickness of the cuticle and why a rotary tool was necessary for the dissection. B. Diagram detailing anatomy of the preparation showing the same pinnate arrangement of the opener muscle and its attachment to the apodeme. Drawing by Rachel Holsinger.

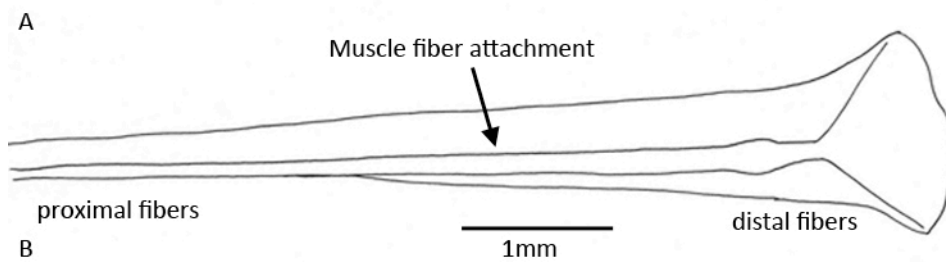
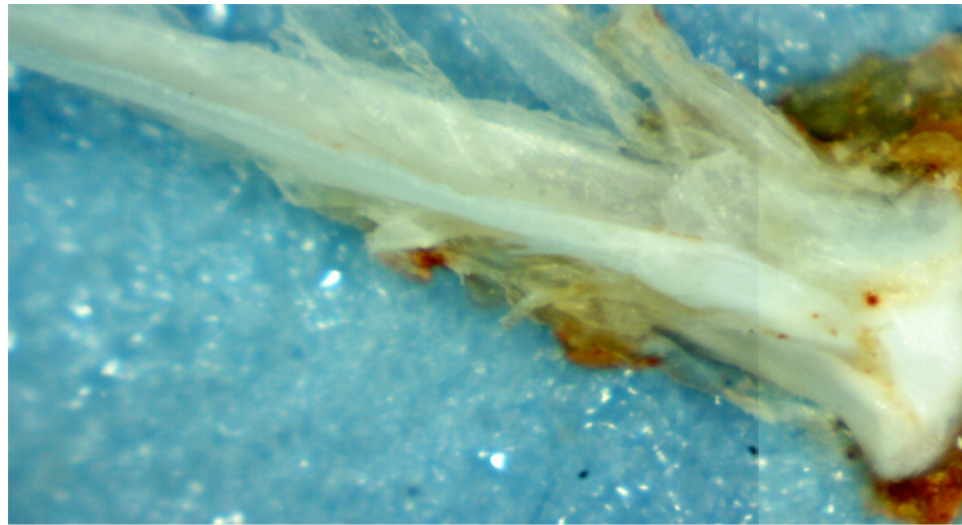


Figure 10. Pyramidal structure of apodeme.

A. Photograph of apodeme showing the location of muscle fiber attachment. B. Diagram detailing pyramidal anatomy of apodeme explaining why a string could not be attached to the apodeme to connect it to the force transducer. Drawing by Rachel Holsinger.

The dorsal dissection leaves the entire cheliped intact and as the excitatory nerve is stimulated the claw opens pushing against the force transducer. The intact claw was so strong that it generated more force than the transducer could measure after 3sec at 20Hz and the force generated at 15Hz continued to increase for 57sec until it reached the maximum level the transducer could measure. For this reason, the chelated first walking leg was revisited using the new dorsal dissection leaving the claw fully intact (see figure 11). The first walking leg is a better preparation to answer the questions because (1) the thinner cuticle makes it easier to expose the opener muscle (2) the walking leg opener nerve is easier to dissect to separate the excitatory and inhibitory nerves, (3) the smaller size leg generates force that is within the limits of the force transducer, and (4) the propodite in the 1st walking leg is wider allowing for easier ablation of the muscle fibers.

An "L" shaped pin was attached to the force transducer, which pressed against the dorsal section of the dactylopodite (see figure 12). To make sure that the pin did not slip off the dactylopodite when the claw opened, a small amount of super glue was added to the pin and etched to add a grip. The dactylopodite has a distinctive curved ridge where the pin was repeatedly rested at the beginning of each experiment (see figure 5). The pin was pressed slightly against the claw to create passive tension between 0.030mV and 0.070mV. This starting position caused the muscle fibers to be stretched slightly to almost a closed position. Then the stimulation paradigm of 5, 10, 20, 30, 40, 50, 60, 70Hz for 15sec each was used to generate force by the opener muscle. Force of the opening claw was recorded using Lab Chart set to 2mV and an acquisition rate of 2k/s. The voltage on the stimulator varied depending on each preparation but the minimum voltage required to open the claw was used.

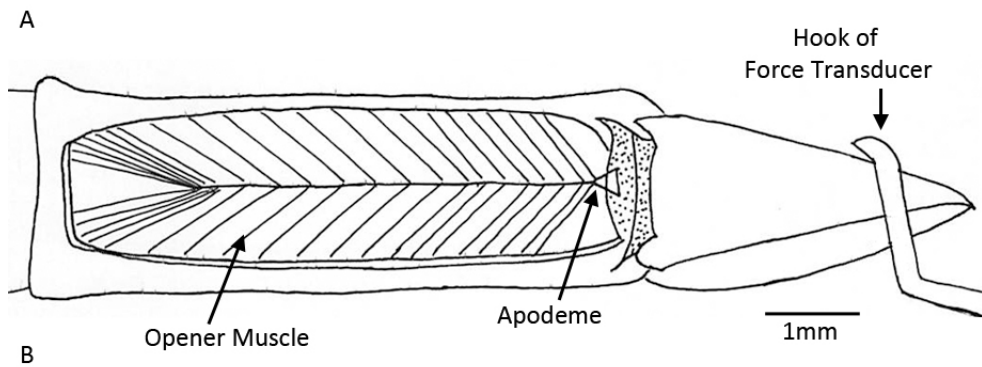


Figure 11. Dorsal dissection of first walking leg with force transducer location.

A. Photograph of first walking leg showing location of the force transducer hook resting on the dactylopodite. B. Diagram detailing anatomy of the preparation showing the pinnate arrangement of the opener muscle and its attachment to the apodeme. Drawing by Rachel Holsinger.



Figure 12. Force transducer “L” shaped pin design.

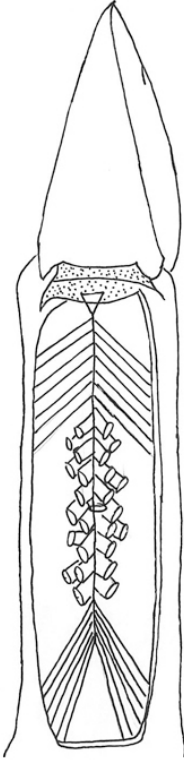
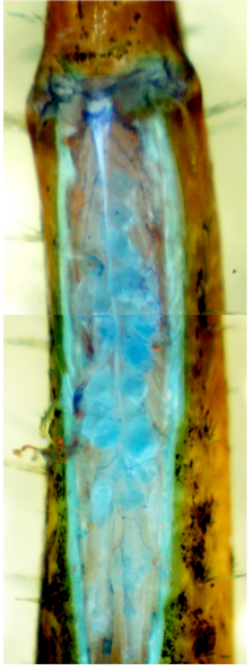
This pin rests on the bump on the dactylopodite and measures the force as the cheliped opens with stimulation.

After the first stimulation paradigm of the intact opener muscle, then muscle bundles corresponding to the proximal, central, and distal regions were systematically cut against the cuticle using a thin curved piece of razor blade (see figure 13). The proximal muscle fibers are closest to the carpopodite and are the innervated first by the opener nerve. Both first walking legs were removed from the crayfish used in these experiments but different tissue (proximal, central, or distal) was ablated from each leg. First, only the distal fibers were detached from the cuticle and the force the opening claw could generate with the stimulation paradigm was recorded (see figure 13a). Then 100 μ L of 1.0 μ M serotonin was added directly to opener muscle and after 30sec the stimulation paradigm was repeated.

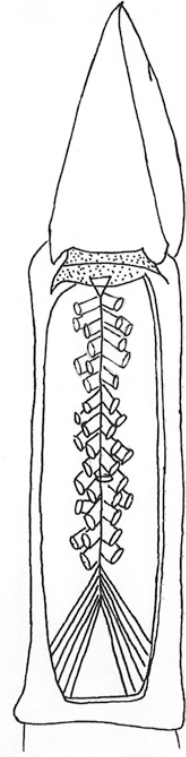
Then in another five preparations only the central fibers were ablated and 100 μ L of 1.0 μ M serotonin was added directly to the remaining muscle (see figure 13b). The same methods were used for an additional five preparations where only the proximal fibers were ablated and serotonin added. When the proximal fibers were cut no force could be measured by the distal and central alone; therefore, to try to determine the contribution of the proximal fibers the central and distal fibers were cut at the same time in another 5 preparations (see figure 13c). Next in five preparations the distal fibers were ablated, tension recorded and then the central fibers of the same preparation were cut and tension recorded. Serotonin was added only after the fibers in both regions were removed from the cuticle. Finally five controls were recorded, the paradigm 5, 10, 20, 30, 40, 50, 60, 70Hz for 15sec each was completed twice and the force was measured before adding the 100 μ L of 1.0 μ M serotonin directly to the preparation (see figure 13d).

There were a few crayfish and data from several preparations that were excluded from the results for the following reasons. In two crayfish the opener nerve was cut during the dissection and the remaining tissue generated no force. The data from the first eight preparations that used the ventral dissection and the five claw dissections were excluded since their treatment was different. Four leg preparations on

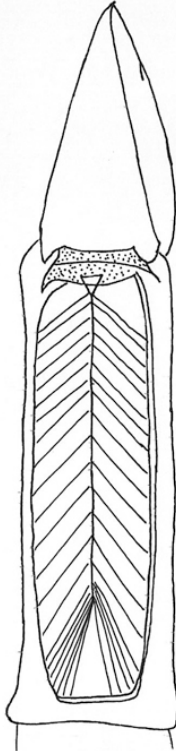
two organisms had major synaptic depression during the first paradigm causing the measurement of force generation to be impossible.



C

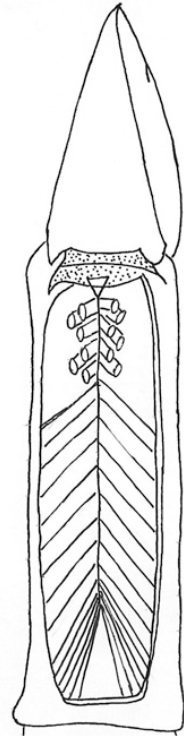
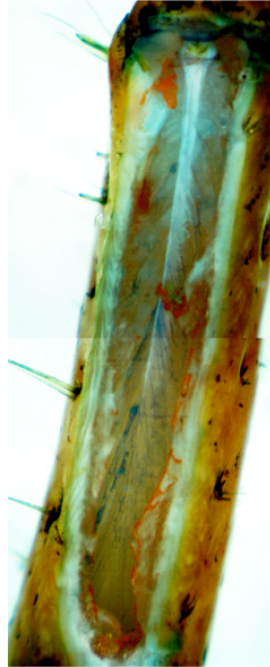


D



A

1mm



B

Figure 13. Dorsal dissections of the first walking leg showing ablated tissue.

The photographs in A-D show the ablated fibers are stained more darkly by methylene blue than the intact fibers. Four different dissections were performed: (A) intact opener muscle control, (B) ablated distal fibers, (C) ablated central fibers, and (D) ablated distal and central fibers. Drawing by Rachel Holsinger.

Electrophysiology

The excitatory axon innervating the opener muscle in the crayfish was stimulated in the meropodite by placing a branch of the leg nerve into a suction electrode connected to a Grass stimulator (Dudel and Kuffler, 1961). Stimulation at 40Hz trains of 30 pulses in duration was applied to the excitatory nerve to compare the responses obtained in the three general regions of the opener muscle: distal, central, proximal). Intracellular EPSP recordings were performed by standard procedures (Cooper et al., 1995; Crider and Cooper, 2000), with glass microelectrodes filled with 3M KCl. Electrical signals were recorded to a computer via a PowerLab/4s interface. Preparations were used immediately after dissection and all the experiments were performed at room temperatures (19-21°C).

To be consistent, the EPSPs were recorded in the most distal fibers of the distal region of the opener muscle, in the central fibers in the middle of the preparation, and in the proximal fibers where they attached to the apodeme. The anatomical distinctions are required as physiological properties of opener muscle differ in distal, central, and proximal regions (Cooper et al., 1995; Mykles et al., 2002). For the electrophysiological responses the amplitudes of the EPSPs at the 5th, 10th, 15th, 20th, 25th and 30th pulse were measured (software Chart Version 5; ADInstruments). A facilitation index for train facilitation was determined by comparing the amplitude of a later pulse to the amplitude of a preceding pulse. For example, the 30th pulse is compared to the 10th pulse and the numerical value one subtracted from the result. The subtraction of the numerical value, one, ensures that if no facilitation is occurring, and the amplitudes of the first and last responses are the same, the facilitation index will be zero (Crider and Cooper, 1999).

Because these stimulus pulses are given so close together, often an EPSP will rise on the decay of the previous EPSP. This leads to difficulty in accurately determining the amplitude of the EPSPs. Measuring from the baseline to the peak will result in too large an EPSP amplitude measure. Likewise, measuring from trough to peak represents an EPSP that is too small because of the differences in the timing of the peak in the

response and decay of the previous potential, but it is one approach that is feasible for comparative purposes among the muscle fiber regions.

Muscle Anatomy

After electrophysiological measures the opener muscle fibers were fully stretched in a natural position by closing the chelated ends. The fibers were fixed with Bouin's fixative and a few bundles of fibers from the three regions were removed and mounted on glass slides (see figure 14). The sarcomere lengths were measured five times in each of the five preparations in the three different regions at 100x and an average was taken.

Statistical Analysis

The data being analyzed always starts at 0.000g and has a long tail that stops at 1.471g, the maximum measurement of the force transducer; therefore, this data set is not normal. Although three independent variables were tested (stimulation, ablated tissue, and addition of serotonin), the data do not meet the assumptions for a multivariate analysis of variance (MANOVA). Because of the small sample size and non-normal distribution of data, the statistics employed were nonparametric statistics, specifically Mann-Whitney test, Kruskal-Wallis one-way analysis of variance by ranks, and Wilcoxon Matched-pairs signed-ranks test.

The Mann-Whitney test has three major assumptions: a random sample with an unknown median, samples are independent and not linked, and the dependent variable is continuous and not normally distributed (Daniel, 1990). This test was used to determine if there were a significant difference in the force generated between the control legs, ablated distal, ablated central, and ablated distal/central at 40Hz. Since the probability of finding an invalid significant result increases as the number of comparisons increase, only the force generated from 40-50Hz was used thus reducing the number of statistical tests performed and the chance of erroneous statistical results.

Force development at 40Hz was used for statistical analysis because all intact preparations showed increase in force above the ± 0.003 error of the force transducer at 30Hz and 40Hz. Stimulation frequencies below 30Hz did not cause force generation

above noise in all intact preparations, with 5Hz only having 11% of preparations generating measurable force. Above 40Hz the force generated reached the upper limit of the transducer on 11 of the 35 preparations and higher stimulation caused depression in 5 of the 35 preparations (see figure 15). Force was maintained at 40Hz for at least five minutes in this study, and EPSPs have been measured for well over an hour in other studies (Wu and Cooper, 2012b).

The Kruskal-Wallis has the same major assumptions as Mann-Whitney: random samples that are independent and not linked, and the dependent variable is continuous and not normally distributed. When only two samples are being compared, the Kruskal-Wallis test simplifies to the Mann-Whitney test; however, the Kruskal-Wallis test is more powerful because more data is used in the analysis (Daniel, 1990). After rejecting the null hypothesis using the Kruskal-Wallis test and concluding that the force generated after tissue removal is not identical, then a multiple comparison test with use of an experimentwise error rate is applied to determine which tissue region is different from which other tissue region. This experimentwise error rate helps to make only correct decisions when multiple comparisons are being made and can be larger because significance has already been shown (Daniel, 1990).

The Wilcoxon Matched Pairs test assumes that data is taken on the same subject, the dependent variable is continuous, and the distribution of the differences is symmetric about the median. This test was used to determine the level of significance between force generated by the repeated stimulation of the control legs, and between the force generated with and without addition of serotonin to each of the ablated legs.

The variability in force generation in the different crayfish cannot be removed; however, since the selection of crayfish for these tests was random there should be the same number of weak crayfish as strong crayfish in every group. This variability makes statistical significance more difficult to obtain, but not inaccurate. A percent difference could be used to reduce this variability within the data set, but could augment the importance of small differences that are within the error of the measuring device (0.000

to $1.471\text{g} \pm 0.003$). If percent differences are not used then the variability within the data set cannot be removed.

The data could be heavily massaged and rules could be made for throwing out data points. For example (1) once a negative percent change was obtained all data collected with a higher stimulation would be omitted. (2) The error of the device could be removed by only using data points that were above a specific number. Assuming the device error should not be more than 5% of the percent difference, then all data below 0.02g would be omitted. Unfortunately, there is not enough data in this study to remove that many data points.

Summary

By conducting these three major experiments in the different regions of the opener muscle, force generation, sarcomere length, and effect of serotonin on force generation I will add to the large amount of data already known about the opener muscle. The results help to tie together already know data with muscle output of force. This thesis will also publish data about input resistance and fiber diameter that have not already been recorded in the literature.

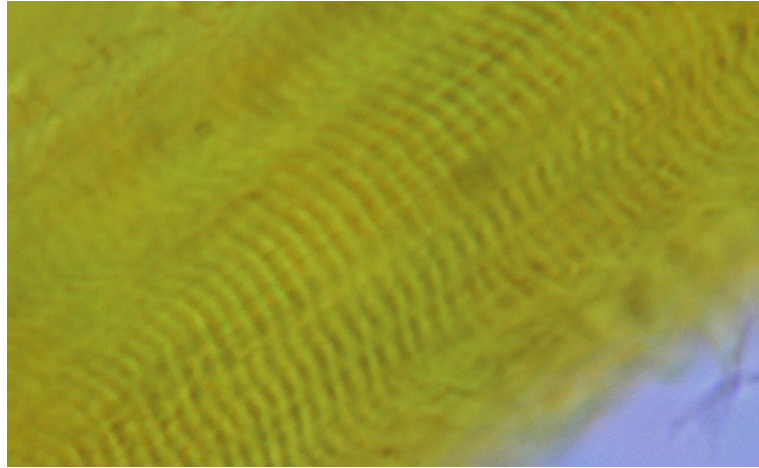


Figure 14: Striated skeletal muscle.

The striated appearance of the skeletal muscle is caused by the regular arrangement of the thick and thin filaments within these myofibrils coupled with the highly organized alignment of adjacent myofibrils. This fiber was fixed with Bouin's fixative and magnified 100x before a photograph was taken and the sarcomeres measured. Photograph by Robin Cooper.

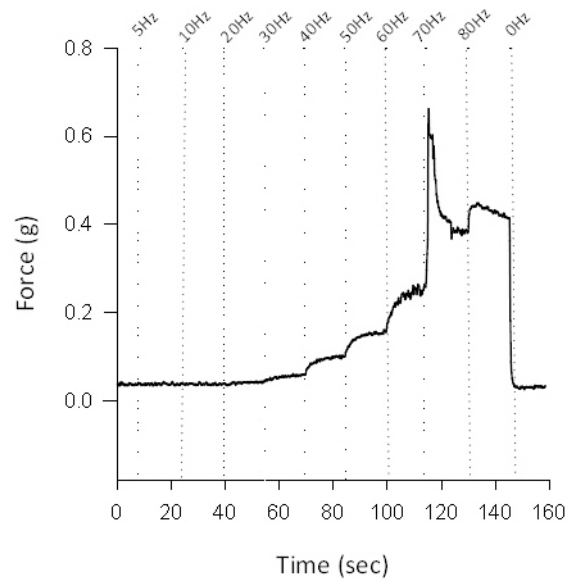


Figure 15: Example of force generation failure at high frequencies

This graph shows why stimulation above 70Hz was not used and why some of the data was removed from the study. If failures like the one that occurred at 70Hz were seen, all the data from that leg was removed from the study.

CHAPTER 4

RESULTS

The result section is divided into three distinct sections reflecting the three different experiments that were performed to be able to answer my original questions. (1) Are there regional differences in the development and maintenance of force across the opener muscle based on the phenotypic differences of the muscles? (2) Do the sarcomere lengths vary based on the phenotypic differences of the muscles? (3) Will serotonin show regional differences in development and maintenance of force based on phenotypic differences across the opener muscle?

Force generation in three regions of opener muscle

Force was generated by the control legs by fully extending the opener muscle and stimulating the motor neuron at 5, 10, 20, 30, 40, 50, 60, and 70 Hz for 15 seconds each. Then the force was measured at the plateau of each stimulation. Figure 16A and 16D show two different representative force traces. Then the leg was stimulated again with out removing any muscle fibers and the force was measured to make the force generation was reproducible. Figure 18A shows data from all 34 crayfish used. Before any tissue was ablated I recorded the force generated by the intact muscle, then determined the change in force from 0-5, 5-10, etc.

As you can see the variability is very high; however, since the selection of crayfish for these tests was random there should be the same number of weak crayfish as strong crayfish in every dissection group. This variability makes statistical significance more difficult to obtain, but not inaccurate. A percent difference could be used to reduce this variability within the data set, but could augment the importance of small differences that are within the error of the measuring device (0.000 to $1.471\text{g} \pm 0.003$). The variability in the data could be related to different life histories. Although the crayfish used in this study are housed consistently in the lab, they were wild caught;

therefore, parasite load, energy level in the cells, and regenerated legs could not be controlled.

In the first set of ablations I removed the distal muscle fibers. Figure 16B shows a representative force trace after the tissue was ablated. Figure 18B and 19B show the change in force the proximal and central fibers could generate on 13 different legs (7 and 6 respectively). I tried to dissect the same number of fibers on each preparation and then stained each dissected leg to determine the correct number were ablated. Several trials were omitted because staining showed bundles not cut or too many bundles cut.

On the preparations represented in figure 19B the central fibers were removed in addition to the already ablated distal leaving only the proximal fibers that were stimulated in the same way. Figure 16C shows a representative force trace after the tissue was ablated. Figure 19C shows the change in force the proximal fibers alone could generate and figure 17A shows the representative set of force traces from the control, ablated distal, and then ablated central fibers. No additional ablations were performed on the preparations represented in figure 18B. The distal and central were also ablated concurrently and figure 18D shows the change in force the proximal tissue could generate. Finally the central muscle fibers were removed. Figure 16E and 17B show representative force traces after the tissue was ablated. Figure 18C show the change in force the proximal and distal fibers could generate.

Only force development at 40Hz was used for statistical analysis because all intact preparations showed increase in force above the error of the force transducer at 30Hz and 40Hz (see figure 20). Stimulation frequencies below 30Hz did not cause force generation above noise in all intact preparations, with 5Hz only having 11% of preparations generating measurable force. Above 40Hz the force generated maxed out the transducer on 11 of the 35 preparations and higher stimulation caused depression in 5 of the 35 preparations. Force was maintained at 40Hz for at least five minutes in this study, and EPSPs have been measured for well over an hour in other studies.

As expected there was no significant difference in force generated at 40Hz between the two stimulations of control preparations by the Wilcoxon Matched Pairs

signed rank test ($z=0.813$, $p<0.05$). There is a significant difference between all other preparations except between the control and ablated distal preparations. One sided test was used for the control preparations versus all ablated data because I know with fewer muscle fibers less force will be generated, but I do not know which region will generate more force and thus used a two sided test for determining significance between the different ablated tissues. By the Kruskal-Wallis test, there was a significant difference in the force that was generated at 40Hz between the control and ablated central ($H=12.071$ $p<0.05$), the control and all 13 ablated central/distal ($H=9.929$ $p<0.05$), the ablated central and ablated distal ($H=10.929$, $p<0.05$), the distal to all ablated central/distal ($H=8.786$, $p<0.05$). However no significance was found between ablated central to ablated distal/ central ($H=2.143$), nor the control and ablated distal ($H=1.143$). The decision rule was different for each test because of the different number of preparations in each group. I was able to group together the ablated distal then central preparations with the concurrently ablated distal and central because by the Mann-Whitney test there was no significant difference in force generation at 40Hz ($T=20.50$).

Because I could not cut the proximal fibers without causing major damage to the nerve, I was unable to directly measure the force generated by the distal and central regions alone. However, by subtracting different measurements I could look at the contributions of the central fibers and distal fibers (see table 2). Therefore, I can conclude the distal fibers contribute the least to the force generation at 40Hz (0.004g), the central fibers contribute the most (0.058g). The proximal fibers are necessary for proper alignment of the muscle on the apodeme and contribute more to the total force generation than the distal but less than the central (0.015g).

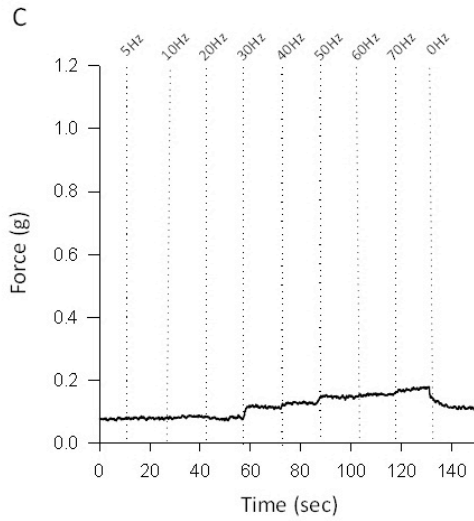
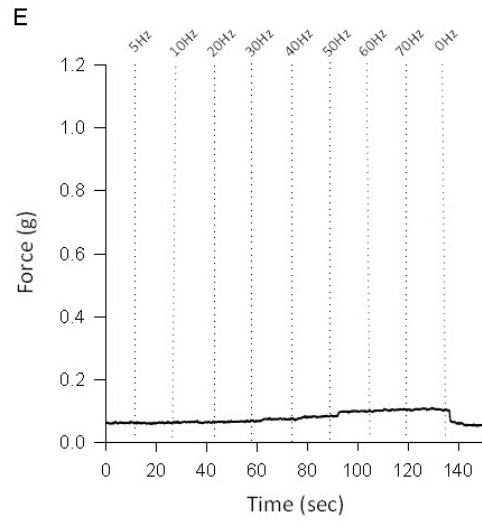
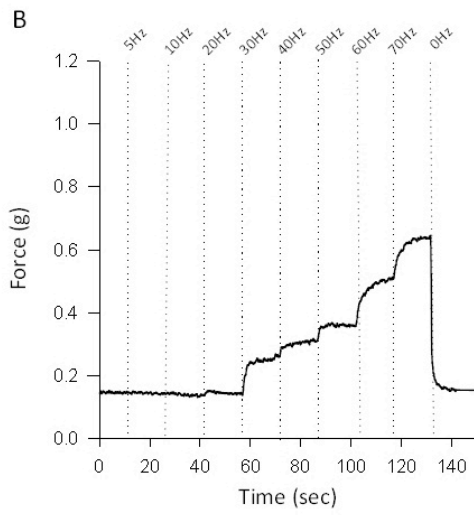
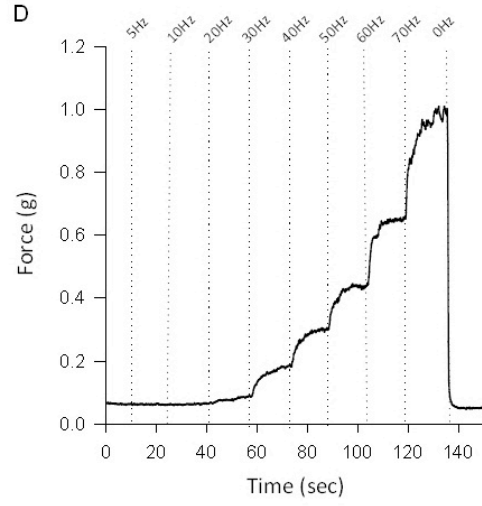
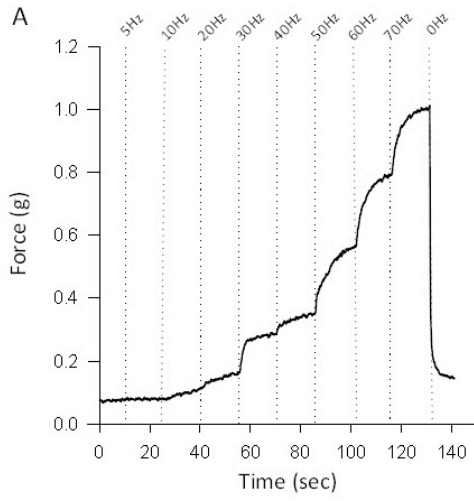


Figure 16. Representative traces of force generated by the opener muscle.

A and D are traces of intact muscle from preparations 53 and 45 respectively. Force is generated by the opener muscle once the excitatory nerve is stimulated at a frequency of 10Hz and increases with each increase in frequency with the maximum amount of force (1.02 g) being generated at 70Hz. B. Ablated distal fibers from preparation 53 show a reduction in force but a similar pattern of force generation. C. When only proximal fibers remain the amount of force generated is less but the pattern of force generation is different. There is a small increase in force at 30Hz and then the amount of force generated only increases slowly. E. Ablated central fibers from preparation 45 show less force generation and the pattern of force generation is similar to ablated central and distal fibers. There is a small increase in force at 30Hz and then the amount of force generated remains consistent as the stimulation is increased.

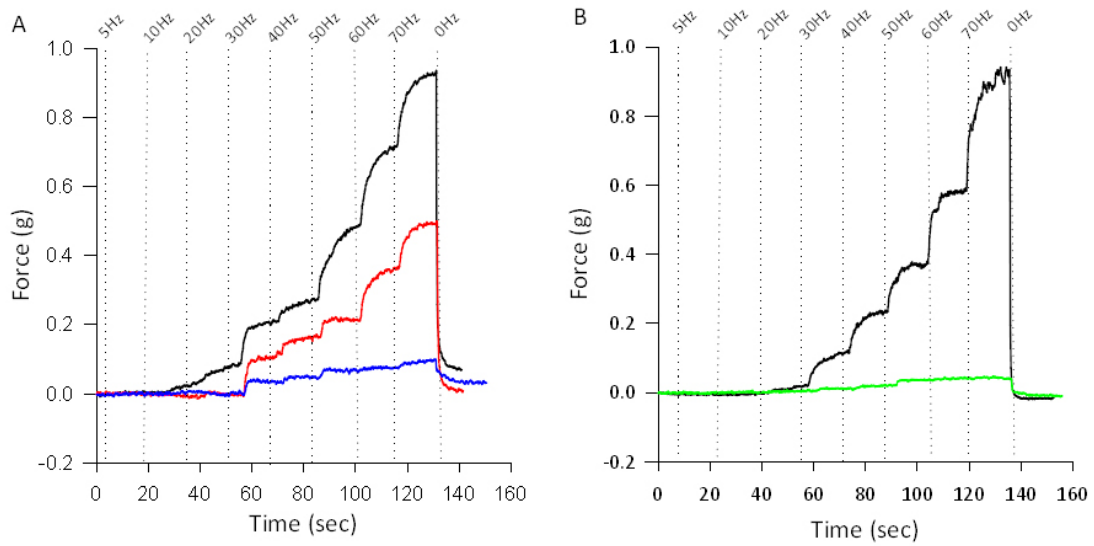


Figure 17. Compiled traces with passive tension subtracted out

Graph A represents the force generated by one preparation stimulated over 145 seconds: the black trace shows force generated by the intact opener muscle, the red trace is the force generated with the distal fibers ablated, and the blue trace is the force generated by the same preparation when the central fibers were ablated in addition to the distal fibers. Graph B represents the force generated in a different preparation: the black trace shows force generated by the intact opener muscle, and the green trace is the force generated with central fibers ablated.

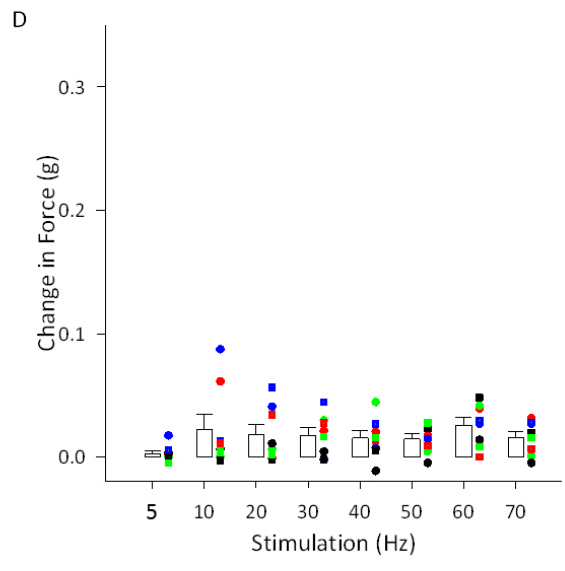
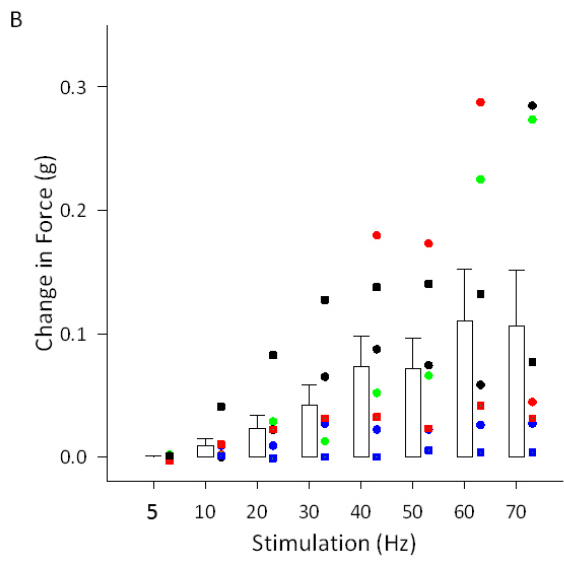
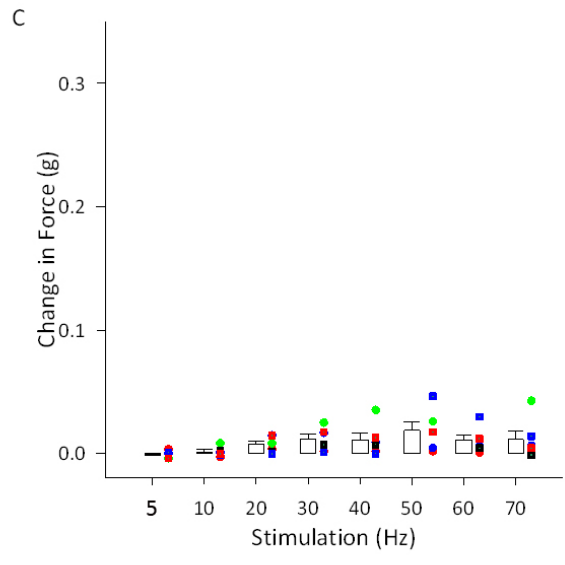
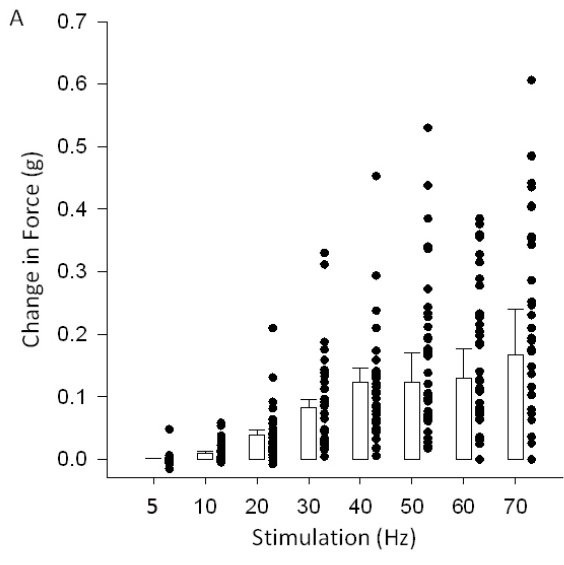


Figure 18. Change in force over stimulation paradigm

This data was gathered by using the traces similar to the representative traces in Figure 16 and 17. The force was recorded in mV at each plateau after about 10 seconds of the stimulation, and then converted into grams using the calibration $1\text{mV} = 0.931\text{g}$. These bar graphs show the mean and standard error of the mean at each of the 8 stimulations. Each dot represents the increase in force from the prior stimulation for each preparation; the dots are color coded for each preparation. A. The increase in force generated by the intact opener muscle for all 34 preparations prior to ablating the tissue. B. The increase in force generated by the central and proximal fibers when the distal fibers were ablated. C. The increase in force generated by the distal and proximal fibers when the central fibers were ablated. D. Increase in force that can be generated by the proximal fibers alone when both central and distal fibers were ablated at the same time.

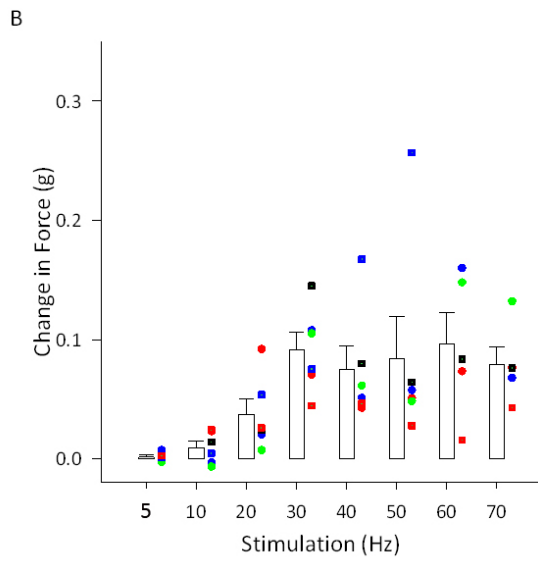
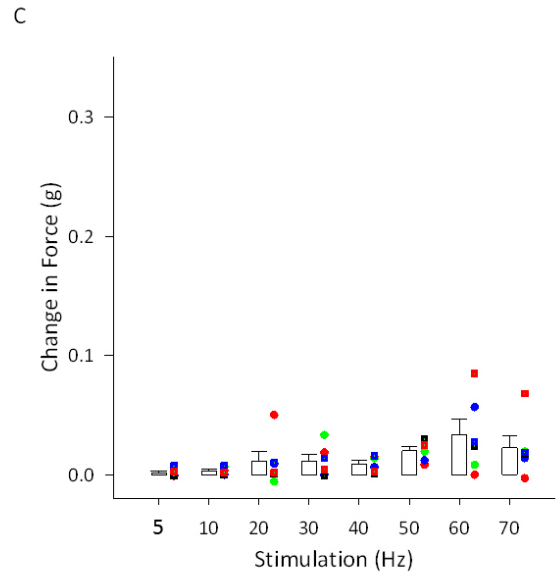
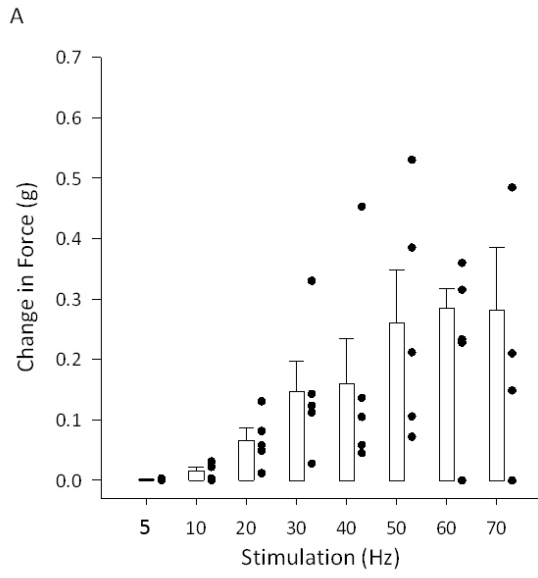


Figure 19. Change in force over stimulation paradigm of preparations with distal then central fibers ablated

Bar graphs show the mean and standard error of the mean at each of the 8 stimulations. Each dot represents the increase in force from the prior stimulation for each preparation; the dots are color coded for each preparation. These three graphs show the force generation of the same five preparations. A. The increase in force generated by the intact opener muscle prior to ablating the tissue. B. The increase in force generated when the distal fibers were ablated. C. Increase in force that can be generated by the proximal fibers alone when the central fibers were ablated after ablating the distal fibers and recording force generation. Similarly to ablating the distal and central fibers at the same time the proximal fibers have an increase in force generation from 50 to 60Hz.

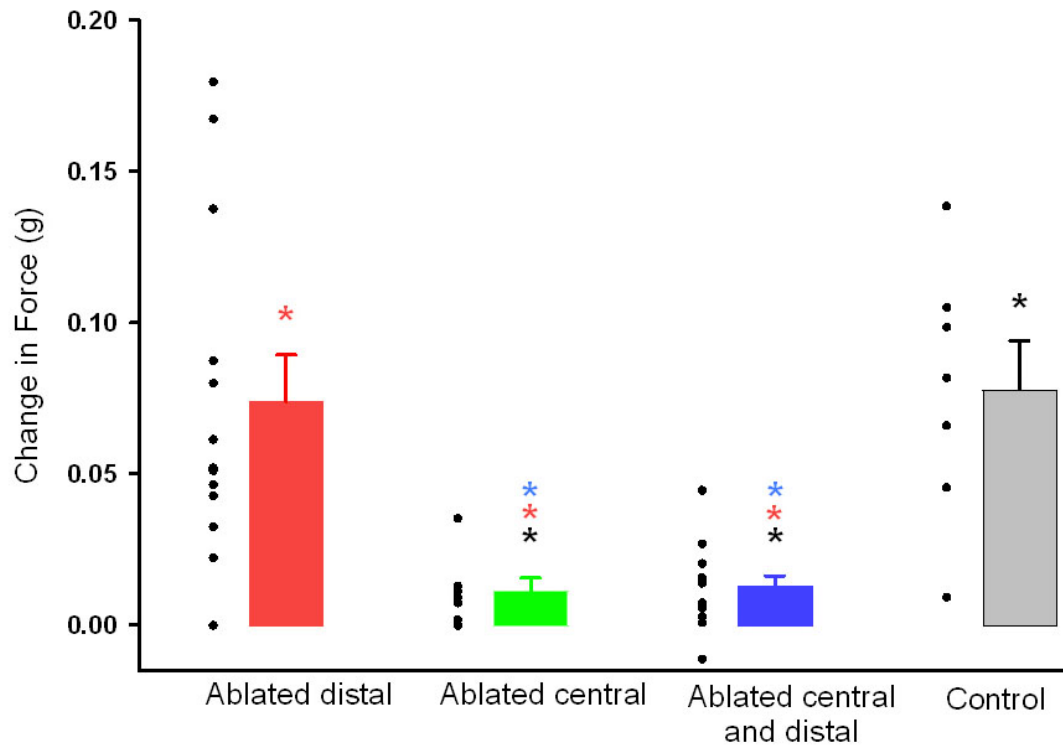


Figure 20: Force development at 40 Hz

The amount of force that can be generated by the central and proximal tissue together is more than the amount just the proximal can generate. With more muscle fibers more force can be generated. What is interesting about this graph is when the central fibers are ablated and similar amount of muscle fibers remain as in the ablated distal preparations there is a significant decrease in the amount of force the muscle can generate. There is no significant difference in the control preparations and the ablated distal preparations because the distal do not appear to be responsible for much of the total force generation.

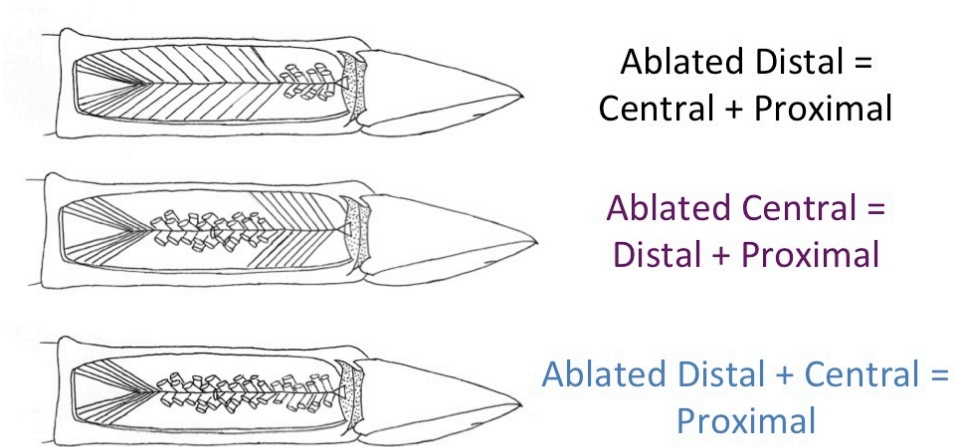


Figure 21: Subtraction technique to determine force generated by distal and central fibers alone.

To determine the amount of force the central fibers contributed I subtracted the ablated distal/central from the ablated distal. Then to determine the force the distal fibers contributed I subtracted the ablated distal/central from the ablated central. Table 3 shows these values. Drawing by Rachel Holsinger.

Amount of calculated force (grams) generated by the intact tissue.

	Frequency of stimulation							
Intact Tissue	5Hz	10Hz	20Hz	30Hz	40Hz	50Hz	60Hz	70Hz
Distal	0.003	0.021	0.013	0.008	0.004	0.005	0.011	0.015
Central	0.003	0.014	0.004	0.024	0.058	0.058	0.085	0.091
Proximal	0.003	0.022	0.019	0.018	0.015	0.015	0.026	0.016

Table 3: The average amount of force generated by the 5 or more preparations was subtracted as explained in figure 21. For the sake of simplicity only 40Hz will be discussed in this thesis. See page 50 for an explanation on why 40Hz was used.

Sarcomere length in three regions of opener muscle

Prior research has reported that the crayfish phasic fibers have sarcomere lengths of 2-3 micron, and tonic fibers are 6-12 microns. Vertebrate phasic and tonic fibers do not show this degree of difference in sarcomere and some animals show no difference in sarcomere lengths. Figure 22 shows representative sarcomere size differences in the different regions of the crayfish opener muscle.

I hypothesized that central sarcomeres will be longer when passively stretched because the central fibers are tonic like. I measured the sarcomeres 5 times in each preparation for a total of 25 measurements in each of the three regions. Figure 23 shows that the central fibers have longer sarcomeres than the distal or proximal. Each dot represents the average measurement in each of the 5 preparations and the bar represents the average of those preparations that were found to be significantly different. The Friedman Two-way analysis of variance by aligned ranks was employed and I rejected the null hypothesis, $M_a=M_b=M_c$, because at least one equality is violated. The test statistic was compared for significance with the tabulated chi-square for 2 degrees of freedom ($T= 7.50, p< 0.025$).

The data shows the sarcomeres in the central region are longest when passively stretched supporting muscle fibers in this region are more tonic-like. The sarcomeres in the proximal region are the shortest supporting muscle fibers in this region are more phasic-like, and the sarcomeres in the distal region are in between the proximal and central length.

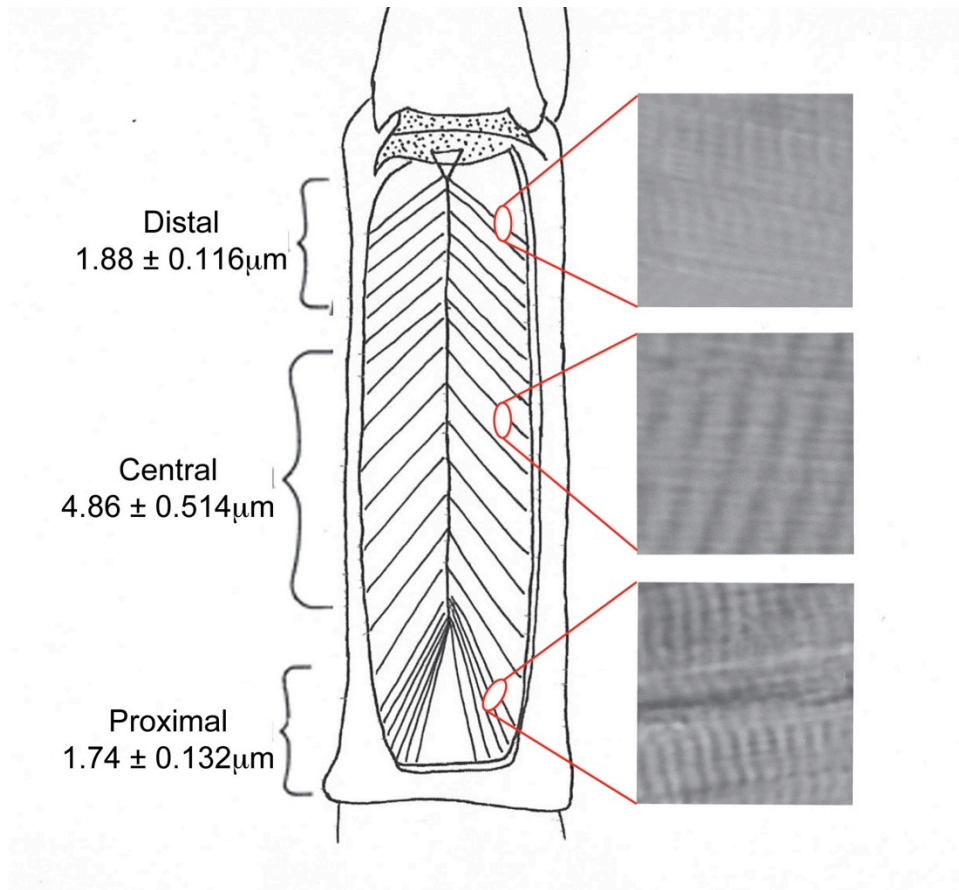


Figure 22: Sarcomere lengths

The sarcomeres of each of the three regions were passively stretched by pushing the claws of 5 different legs closed. Then the sarcomeres were fixed with Bouin's fixative, removed, mounted on slides and photographed at 100x. Each region was measured 5 times in each preparation for a total of 25 measurements. Highlighted to the right of this figure are sample photographs. The central region had larger sarcomeres than the proximal or distal.

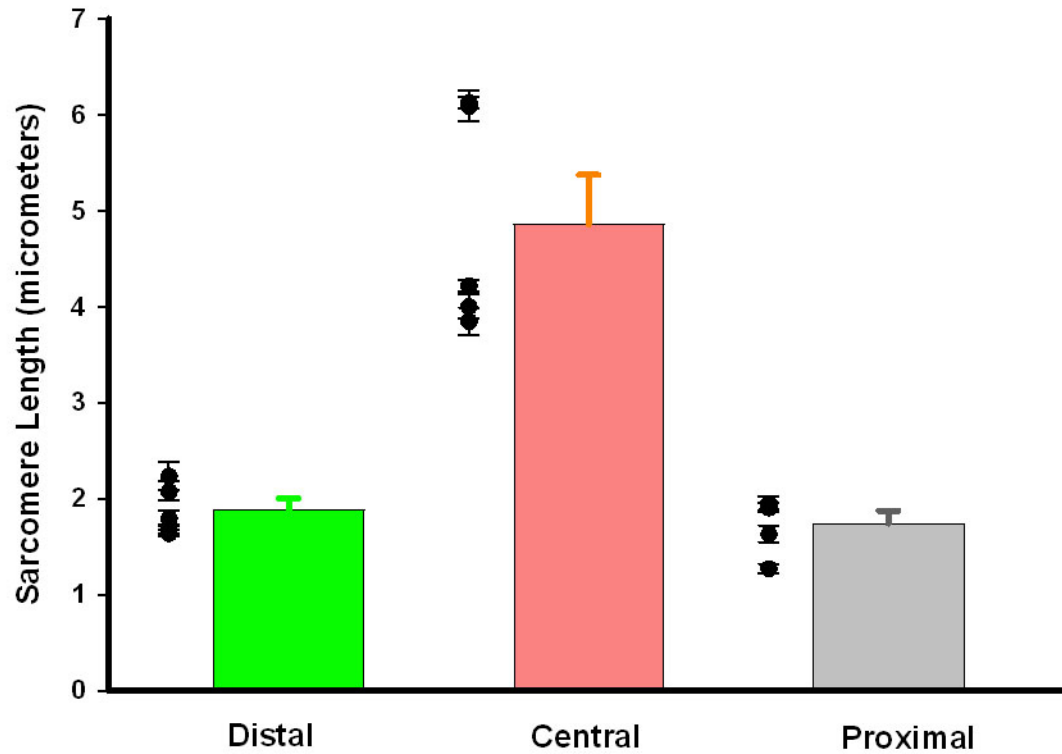


Figure 23: Regional differences in sarcomere lengths

Each dot represents the average measurement in each of the 5 preparations. The bar represents the average of those preparations. The sarcomeres in the central region are larger than those in the proximal and distal ($T=7.49$, $p<0.025$).

Effect of serotonin on force generation

Serotonin enhanced synaptic transmission at the neuromuscular junction (Dudel, 1965; Dixon and Atwood, 1989) and therefore I hypothesized that would translate to increase in force generation. Crider showed that serotonin had little effect on the facilitation of EPSPs in the proximal region (Crider and Cooper, 2000); therefore, I expected the central fibers to give rise to more force with serotonin.

After stimulating the opener muscle twice with the same paradigm, I added 100 μ L of 20 μ M serotonin directly to the muscle fibers. I then waited 1 minute and stimulated the muscle again. Figure 24A show a representative trace depicting an increase in force generated when serotonin was added. Figure 25A shows the mean and standard error of the mean at each of the 8 stimulations. Each dot represents the increase in force from the prior stimulation for each preparation; the dots are color coded for each preparation. Same procedure was repeated for the ablated distal fibers (see figures 24B and 25B). Then only the central fibers were ablated and serotonin was added (see figures 24C and 25C). Lastly distal and central fibers were ablated concurrently leaving only the proximal fibers (see figures 24E and 25E). Again, I was able to merge the data of distal then central ablation and concurrent ablation of distal and central fibers because there was no significant difference in force generated by these 13 preparations ($T=20.50$).

Figure 26 shows how much more force (not total force) was generated by the fibers when the serotonin was added at 40Hz. By the Wilcoxon Matched Pairs signed ranks test there is a significant difference between the amount of force generated at 40Hz between control and added serotonin ($p=0.047$), ablated central and added serotonin ($p=0.05$), and ablated central/ distal and added serotonin ($p=0.027$). There is no significant difference between ablated distal and added serotonin ($p=0.219$). The graph shows that not all the preparations showed an increase in force generation.

I expected to see that central fibers were most affected by addition of serotonin, but I saw the reverse (see table 4). As with the initial force generation in experiment 1, I subtracted the force generated by the proximal fibers from the ablated distal and

ablated central. The distal fibers were most affected by the addition of the serotonin and generated the most force (0.015), then the proximal (0.009) and the least affected was the central (0.006). But the graph shows there is a tremendous amount of variability among the preparations and these values are so low that they are not significant.

Summary

In conclusion the data for force generation and sarcomere length supported the statement that central fibers are tonic-like fibers by generating the most force with the longest sarcomeres. However, the effect of serotonin on force generation was not consistent with prior research that showed the central fibers having the greatest increase in EPSPs after the addition of serotonin. I will address reasons for this contradictory data in Chapter 5: Conclusions and Future direction.

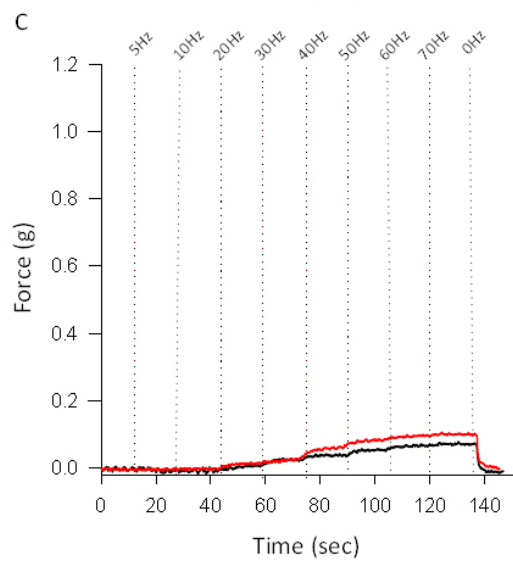
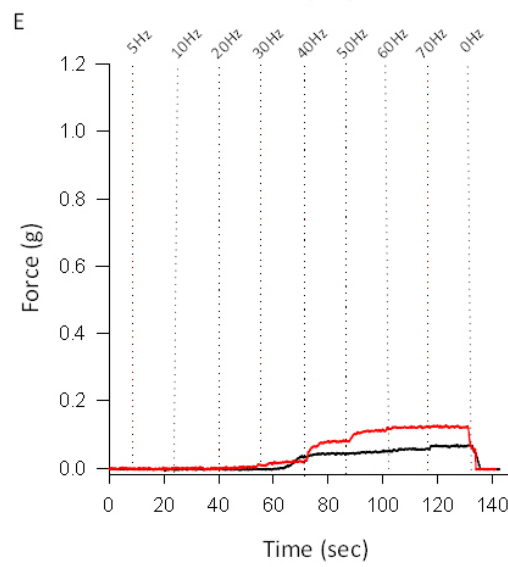
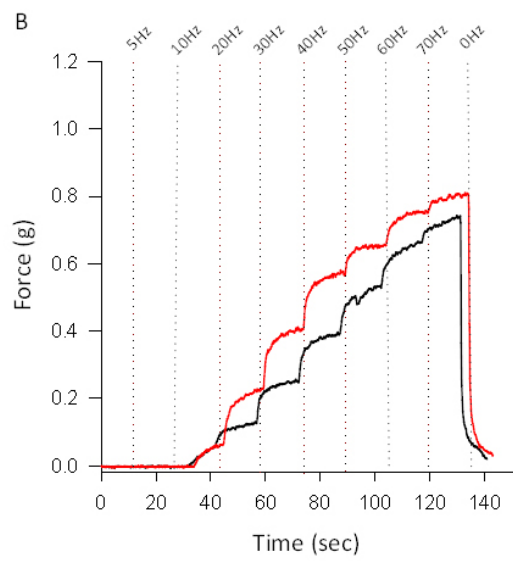
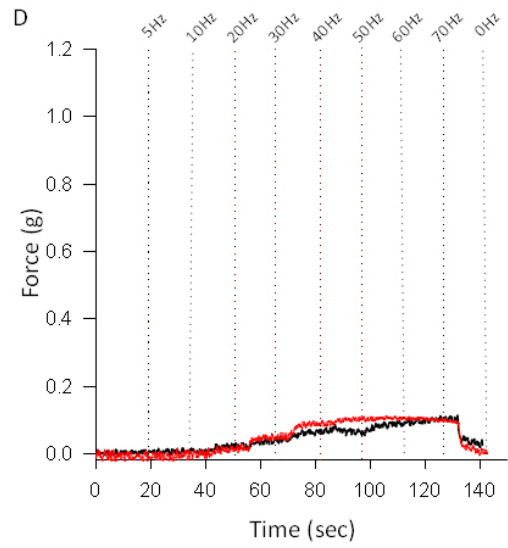
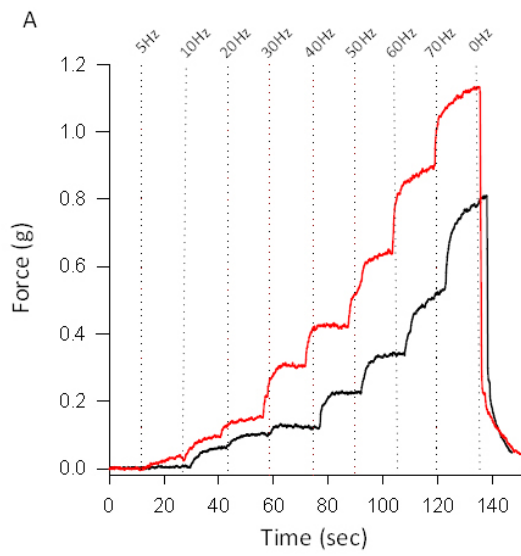


Figure 24. Representative traces of force generation with and without serotonin

In each graph the black trace represents the force generated by fibers without serotonin and the red trace represents the force generated in the presence of serotonin. Serotonin increased the force generated in each of the different preparations: (A) the control (B) ablated distal fibers (C) ablated central fibers (D) concurrently ablated distal and central fibers, and (E) ablated distal then central fibers.

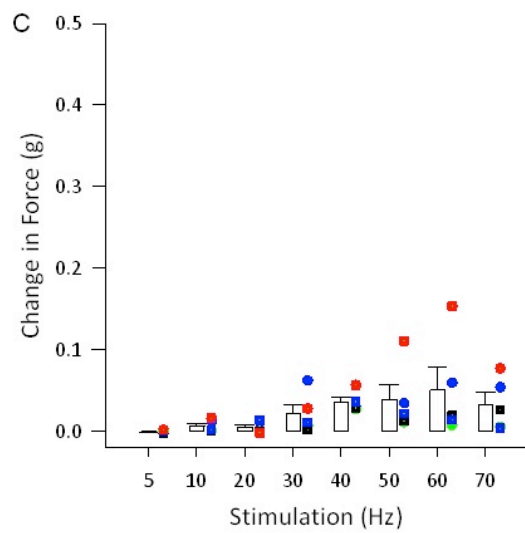
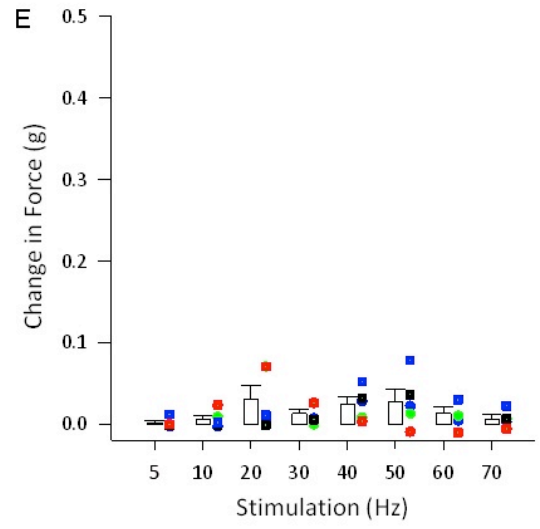
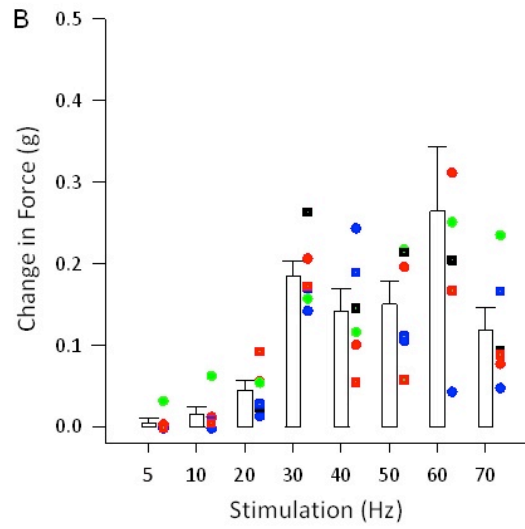
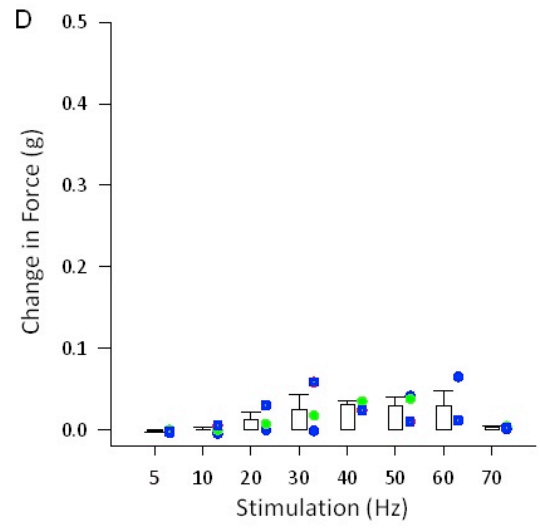
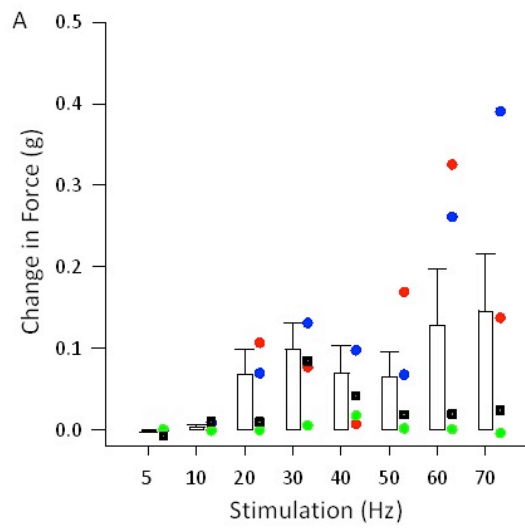


Figure 25. Change in force over stimulation paradigm with serotonin

These bar graphs show the mean and standard error of the mean at each of the 8 stimulations. Each dot represents the increase in force from the prior stimulation for each preparation; the dots are color coded for each preparation. Serotonin increased the force generated in each of the different preparations: (A) the control (B) ablated distal fibers (C) ablated central fibers (D) concurrently ablated distal and central fibers, and (E) ablated distal then central fibers.

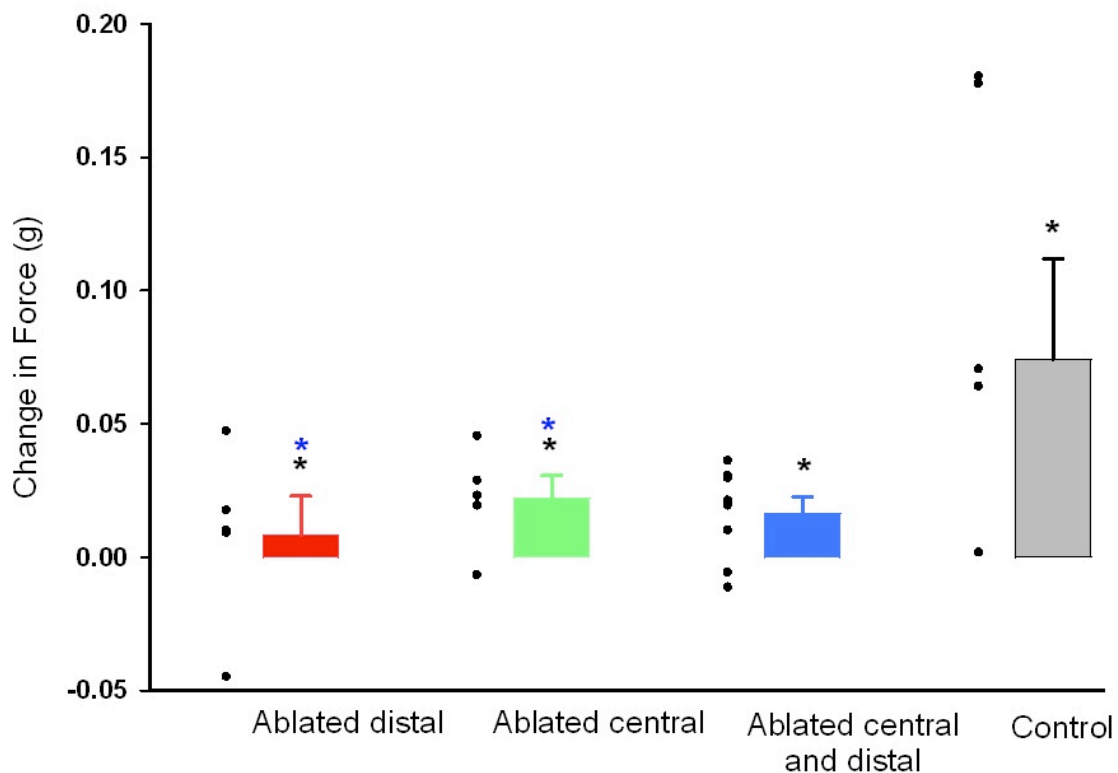


Figure 26: Effect of serotonin on force development at 40 Hz

This graph shows how much additional force (not total force) was generated by the fibers when the serotonin was added at 40Hz. There is significantly more force generated by the control than the ablated distal, ablated central and concurrently ablated central and distal. There is also a significant difference between the increase in force the ablated central generated in comparison to the ablated distal ($p < 0.05$). No significance was found between the ablated distal and ablated central/distal or the ablated central and ablated central/distal.

Increase in calculated force (grams) generated by the intact tissue after serotonin was added.

	Frequency of stimulation							
Intact Tissue	5Hz	10Hz	20Hz	30Hz	40Hz	50Hz	60Hz	70Hz
Distal	0.001	0.0216	-0.013	0.0167	0.015	0.005	0.048	0.012
Central	0.001	0.011	0.033	0.061	-0.006	-0.020	0.031	0.049
Proximal	-0.002	-0.017	0.013	-0.005	0.009	0.013	-0.013	-0.009

Table 4: The average amount of force generated by the 5 or more preparations was subtracted as explained in figure 21. For the sake of simplicity only 40Hz will be discussed in this thesis. See page 50 for an explanation on why 40Hz was used.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTION

The findings in this thesis are important as they increase our understanding of the basic mechanisms used in muscles for biomechanics and how the nervous system can control muscle with local (synaptic) modifications. The work is both comparative and interdisciplinary, combining neurobiology, biomechanics, modulation of motor unit function and anatomy. This study demonstrates how malleable the motor unit is with modulation and frequency of stimulation. While conducting this experiment I questioned why there might be different phenotypes in the different regions of the opener muscle. It might be that the proximal region is needed to open the claw quickly at the beginning, which would require the short sarcomeres and EPSPs that facilitate quickly. But to maintain the claw being open for any length of time would require the tonic like fibers in the central region.

Conclusions

My data for force generation and sarcomere length supported the statement that central fibers are tonic-like fibers that is found in the literature. The data also supported the statement proximal fibers are phasic like by generating the least force with the shortest sarcomeres. In this chapter I will tie together my findings about force development and sarcomere length with the data on the opener muscle reported in the introduction of Chapter 3: EPSP amplitude and facilitation, input resistance, fiber diameter, and synaptic structure.

Animals used in this study had orbital thorax lengths of 32-44mm and the propodite segment ranged in length from 6.5 to 8mm and ranged in weight from 0.13 to 0.25g. These differences have been found not to change the input resistance of the muscle fibers (Lnenicka and Mellon, 1983), which may indicate that the EPSP amplitude remains constant, and therefore would not affect the outcome of the force development. However, a response curve based on size could determine if the kinetics

of synaptic transmission change as the muscle grows and help to determine if the variation seen in my force data might be related to size of the crayfish at the time of experimentation.

The effect of serotonin on force generation was not consistent with prior research that showed the central fibers having the greatest increase in EPSPs after the addition of serotonin. My data showed the distal fibers were affected the most by the addition of serotonin at 40Hz. In this chapter I will give explanations for why this serotonin experiment should be repeated before stating that the data were an accurate representation of what is occurring in the muscle, make suggestions for other experiments to be conducted, and explain some new research about the cellular mechanism.

As the size of the crayfish might be a concern so is the gender. It has been reported that serotonin has no effect on synaptic transmission on gravid females (Pagé and Cooper, 2004). It would follow that levels of hormones would be substantially altered in crustaceans at different developmental times, such as when storing spermatozoa or when gravid. Although no gravid females were used in this study, the gender of the crayfish, social status, and developmental timing might be factors in the data variation seen in the effect of serotonin on force development experiments.

Force generation and sarcomere length

The data in this investigation show the distal fibers contribute the least to the force generation, the central fibers contribute the most, but the proximal fibers are necessary for proper alignment of the muscle on the apodeme and contribute more to the total force generation than the distal but less than the central. There is a significant difference between all preparations except between the control and ablated distal preparations. This lack of difference in force generation when the distal fibers were ablated could show that these fibers contribute a small amount of the total force, but before making that conclusion I think determining the force generated by individual fibers and not the region as a whole is necessary. This could be done by determining the total ablated cross sectional area or determining force generated by individual fibers. It

is interesting that the distal fibers appear to contribute the least to the total force generated because the tension receptor neurons that sense force development of the muscle are located in the distal region of the apodeme. I will discuss this idea further in the future direction section of this chapter.

A study with individual fibers would also address the issue that the proximal fibers could not be cut without damaging the nerve. At the end of this investigation a proximal fiber damage study was conducted to determine if EPSPs could still be recorded in the distal region before and after the proximal tissue was ablated. No matter the stimulation frequency used the EPSPs stopped in the distal region once the proximal fibers were ablated. These fibers appear to hold the entire muscle in proper alignment with the apodeme and when ablated allow the rest of the muscle fibers to shift affecting the neuromuscular junctions or the nerve itself. Back filling the nerve might help to determine where the damage occurred.

The proximal region's synapses contain two or more active zones and are more complex than the central region; therefore, the proximal fibers are able to release more neurotransmitter which leads to a large initial EPSP. However as facilitation occurs, the vesicles in the ready releasable pool (RRP) become depleted faster in the proximal region because a large amount of neurotransmitter was released at one time (Millar et al., 2005).

The high-output proximal region has larger enhancement of EPSP amplitude and larger facilitation index when compared to the low-output central region. Vesicles release neurotransmitter due to influx of Ca^{2+} and the amount is proportional to the length of depolarization or amount of Ca^{2+} entering the cell. There are several mechanisms that return the intracellular Ca^{2+} levels to normal after a depolarization event; for example Ca^{2+} binding proteins, Ca^{2+} being pumped out of cell via calcium pumps, or Ca^{2+} being pumped back into the sarcoplasmic reticulum by SERCA. These mechanisms affect the short-term facilitation because if the time between depolarizations does not allow the cell to return the Ca^{2+} to basal levels then the second depolarization will ride on the first, increasing the EPSPs produced. In the proximal

region, the high-output terminals release a large amount of neurotransmitter when given the first pulse (see figure 3) but the amplitude of EPSP plateaus quickly and therefore the facilitation index is less than the central region low-output terminals (see figure 4). The central region contributed the most to the total force generation, but it would be interesting to determine if the slope of force generation is different for the proximal and central fibers. I would expect the proximal region would generate force more quickly because of the large amplitude of the initial EPSP but this force would not be maintained and therefore would not contribute as much to the overall force generated.

The fiber diameter and input resistance correlate for the three regions. The larger diameter fibers have a lower input resistance. These larger fibers have a greater surface area and if one assumes the leak channels of the membrane are uniform then one would expect the larger surface area to have a lower input resistance (Law and Atwood, 1971). The specific membrane resistance can be estimated based on the input resistance and specific internal resistance of the muscle fiber (Battelle and Kravitz, 1978; Kravitz et al., 1980). The input resistance alone does not account for the large differences in EPSP amplitudes between the proximal fibers and the central or distal fibers. There is a two fold difference between the proximal fiber input resistances and the central; however, the last EPSP amplitude in the 30 pulse train shows a five fold difference from proximal to central or distal (see Figure 3). Thus, the differences in input resistance of the muscle fibers alone cannot account in the entire EPSP amplitude differences.

As mentioned in Chapter 3, there is likely a difference in synaptic efficacy with respect to mean quantal content on the different muscle fibers. The large differences in amplitudes of the EPSPs between the regions would suggest that the amount of Ca^{2+} that enters the muscle cells through the voltage gated ion channels on the plasma membrane would also be varied. A prediction would be that more calcium would enter into the proximal fibers, and given that they are narrow in diameter, the Troponin-C proteins would sense the high Ca^{2+} influx for initiating strong contractions. There are

fewer proximal fibers and therefore potentially fewer total contractile proteins as a whole for the proximal region as compared to the central or distal fibers causing the total force generated to be less. The potentially large Ca^{2+} influx in the proximal fibers could account for a range fractionation in the development of force with the proximal having the rapid development while the central and distal take over in time and amount of force during contraction of the whole opener muscle.

With the amount of data that was generated and not used in this study, there are many other manipulations of the data that could be interesting. For example at what frequency was the greatest change in force? There appear to be differences in the pattern of force increase when the opener was stimulated at different frequencies (see figure 18 and 20.) For example there appears to be an increase in force from 50 to 60Hz in the proximal fibers alone, both when the distal and central fibers were ablated concurrently and when they were ablated consecutively. As mentioned earlier, the rate of force development at 40Hz could also be determined by looking at the initial slope of the force curve. To address Chapple's idea that force is a function of length and length should be expressed in terms of sarcomere length, it might be interesting to see if reporting the data as the force generated per length of sarcomere changed the results of this study.

There are several other ways this experiment could be designed, either *in situ* (with different force transducers and different stimulation paradigms) or *ex situ* (using individual fibers). The force transducer used in this experiment worked well but using one that could measure greater and lesser forces might give more precise measurements. The 15sec stimulation paradigm used in this experiment was used because with longer than 15sec stimulation there was a loss of muscle tension and EPSPs disappeared in higher frequencies. However, the paradigm shown in figure 27 might show different results since the muscle is allowed to relax before the next stimulation. Muscle fatigue is defined by an increase in stimulation to exert a desired force and eventually an inability to produce the force; however, fatigue can be reversed by allowing the muscle to rest (Nordstrom et al 2006). This paradigm was not used

because the preparation tended to jerk when the stimulation was turned on and off affecting the placement of the force transducer pin and electrode; therefore continual stimulation was employed.

Effect of serotonin

Serotonin is thought to enhance synaptic transmission independent of Ca^{2+} levels in the cell. Dudel showed that serotonin caused an increase in the frequency of spontaneous events and therefore increases probability of neurotransmitter release and increased EPSP amplitude (Law and Atwood, 1971). Later Glusman and Kravitz were able to show that the number of failures decrease when serotonin is added (Glusman and Kravitz, 1982). The mechanism of serotonin effect when applied directly on muscle might be related to the activation of two different second messenger systems: activation of protein kinase C (PKC) and production of inositol triphosphate (IP_3) (Goy et al., 1984; Goy and Kravitz, 1989). This action could increase the input resistance and account for some of the increase in EPSP amplitude.

A possible mechanism for serotonin to increase force generation in the opener muscle is through activation of G coupled receptors and the 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) pathway. The production of IP_3 can directly result in Ca^{2+} release from the sarcoplasmic reticulum through IP_3 receptors on the SR (Yang et al., 1999). It has also been shown in *Aplysia*, that serotonin could cause the phosphorylation of synapsins thus increasing the likelihood that vesicles would become untethered from the cytoskeleton and dock with the presynaptic membrane (Fiumara et al., 2004). This could account for the increase occurrence of spontaneous quantal events and enhanced evoked responses with serotonin exposure (Wu and Cooper, 2012a). When neurons are exposed to phosphatases, serotonin action is enhanced showing the importance of phosphorylation in serotonin action (Swain et al., 1991). There is also a slight increase in input resistance when serotonin is added that could account for the increase in EPSP amplitude which might suggest a serotonin has some effect on the presynaptic motor nerve terminal (Strawn et al., 2000).

The data from my experiments with serotonin did not support my hypothesis. It is interesting to note that the force generated at 30Hz is more in line with the expected results (see table 4), with higher forces being generated in central fibers than the proximal fibers. Obviously these experiments should be run again taking into account the size of the crayfish, the gender of the crayfish, the social class to reduce the variability in the data. The amount of serotonin that is present in the hemolymph before experimentation could be determined to make sure that it is consistent. As mentioned earlier the cross sectional area of the bundles should be determined and the force should be reported as force per fiber. All these additions could allow for more accurate representation of the increase in force the animal is able to produce when serotonin is present. A dose response might also be helpful. I only used one concentration of serotonin, 100 μ M, which might not be enough to cause an increase in force in the central fibers. It would be interesting to determine if the different regions have different responses to different concentrations of serotonin. The literature does not seem to currently address if there are differences in the cellular action of serotonin on the different fiber types.

Future direction

In conducting these experiments and documenting my findings, I have realized there are several other follow up experiments to this thesis that could be completed. Some projects are the simple manipulation of the vast amount of data that I collected but did not use in this study, while others are more time consuming and proceed deeper into the understanding of the crayfish ability to sense force generation. I will discuss a few of these in this section.

I have shown that the distal muscle fibers don't appear to participate much in the total force generation of the opener, but the tension receptors are likely located in the distal region of the apodeme if the same trend in the anatomical location of tension receptors holds for the crayfish as in crabs (Cooper and Hartman, 1994; Hartman, 1985; Tryba and Hartman, 1997; Macmillan and Dando, 1972; Parsons, 1980). So how are these neurons able to measure the force generated by proximal or central fibers? These

tension receptors are too small and inaccessible without damaging the muscle to be physiologically recorded crayfish, but have been described in blue crabs, which I would suggest for use as the model organism to follow up in addressing the same questions that I proposed in the crayfish (Tryba and Hartman, 1997).

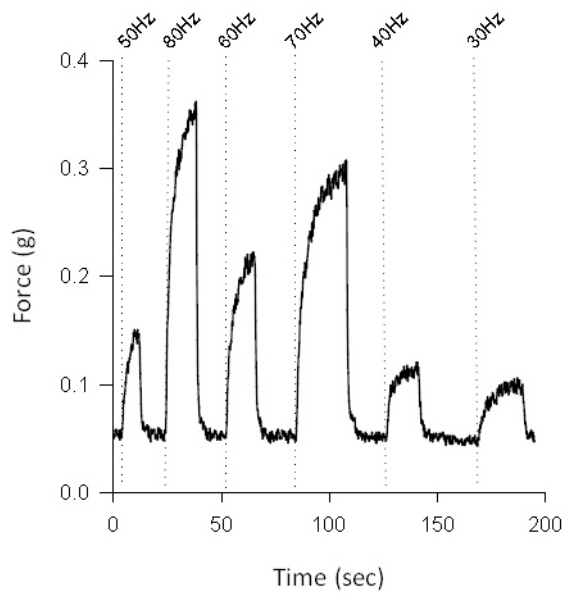


Figure 27: Unused stimulation paradigm.

Depicts force generated by the opener muscle as the frequency of stimulation was changed. Stimulation was stopped before increasing the frequency. There is more force generated when higher frequencies are used regardless of the order of the stimulation.

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VITA

EDUCATION

- ✿ Post Baccalaureate course work. Miami University. Oxford, OH. Summer 2001, 2003. Awarded Howard Hughes Foundation grant for Education course work.
- ✿ Bachelor of Science in Biology. Dickinson College. Carlisle, PA. Aug 1993-May 1997. Minors in Chemistry and Studio Art. *Magna Cum Laude*.

HONORS

- ✿ Recipient of the Sayre School Board of Trustees Award for Excellence in Teaching, May 2007.
- ✿ Recipient of the Sayre School Short Award for excellence in teaching Math and Science, May 2003.

PROFESSIONAL DEVELOPMENT

- ✿ Attended AP Chemistry Workshop, 1997. Attended AP Biology Workshop, 1998.
- ✿ NSTA Member, 1998-2004, 2006, 2008. Attended yearly regional conferences, 1998, 2002, 2008, 2012.
- ✿ Participated in National Association of Independent Schools David Mallory Leadership Conference, 2000.
- ✿ Recipient of the Sayre School Summer Sabbatical, June 2002. Studied rainforest, mountain, beach ecosystems and learned new conservation techniques for native endangered wildlife in New Zealand.
- ✿ Attended yearly Laptop Institute conference, Lausanne Collegiate School. Memphis, TN. 2005, 2006.
- ✿ Attended 6-day summer Vernier Conference. Biology, Chemistry, and Physics, 2007.
- ✿ Attended the Amaz!ng Meeting. I, Skeptic: Modern Skepticism in the Internet Age, Las Vegas, NV, 2008.
- ✿ Participated in *Kauai Science of Ecosystem* 10 day Conference. Kauai, HI, 2009.

PRESENTATIONS:

- ✿ “Paper to Paperless” at Laptop Institute, Lausanne Collegiate School. Memphis, TN, 2006.
- ✿ “The Electronic Science Classroom” at Kentucky Association of Independent Schools Science and Math conference, 2007.
- ✿ “Effects of Environmental and Modulators on GI and Heart Function in Invertebrates: Shrimp and *Drosophila*” at Association for Biological Laboratory Education Conference. Las Cruces, NM, 2011.
- ✿ “STEM and Health: Stressors on the Circulatory System Related to Excess Body Fat” at National Science Teacher Association meeting. Louisville, KY 2012.

REVIEWS AND EVALUATIONS

- ✿ Participated in Independent Schools Association of the Central States evaluation team of Cincinnati Hills Christian Academy. Middle and Upper School Science and Math, and K-12 Technology, 2005.
- ✿ Reviewed article for Brain Research, 2011.

ABSTRACTS PRESENTED:

- ✿ Holsinger, R.C., Cooper, R.L. "The effect of regional phenotypic differences of *Procambarus clarkii* opener muscle on sarcomere length, fiber diameter, and force development" at Society for Integrative and Comparative Biology. San Francisco, CA. 2013
- ✿ Holsinger, R.C., Potenza, J.B., Mercier, A.J. and Cooper, R.L. (2012) Physiological investigations with the crayfish hindgut. Society for Integrative and Comparative Biology Annual Meeting. January 3-7. Charleston, SC.
- ✿ Holsinger, R.C., Potenza, J.B., LeBlancq, M.J., Mercier, A.J. and Cooper, R.L. (2011) Modulating the neural control and direct actions on the crayfish hindgut: Serotonin, octopamine and dopamine. Ohio Physiological Society Annual Meeting. October 6-7. Cincinnati, OH.
- ✿ Holsinger, R.C. (1993) The Effect of Phosphorus and Mycorrhizal Symbionts on Sorghum Growth. The Virginia Academy of Science Annual Meeting. Richmond, VA.
- ✿ Holsinger, R.C. (1990) North American Squirrels Chose Acorns due to Availability and not Preference. Virginia Junior Academy of Science. Richmond, VA. Won First Place in Biology.