ANATOMY AND LENGTHENING VELOCITY OF MUSCLES IN THE LOBSTER
STOMATOGASTRIC SYSTEM

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Jeffrey B. Thuma
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by

JEFFREY B. THUMA

has been approved for

the Department of Biological Sciences

and the College of Arts and Sciences by

Ralph A. DiCaprio
Professor of Biological Sciences

Benjamin M. Ogles
Dean, College of Arts and Sciences
Abstract

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ANATOMY AND LENGTHENING VELOCITY OF MUSCLES IN THE LOBSTER STOMATOGASTRIC SYSTEM (45 pp.)

Director of Thesis: Ralph A. DiCaprio

Muscle shortening/lengthening velocity is an important component of muscle response to motor neuron input. What structural features help determine lengthening velocity is thus important to understanding how nervous systems generate behavior. Using data from muscles with similar electrical properties and a wide range of sarcomere and muscle lengths, I verify here Huxley and Neidergerke’s hypothesis that shortening (and lengthening) velocity depends on sarcomere number. Using a fluorescent marker for F-actin, muscles from the lobster (*Panulirus interruptus*) stomatogastric system, one of the best studied motor pattern generating model systems in neurobiology, were stained. Prior work has shown that the widely differing dynamics of these muscles play a crucial role in determining how these muscles respond to neural input. Therefore, this work not only verifies that sarcomere number is a fundamental determinant of muscle shortening velocity, but also the important role that shortening/lengthening velocity can play in determining motor output.

Approved:

Ralph A. DiCaprio
Professor of Biological Sciences
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Introduction

Understanding how organisms produce behavior is a fundamental goal of neurobiology. A key component to this goal is understanding how neural activity is transformed into muscle response. A recurring theme in this work is attempts to relate muscle anatomy to muscle shortening velocity. The first such effort was by Jasper and Pezard (1934), who used data from four muscles in *Carcinus maenus* to suggest that differences in muscle contraction velocity (i.e., shortening velocity) were due to differences in sarcomere length. At this time the sliding filament theory of muscle contraction was unknown, but it was known that action potentials traveled more rapidly in larger axons. In their introductory comments, Jasper and Pezard refer to this observation and ask whether a similar relationship “between histological structure and the velocity of excitability and of contraction” (“entre la structure histologique et la vitesse d’excitabilité et de contraction”) might exist. Exactly how muscles contracted was unknown, therefore Jasper and Pezard could not predict from theory which morphological features might be important. Their data showed that muscle shortening speed varied linearly with sarcomere length ($R = 0.99$, replotting of their data), and they therefore hypothesized that shortening speed depended on sarcomere length.

In 1954, Huxley and Neidergerke provided a theoretical explanation for this observation by noting that the sliding filament hypothesis (Huxley and Hanson, 1954) predicts that muscle shortening velocity should depend on sarcomere number, and in muscles of approximately equal length, sarcomere
number will vary with sarcomere length. Figure 1 schematically illustrates Huxley and Neidergerke’s hypothesis. A critical point in it is that the sliding filament theory predicts that muscle shortening speed depends on how rapidly the myosin heads bind and unbind the actin filament. Maximum shortening speed should thus be independent of thick and thin filament overlap. Therefore, even though the absolute amount of thin:thick filament overlap will, in general, differ in sarcomeres of different length, provided the kinetics of actomyosin cycling are the same in both muscles, the contractile speed of individual long and short sarcomeres should be the same.

Figure 1A compares muscle shortening velocity in two muscles of equal length. The top muscle has ten 1 µm long sarcomeres and the bottom muscle has five 2 µm long sarcomeres. If in both muscles each sarcomere shortens 0.5 µm per sec, then the top muscle, with its ten sarcomeres, will shorten 5 µm/sec while the lower muscle, with its 5 sarcomeres, will only shorten 2.5 µm/sec. Figure 1B emphasizes that sarcomere number is the important factor by comparing muscles with different lengths (top muscle, 10 µm; bottom muscle, 5 µm) but equal (1 µm) sarcomere lengths. Each sarcomere again shortens 0.5 µm per sec, so the muscle with more sarcomeres—the longer muscle—will again shorten faster. Figure 1C further demonstrates how muscle shortening speed should depend on sarcomere number by comparing two muscles whose total lengths and sarcomere lengths have been arranged such that the muscle with the shorter sarcomeres also has fewer sarcomeres. The top muscle has six 2
µm long sarcomeres and the bottom muscle has four 1 µm long sarcomeres. Again, each sarcomere shortens 0.5 µm per sec. Despite the fact that the top muscle’s sarcomeres are twice as long as the bottom muscle’s, the top muscle shortens faster (3 µm/sec vs. 2 µm/sec) because it is longer and thus has more sarcomeres. This shows that sarcomere number is the fundamentally important parameter in determining muscle shortening velocity (as predicted by Huxley and Niedergerke). As such, the dependence on sarcomere length observed in the four muscles of Jaspar and Pezard presumably arose from the case shown in Fig. 1A, in which the muscles had sufficiently similar muscle lengths that sarcomere length served as a proxy for sarcomere number.

Limited experimental verification of Huxley and Niedergerke’s hypothesis has been provided by studies of fibers from single muscles. In these cases, all the fibers have the same length (as in Fig. 1A) and thus sarcomere length is a direct proxy for sarcomere number—fibers with large (and hence few) sarcomeres elicits slow muscle shortening whereas fibers with small (and hence many) sarcomeres elicits fast shortening (Atwood, 1963; Atwood & Dorai Raj, 1964; Dorai Raj, 1964; Atwood, 1965; Fahrenbach, 1967; Hoyle, 1967; Franzini-Armstrong, 1970; Jahromi & Atwood, 1971; Hoyle, 1973; Costello & Govind, 1983; Read & Govind, 1993). Although these data seem to support Huxley and Niedergerke’s sarcomere number based hypothesis, since all this work was done in same length fibers, it is unclear from these articles whether these workers appreciated that the fundamental characteristic of interest was sarcomere
number. For instance, in all figures in these papers only sarcomere length is
reported and in the text sarcomere number is never mentioned. It should also be
pointed out that all this work was conducted in fibers that were electrically
different, ranging from fibers that can generate action potentials to ones that
cannot within the same muscle. Therefore, the effect that sarcomere
number/length had on the differences in shortening velocities between fibers
cannot be known for certain.

I could find only one article in which the sarcomere number theory was
correctly applied to the data (Jahromi & Atwood, 1967). In this work, the authors
explicitly state that “the total shortening of the phasic fiber would be greater
because of the larger number of sarcomeres”. However, this work again
compared two electrically different muscle fibers—the fast “phasic” fibers and the
slow “tonic” fibers—so this correlation is again suspect at best. In much other
work, however, the importance of sarcomere number has clearly been
misunderstood. For instance, many researchers (Parnas & Atwood, 1966;
Atwood, 1967; Hoyle, 1967; Jahromi & Atwood, 1969a; Jahromi & Atwood,
1969b; Hoyle, 1969; Atwood, 1971; Jahromi & Atwood, 1971; Atwood, 1972,
1973; Govind et al., 1975; Atwood, 1976; Jahromi & Govind, 1976; Lang et al.,
1977; Komuro, 1981; Costello & Govind, 1983; Royuela et al., 1998; Royuela et
al., 2000) have asserted that one muscle should shorten faster than another
solely because the muscles have different sarcomere lengths without taking into
account muscle length and sarcomere number—the equivalent of asserting that
the lower muscle in Fig. 1C should shorten faster than the upper muscle. Perhaps most remarkable is a proposal by Hoyle (1969), without any theoretical basis and in contradiction to the data on which the “relationship” was supposedly based (see Discussion), that relaxation time and sarcomere length are logarithmically related. The above work shows that a misinterpretation of Huxley and Niedergerke’s hypothesis has been and continues to be present in the literature. Moreover, with the exception of the work in equal length fibers from single muscles noted above, their hypothesis has never been experimentally verified and because the equal length fiber work compared electrically different fibers, this work also cannot be considered. A final problem with all of the work above (different fibers from the same muscle and different muscles) is that the sarcomere lengths were never standardized. I show that even muscles at rest length can have different contraction states resulting in different sarcomere lengths. Without a method to standardize these varying lengths (see Materials and Methods), comparison across different fibers and different muscles is flawed.

I have therefore examined the relationship between sarcomere number, sarcomere length, and muscle relaxation time (measured using the time constant) in the lobster (*Panulirus interruptus*) stomatogastric system. This work is important for two reasons. First, because these muscles are all electrically similar (they are all slowly contracting, non-spiking muscles) (Jahromi & Govind, 1976; Govind et al., 1975) and have many different sarcomere and muscle lengths (present work), they can provide a complete test of Huxley and
Niedergerke’s hypothesis. Second, this system is one of the best understood systems in neurobiology, particularly the pyloric neural circuit, which controls the filtration portion of digestion (Maynard and Dando, 1974; Mulloney, 1977; Russell, 1979; Selverston, 1974; Selverston et al., 1976). A notable characteristic of this system is that its muscles non-intuitively respond to their driving neural input. For instance, as a result of their different response properties, two of its muscles respond very differently, one contracting predominantly tonically and the other phasically, in response to identical (from the same motor neurons) neural input (Morris et al., 2000). Similarly, several of the muscles contract in time with neural networks that do not even innervate the muscles, and the extent to which this occurs varies from muscle to muscle, even among muscles innervated by identical motor neurons (Morris et al., 2000; Thuma et al., 2003). Therefore, studying how muscle cell morphology contributes to the behavior of this system’s muscles is important in its own right.

In this study I used a fluorescently labeled phallotoxin which tightly and specifically binds to F-actin (Wulf et al., 1979; Bukatina, et al., 1984), to stain and measure sarcomeres in muscles of the lobster, *Panulirus interruptus*, stomach. I show here that sarcomere lengths of nine muscles in the stomach have widely different muscle lengths (5.9 to 21.1 mm) and sarcomere lengths (3.3 to 12.3 μm) and, consequently, have widely different sarcomere numbers (648-3036). For five of these muscles I compare these data with muscle relaxation velocity, and verify that velocity depends on sarcomere number.
Materials & Methods

Sarcomere Length

Lobsters of both sexes were obtained from Don Tomlinson Commercial Fishing (San Diego, CA) and maintained in aquaria at 10-15°C with circulating artificial seawater. The tail, legs, and antennae of the animals were removed and much of the dorsal carapace was removed. The dorsal ventricular nerve, which carries all motor neuronal input to the stomatogastric muscles, was then cut. The gonads and the hepatopancreas were then removed. Care was taken throughout the dissection to ensure all stomach muscle attachments were left intact. The entire animal was then immersed in 10% buffered formalin for 4-5 hours where it had full access to all stomach muscles. Afterwards, the animal was removed from the fixative and 1L of lobster saline (479 mM NaCl, 12.8 mM KCl, 13.7 mM CaCl₂, 3.9 mM Na₂SO₄, 10 mM MgSO₄, 10.9 mM dextrose, 11.1 mM Tris base, 5.1 mM maleic acid, pH 7.5-7.6; Fisher Scientific, Pittsburgh, PA) was poured over the animal to rinse.

Before each muscle was removed from the animal, its length was measured using digital calipers. Seven muscles (p1, p2, p8, cv1, cv2, cpv1b, cpv2b) innervated by pyloric neurons and two muscles (gm6b, cpv1a) innervated by gastric mill neurons were then removed from the stomach (Fig. 2). During this process any large pieces of fat and/or connective tissue adhering to the muscles were removed and care was taken not to stretch the muscles. In all cases one
end of the muscle was carefully teased away from its carapace or ossicle attachment while keeping the thin membrane between the muscle and the attachment site intact, so that sarcomere length at the end of the muscle could be measured. The muscles were then placed and washed twice in an Eppendorf tube with 0.1M phosphate buffer, pH 7.4. After washing, the buffer was replaced with 400 µl of phosphate buffer containing 1% Triton-X. 15 µl of a fluorescently conjugated phalloidin (excitation wavelength 488 nm, Invitrogen, Carlsbad, CA) were then added. Each tube was inverted once for mixing and left for 15 minutes, covered to protect from light. The phalloidin mixture was then removed and each muscle washed twice with phosphate buffer for 15 minutes. The muscles were then stored in phosphate buffer at 4°C until they were viewed using a confocal microscope.

For viewing, each muscle was placed on a slide with a well deep enough to prevent the muscle from being squeezed when the cover slip was applied. The muscle was scanned at one end and in the center using an LSM510 confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY) with a 100x, 1.45 NA objective, a 488 nm wavelength laser, and a 505 nm long pass filter.

**Procedure to Standardize for Differing Thin-Thick Filament Overlap**

Figure 3 shows a schematic of two sarcomeres and scanned lobster stomach muscle sarcomeres. The sarcomere’s left and right edges are defined by the Z lines where the thin filaments are anchored. The Z lines appear as very
bright lines in the scan because of thin filament overlap (Szczesna and Lehrer, 1993). The thin filaments extend from the Z line towards the center of the sarcomere where they overlap the thick filaments. The thin filaments appear as less bright bands on both sides of the Z lines in the scan. The area with only thin filaments is called the I band and the area with only thick filaments is called the H band. The thick filaments contain no F-actin and therefore the H bands appear as dark bands in the scan. The area that includes the entire length of the thick filaments (in the schematic this area also includes a slight overlap with the thin filaments) is called the A band and since the thick filaments are not stained, it is not possible to label the A bands in the scan.

Initial stainings showed that, despite the muscles being at their nominal “rest” lengths during fixation, the same muscle from different animals showed varying contraction states. Figure 4 shows drawings of three sarcomere contraction states, and the corresponding muscle scans, for three cv1 muscles. Fig. 4A shows sarcomeres with clear Z lines, I bands, and H bands. Fig. 4B shows slightly contracted sarcomeres where the Z lines and I bands are visible but the H bands are now hidden because the thin filaments completely cover the thick filaments. Very contracted sarcomeres are shown in Fig. 4C. These sarcomeres still have clear Z lines, but now also show thicker, bright bands due to thin filament overlap (see diagram). Without standardization this variation in muscle contraction state would lead to very different measurements of sarcomere length between different preparations. This problem is particularly
evident when looking at the cpv1a and gm6b muscles whose unstandardized sarcomere lengths have more than a 3 fold range (4.32 to 13.52 µm and 3.89 to 13.02 µm, respectively).

Figure 5 shows how measurements from sarcomeres in different contraction states were standardized. The sarcomere configuration in Fig. 5A, where the H band is not visible and there is no thin filament overlap (thick, bright bands in Fig. 4C), was defined as my “standard” sarcomere. Measurements taken from muscles not in this configuration were adjusted to obtain the sarcomere length they would have had in the configuration shown in Fig. 5A. Fig. 5B shows this standardization when H bands were present. A measurement from Z line to Z line was taken and the width of the H band was also measured. The H band measurement (gray box) was then subtracted from the Z line to Z line length to obtain the “standard” sarcomere length shown in Fig. 5A. Fig. 5C shows this standardization when thin filament overlap was present. For this configuration, a measurement from Z line to Z line was again taken and the width of the thin filament overlap (gray box in Fig. 5C; thick, bright bands in Fig. 4C) was also measured. To adjust this muscle configuration to my “standard” configuration, the width of the overlap was added to the Z line to Z line length. This adjustment procedure is similar to the method used by Huxley & Peachey (1961) and allowed me to standardize sarcomere length regardless of contraction state. For each muscle, five sarcomeres at the center of the muscle, and the first ten sarcomeres at one end of the muscle, were measured and standardized.
Once the data had been measured and standardized, the five values for the sarcomeres in the center of each muscle were averaged and an ANOVA was performed on the mean middle sarcomere lengths (SPSS Inc., Chicago, IL). Since the ANOVA showed the different muscles had unequal variances, a Games-Howell *post hoc* test was performed to determine significance. For the sarcomeres at the end of the muscle, a paired *t*-test (SPSS) was performed to test for significant differences between the muscle's first sarcomere, its tenth sarcomere, and the mean of its five middle sarcomeres (i.e., between the first and tenth, first and middle, and tenth and middle).

To calculate the number of sarcomeres per muscle fiber, each muscle’s length was divided by its unstandardized mean sarcomere length since the number of sarcomeres is dependent on the actual sarcomere length, not the standardized length. A paired *t*-test on the unstandardized sarcomere lengths was performed in the same manner as the standardized data. In cases where there was no significant difference between the unstandardized lengths of the tenth and mean middle sarcomere (p2, cpv2b, gm6b, cpv1a, cv1, cpv1b), mean sarcomere length was determined by averaging the unstandardized six measured values (i.e., the lengths of the tenth and the five middle sarcomeres). For muscles where there was a significant difference between the unstandardized lengths of the tenth and the mean middle sarcomere (p1, p8), a range of sarcomere numbers was calculated. The unstandardized length of the tenth sarcomere and the unstandardized length of the mean middle sarcomere
were both divided into total muscle length to arrive at two sarcomere number values. The sarcomere number determined using the length of the tenth sarcomere represents the number of sarcomeres if all sarcomeres maintained this length until they reached the middle of the muscle; the sarcomere number determined using the mean middle sarcomere length is the number of sarcomeres if sarcomere length decreased to the middle value immediately after the tenth sarcomere. Any difference in sarcomere number was again determined using an ANOVA with a Games-Howell post hoc test.

All plots were created in Kaleidagraph (Synergy Software, Reading, PA) and all figures were created in Canvas (Deneba Software, Miami, FL).

**Muscle Relaxation Times**

Relaxation times were measured for five muscles (p1, cpv2b, cpv1a, cv1, cpv1b). Complete methods are provided in Morris & Hooper (1997), but a brief description follows: an isotonic transducer was attached to the muscle and muscle contractions triggered by extracellular nerve stimulation were recorded. Muscle loading was adjusted to achieve optimal contractions and overstretching was prevented using a bar under the transducer arm. Data were transferred to a computer using an analog-to-digital converter (1401plus, Cambridge Electronic Design (CED), Cambridge, England) and Spike 2 software (CED).

For each of the five muscles, the largest amplitude contraction from five preparations (a total of 25 contractions) was identified, exported as raw data, and
plotted using KaleidaGraph. A double exponential fit was used to determine the relaxation time for each contraction from each muscle. In the data reported here only the fastest relaxation time ($\tau$) from the double fit is used (Figs. 11, 13). However, when these analyses were re-performed using the slow relaxation time from the fit an equivalent dependency on sarcomere number was observed. The fast relaxation times were averaged and significance was determined using an ANOVA with a Games-Howell post hoc test.

**Results**

Figure 6 shows the nine investigated muscles after staining and scanning with the confocal microscope. All panels are at the same scale and show an area from the center of the muscle. Clear Z lines, I bands, and H bands can be seen in each muscle and the wide range of sarcomere lengths present in the muscles is also apparent. This is particularly evident when comparing p1 (top row, left panel) and cpv1b (top row, middle panel) where 30 sarcomeres are visible for p1 but only 10 for cpv1b. This range of sarcomere lengths across muscles is quantified in Figure 7. In this figure each data point is the mean length of five (standardized) sarcomeres measured from the middle of an individual muscle. The standard deviations for the individual data points were very small and therefore not plotted. The circles to the right of each data column show the overall mean and standard deviation for the muscle. The p1 muscle had the smallest sarcomeres with a mean standard length of $3.25 \pm 0.32 \, \mu m$
while the cpv1b muscle had the largest sarcomeres, 12.29 ± 0.92 µm—an almost 4 fold difference. The stars and open circles at the bottom of the plot give the $P$ values from an ANOVA on these data. In each row, the muscle on the leftmost end of the horizontal line is compared to all muscles to the right, with an open circle showing no significance ($P > 0.05$), one star $P \leq 0.05$, two stars $P \leq 0.01$, and three stars $P \leq 0.001$. The sarcomere lengths of almost all the muscles differ at $P \leq 0.01$ or greater.

In frog semitendinosus muscle, sarcomeres at the end of a muscle are 2-3 times smaller than in the center (Huxley and Peachey, 1961). Similar measurements have never been reported for an invertebrate muscle, and this issue is clearly important for calculating sarcomere number. I therefore performed such measurements on the first ten sarcomeres in each of my muscles (Fig. 8). Figures 8A1, A2 show actual data from a p8 muscle (Fig. 8A1, A2 are at the same scale). The length of the muscle’s first sarcomere is 9.6 µm (11.4 µm standardized) which decreases to 7.1 µm (9.0 µm standardized) by the twelfth sarcomere and to 4.0 µm (4.65 µm standardized) by the middle of the muscle, a decrease of almost 2.5 fold. Figure 8B shows the mean standardized lengths of the first and tenth sarcomeres from at least six muscles and the middle means shown in Fig. 7. Paired $t$-tests (lines with stars above the bars) showed no significant difference between the first, tenth, and middle lengths for gm6b, cpv1a, and cpv1b, while cpv2b showed a significant difference between only the first and tenth ($P \leq 0.001$) and cv1 between only the tenth and middle ($P \leq 0.01$).
The four remaining muscles (p1, p2, cv2, p8) all showed significant differences between each comparison except for the tenth and middle sarcomeres of cv2 where there was no difference.

Both sarcomere length and muscle length must be known to calculate sarcomere number. Figure 9 shows mean length for each muscle except cv2 (at least 12 muscles were averaged for each muscle type). cv2 is an irregularly shaped muscle with fibers at one end longer than fibers at the other (see Fig. 2). Because of this, cv2 muscle length and sarcomere number will differ depending on where the muscle length measurement is taken, and this muscle is therefore not considered further here. p1, p2, p8, cpv1a, and cpv1b have very similar lengths. cpv2b, gm6b, and cv1 are longer and significantly different from all other muscles and each other at the $P \leq 0.001$ level. These lengths were used to calculate sarcomere number (muscle length divided by sarcomere length) per muscle fiber (Fig. 10). p1 and p8 have two values because their unstandardized tenth and middle sarcomere lengths differed statistically (see Materials & Methods). The muscles showed a very wide range of sarcomere numbers, approximately half of which were statistically different. These different numbers arose from changes in both sarcomere length and muscle length. For instance, p1, p2, p8, cpv1a, and cpv1b have very similar muscle lengths, but because of the differences in their sarcomere lengths, these muscles have a wide range of sarcomere numbers (for example: cpv1b, length = 7.4 mm, 648 sarcomeres per fiber; p8, length = 7.7 mm, 1068 – 1534 sarcomeres per fiber; p1, length = 8.0
mm, 1646 to 2302 sarcomeres per fiber). For other muscles, the difference in sarcomere number was primarily due to changes in muscle length (cpv2b, sarcomere length 7 µm, 3,036 sarcomeres per fiber; cpv1a, sarcomere length 7.8 µm, 974 sarcomeres per fiber).

One goal of this work was to test whether sarcomere length and muscle contraction/relaxation times were correlated. I therefore measured relaxation times (using the time constant, \( \tau \)) for p1, cpv1a, cpv1b, cpv2b, and cv1 (Fig. 11C). The time constant is the time required for the muscle to relax to 37% of its initial amplitude. We defined our initial amplitude as the maximum amplitude the muscle achieved. Fig. 11A shows a muscle contraction from cpv1b (black line) and cpv2b (red line) and the arrow shows the time at which cpv1b has relaxed to 37% of its maximum amplitude (approximately 3.06 sec). Fig. 11B is the same two muscles at a much smaller time scale—scale bar is equal to 1 sec instead of 10 sec as in Fig. 11A. The arrow denotes the time at which muscle cpv2b reaches 37% of its maximum amplitude (approximately 0.18 sec). Consistent with the wide range of sarcomere numbers shown in Fig. 10, a wide range of muscle relaxation times (most of which significantly differed) were present (Fig. 11C). It is valuable to point out here that these changes in relaxation time vary with sarcomere number in the expected direction (greater sarcomere number = faster contraction). cpv1b has a very long relaxation time (2.07 ± 0.48 sec) and a small number of sarcomeres (648). cpv2b relaxes almost fourteen times faster (0.15 ± 0.09 sec) and has a large number of sarcomeres (3,036). The difference
in relaxation time between these two muscles is most apparent in Fig. 11A. p1 and cv1 have similar relaxation times (p1, 0.77 ± 0.06 sec; cv1, 0.70 ± 0.16 sec) and similar sarcomere numbers (p1, 1646-2302; cv1, 2031). It is also important to note that cpv1a and cpv2b have very similar sarcomere lengths (cpv1a, 7.8µm; cpv2b, 7µm), but very different relaxation times (1.07 ± 0.05 and 0.15 ± 0.09 sec, respectively), clearly showing the fallacy of equating sarcomere length with shortening/lengthening speed.

Discussion

Jasper and Pezard (1934) first suggested that sarcomere length helped determine muscle shortening speed. Huxley and Niedergerke (1954) provided a theoretical basis for this observation by noting that the sliding filament theory predicted that contraction dynamics would depend on sarcomere number, and that in muscle fibers of constant length, sarcomere number depends on sarcomere length. Building on these two ideas, many papers then demonstrated that fibers from a single muscle (and which were therefore the same length) showed slow muscle shortening with large sarcomeres (i.e., small number of sarcomeres) or fast shortening with small sarcomeres (i.e., large number of sarcomeres) (Atwood, 1963; Atwood & Dorai Raj, 1964; Dorai Raj, 1964; Atwood, 1965; Fahrenbach, 1967; Hoyle, 1967; Franzini-Armstrong, 1970; Jahromi & Atwood, 1971; Hoyle, 1973; Costello & Govind, 1983; Read & Govind, 1993).
Unfortunately, that this dependence was on sarcomere number, and not length, was not explicitly noted in the work above. Much other work has mistakenly asserted that muscle shortening velocity depends on sarcomere length without considering the effect of muscle length and sarcomere number, a particularly important issue when comparing different muscles (Parnas & Atwood, 1966; Atwood, 1967; Hoyle, 1967; Jahromi & Atwood, 1969a; Jahromi & Atwood, 1969b; Hoyle, 1969; Atwood, 1971; Jahromi & Atwood, 1971; Atwood, 1972, 1973; Govind et al., 1975; Atwood, 1976; Jahromi & Govind, 1976; Lang et al., 1977; Komuro, 1981; Costello & Govind, 1983; Royuela et al., 1998; Royuela et al., 2000). This error reached its nadir in a paper by Hoyle (1969) in which he incorrectly proposed a logarithmic relationship between relaxation time (\(\tau\)) and sarcomere length (discussed in greater detail below). All of the above work (both different fibers from the same muscle and different muscles) also failed to correct for differences in muscle contraction state which could result in vastly different sarcomere lengths between preparations.

Consideration of the data reported here for muscles p1 and cv1 well illustrates these issues. p1 and cv1 have standardized mean middle sarcomere lengths of 3.25 \(\mu\)m and 8.61 \(\mu\)m, respectively (Fig. 7). Using sarcomere length to make an \textit{a priori} estimation of shortening speed would predict that p1 should shorten much faster than cv1 because p1’s sarcomeres are more than two and a half times smaller. However, because cv1 is more than twice as long as p1 (17 and 8 mm, respectively; Fig. 9), the muscles have very similar sarcomere
numbers (p1 = 1646-2302, cv1 = 2031; Fig. 10) and, consequently, have nearly identical \( \tau \)s (p1 = 0.77 sec, cv1 = 0.70 sec).

This issue of muscle shortening speed can have great functional importance. Crustacean stomatogastric muscles are slow, graded muscles (Govind, et al., 1975; Hoyle, 1983), and consequently act as low-pass filters of the rhythmic motor neuron input they receive (Morris, et al., 2000; Thuma, et al., 2003). Differences in muscle shortening speed alter the magnitude of this filtering, and thus can greatly alter muscle response. Figure 12 illustrates this issue using data from two stomatogastric muscles, cpv1b and cpv2b, that are innervated by the same neurons (the Pyloric Dilator, PD, neurons) and therefore receive identical neural input. As mentioned at the end of the Results, cpv1b has far fewer sarcomeres than cpv2b (648 vs. 3,036) and is also much slower (2.07 vs. 0.15 sec time constants). As a result of this difference, cpv1b transforms the rapid PD neuron rhythmic burst input (Fig. 12C) into a sustained tonic contraction (Fig. 12A) whereas cpv2b is fast enough to show 1:1 phasic contractions with each PD neuron burst (Fig. 12B).

Particularly given the history surrounding attempts to relate sarcomere characteristics with muscle shortening speed, it is extremely important to test whether the data reported here conform to theoretical expectations. Returning to Fig. 1A we find that shortening velocity is inversely proportional to sarcomere length—as sarcomere length increases, velocity decreases. Fig. 1B shows that shortening velocity is also directly proportional to muscle length—longer muscles
have more sarcomeres which allows velocity to increase. Finally, Fig. 1C combines the previous two figures showing that velocity is directly proportional to muscle length divided by sarcomere length. Muscle length divided by sarcomere length is equal to sarcomere number, hence, velocity is directly proportional to sarcomere number. Moreover, \( \tau \) is inversely proportional to velocity because as velocity increases the time required for the muscle to reach 37% of its initial amplitude, decreases. Therefore, since velocity is proportional to sarcomere number, \( \tau \) is inversely proportional to sarcomere number—as sarcomere number increases, \( \tau \) will decrease because the muscle is able to shorten faster. Thus, \( \tau = \frac{k}{\text{sarc}\#} \) or \( k \times \text{sarcomere number}^{-1} \), where \( k \) is a constant that depends on the shortening rate of an individual sarcomere. Figure 13A plots \( \tau \) versus sarcomere number from Fig. 10 (the midpoint of the range for \( p1 \) is used for plotting purposes). Fitting the data with the equation \( k \times \text{sarcomere number}^{-1} \) (i.e., with \( k \) being the only free parameter of the fit) gives an R value of 0.96 (line with long dashes). When both \( k \) and the exponent are free parameters (line with small dashes) the equation \( \tau = k \times \text{sarcomere number}^{-1.4} \) is obtained (again with an R value of 0.96), an exponent very close to the predicted theoretical value of 1. For comparison, Fig. 13B plots \( \tau \) vs. sarcomere length; as expected, no obvious relationship exists.

It is also important to consider the basis for Hoyle’s contention (1969) that \( \tau \) depended on the logarithm of sarcomere length. Hoyle based this contention on two data sets, one on multiple fibers from a single muscle (Hoyle, 1967) and
another comparing fibers from multiple muscles (Atwood, 1967). Replotting the
data (not shown) Hoyle used and running various fits on them gives logarithmic
fits with R values of 0.77 (Hoyle, 1967) and 0.73 (Atwood, 1967), linear fits with R
values of 0.88 (Hoyle, 1967) and 0.81 (Atwood, 1967), power fits with R values of
0.96 (with an exponent of 2.1) (Hoyle, 1967) and 0.86 (with an exponent of 3.5)
(Atwood, 1967), and exponential fits with R values of 0.99 (Hoyle, 1967) and 0.85
(Atwood, 1967). Hoyle asserted the relationship (logarithmic) with the worst fit of
several commonly used possibilities. A likely possibility is that Hoyle plotted
these two data sets on semi-log paper (as in Fig. 3 of Hoyle, 1967), noted that
they formed a straight-line, and confused which variable was plotted on which
axis. Regardless, it is clear that the equation $\tau = k \log(sarcomere \ length)$ in
Hoyle (1969) is the one least consistent with the data.

With respect to comparing these data to theoretical expectations, unless
muscle length is taken into account so that sarcomere number can be calculated,
there is no theoretical basis for thinking that shortening velocity should depend
on sarcomere length when comparing different muscles. Comparison of the R
values given above of fits to the Atwood data shows that none of them achieve
significance (for four data points the R value for significance at the 0.05 $\alpha$ level
must be $\geq 0.878$). The Hoyle (1967) data, alternatively, are drawn from fibers
from a single muscle. Since all these fibers therefore have the same length,
sarcomere number therefore varies inversely with sarcomere length in them
(since sarcomere number = muscle length / sarcomere length), and thus theory
predicts a linear relationship between sarcomere length and $\text{tau}$. Although the linear fit is indeed significant, it is much worse than the power or exponential fits, a discrepancy likely arising from the fact that Hoyle was comparing fibers with very different electrical properties. A final important point to make about this work is how clearly it demonstrates that Huxley and Niedergerke’s hypothesis has been misunderstood. Although Fig. 1 demonstrates that in equal length fibers contraction time constant should depend linearly on sarcomere length, at no point does Hoyle note that sarcomere number is the important factor, that the correct relationship should be linear, and that the relationship he found was not.

The work presented here has two generally relevant messages. The first is to confirm Huxley and Niedergerke’s hypothesis (1954) that muscle shortening velocity depends on sarcomere number, using muscles with a wide range of sarcomere lengths and muscle lengths. The second is that, despite science’s cornerstone belief in self-correction, error can spread and persist in the literature for long periods of time.
References


Appendix A

Figure 1. Muscle shortening should depend on sarcomere number. Muscles with long sarcomeres will not always shorten slower than muscles with short sarcomeres. In each example, the sarcomeres shorten 0.5 µm per sec. A. Two muscles of the same length, the upper muscle has 1 µm sarcomeres, the lower has 2 µm sarcomeres. Since the muscles are of equal length, the muscle with the longer (and fewer) sarcomeres shortens slower. B. Two muscles of unequal length but with equal length sarcomeres. The longer muscle shortens faster because it has more sarcomeres. C. Two muscles of unequal length with different length sarcomeres. The upper muscle has 2 µm long sarcomeres, the lower has 1 µm sarcomeres. Despite having longer sarcomeres, the upper muscle shortens faster because it has more sarcomeres.
Figure 2. *Panulirus interruptus* stomach muscles. Muscles p1, p2, p8, cv1, cv2, cv1b, and cv2b are innervated by pyloric network neurons, while muscles gm6b and cv1a are innervated by gastric mill network neurons.
Figure 3. Structure of a sarcomere. The top panel shows a diagram of two typical sarcomeres, the bottom panel shows scanned lobster stomach muscle sarcomeres. A sarcomere consists of two Z lines (bright lines in scan) which anchor the thin filaments contained in the I bands (less bright bands in scan on both sides of Z line). In the center of the sarcomere are the thick filaments which comprise the A bands. The H bands (dark bands in scan) are the portion of the A band in which there are no thin filaments.
Figure 4. Contraction state of muscle may differ during fixation. The same muscle (at rest length), in three animals, showing three possible muscle configurations during fixation. Different contraction states can result in more than a three fold difference in unstandardized sarcomere lengths in the same muscle from different preparations (e.g., gm6b 3.89 to 13.02 µm). On the left is a schematic of the sarcomere with the corresponding scan on the right. A. The muscle was not contracted and showed clear Z lines (arrows), I bands (light bands on both sides of the Z line in scan), and H bands (dark bands in scan). B. The muscle was slightly contracted and showed only Z lines (arrows) and I bands. The H bands were now hidden by the thin filaments. C. The muscle was very contracted showing Z lines (arrows) and a thicker, bright band in the scan that was the overlap of thin filaments in the I band. The H bands were again hidden by the thin filaments.
Figure 5. Standardization of sarcomere length. Sarcomere lengths from the same type of muscle may differ between preparations because of different muscle contraction states (Fig. 4). To correct for this, a standardization protocol was applied to each sarcomere length. A. “Standard” sarcomere length was the length of the Z line to Z line measurement when no H bands were visible and there was no thin filament overlap. B. Muscles that showed an H band were standardized by measuring from Z line to Z line and subtracting the length of the H band (gray box) from this measurement. C. Muscles that displayed a thin filament overlap were standardized by measuring from Z line to Z line and adding the length of the thin filament overlap (gray box) to this measurement.
Figure 6. Confocal scans of nine lobster stomach muscles. All scans are at the same scale and taken from the middle of the respective muscle. Z lines, I bands, and H bands are evident in each muscle. Comparing p1 (upper left panel) and cpv1b (upper middle panel) shows the wide range of sarcomere lengths present.
Figure 7. Sarcomere mean length at the center of the muscle. Each data point represents the mean of five standardized measurements from the middle of a single muscle. The sarcomere lengths ranged from 3.25 µm for p1 to 12.29 µm for cpv1b. The filled circle to the right of each range of values is the mean for that range, error bars are standard deviation. The stars above the x axis are the results from an ANOVA. In each row, the muscle at the beginning of the line is compared to all muscles to its right. O = no significant difference, * = significant at 0.05 level, ** = significant at 0.01 level, *** = significant at 0.001 level.
Figure 8. Sarcomere length at the ends of the muscles can differ from the length at the center. **A1.** Scan showing the first twelve sarcomeres of a p8 muscle. The first sarcomere was 9.6 µm (11.4 µm standardized) long, but length decreased to 7.1 µm (9.0 µm standardized) by the twelfth sarcomere. **A2.** The middle portion of the same muscle from A1 at the same scale. Sarcomere length has decreased to 4.0 µm (4.65 µm standardized). Because of this possible change in sarcomere length along each muscle, the first ten sarcomeres at one end of the muscle were measured, in addition to the five center measurements. Using a paired $t$-test, the first and tenth, first and middle, and tenth and middle sarcomere lengths were compared. **B.** The mean length (bars) of the first, tenth, and middle sarcomeres for each muscle type. Paired $t$-test results are shown as lines with stars above, * = significant at 0.05 level, ** = significant at 0.01 level, *** = significant at 0.001 level. Horizontal lines with two vertical lines indicate that the first bar differed (with the marked significance) from both the second and third bars.
Figure 9. Whole muscle lengths. Intact muscles were measured after fixation and before removal from the animal. The cv2 muscle is an irregularly shaped muscle and was not included in further analysis. The results of an ANOVA are again shown as stars below the x axis using the same convention as in Fig. 7.
Figure 10. Approximate number of sarcomeres per muscle fiber. The number of sarcomeres per fiber was calculated for each muscle type by dividing muscle length (Fig. 9) by mean unstandardized sarcomere length (see Methods). For muscles that showed no significant difference between the length of the tenth sarcomere and the mean of the middle sarcomeres (p2, cpv2b, gm6b, cpv1a, cv1, cpv1b), a mean sarcomere length was calculated by averaging all six sarcomere lengths for each muscle (i.e., the tenth plus the 5 middle lengths). This mean was used to calculate sarcomere number. For muscles that showed a significant difference between the length of the tenth sarcomere and the mean of the middle sarcomeres (p1, p8), a range was calculated. Using the length of the tenth sarcomere to determine sarcomere number gives the number of sarcomeres if sarcomere length remained constant until the center of the muscle and then decreased to the middle sarcomere length. Using the middle length to determine sarcomere number gives the number of sarcomeres if sarcomere length decreased to the middle sarcomere length immediately after the tenth sarcomere. The results of an ANOVA are shown below the graph (as in Fig. 7).
Figure 11. Muscle lengthening velocity (tau).  A. Muscle contractions from cpv1b (black line) and cpv2b (red line) which are innervated by the same neurons. The arrow shows the time at which cpv1b had relaxed to 37% of its maximum amplitude (~3.06 sec).  B. The same two muscles at a much smaller time scale, the arrow denotes the time constant for cpv2b (~ 0.18 sec).  C. Mean time constants. Individual time constants were measured using a double exponential fit to the actual muscle relaxation curve. Despite having very different muscle lengths, p1 and cv1 had nearly identical taus because of the similar number of sarcomeres in each muscle. cpv2b and cpv1a differed in relaxation speed by more than 6 fold despite having nearly identical sarcomere lengths. The results of an ANOVA are shown below the graph (as in Fig. 7).
Figure 12. Muscles receiving identical neural input produce very different contraction patterns. **A.** cpv1b muscle contraction (top “wavy” line) and its baseline when fully relaxed. **B.** cpv2b muscle contraction and its relaxation baseline. **C.** Both muscles receive identical neural input from the two PD neurons. The cpv1b contraction had almost no phasic component (the very small oscillations are the muscle’s response to each PD neuron burst) while cpv2b showed a large phasic component riding on the tonic contraction. cpv1b had approximately six times fewer sarcomeres than cpv2b, which would cause cpv1b to relax much more slowly. cpv1b would therefore be a much lower low pass filter than cpv2b, and hence have a much larger tonic component to its contraction. The three large amplitude changes in cpv2b were due to the activity of another stomatogastric rhythmic network, the gastric mill, that modulates PD neuron activity (Thuma & Hooper, 2002); cpv1b filtered out both the rapid PD neuron bursting activity and the slower gastric mill derived modulation of PD neuron activity.
Figure 13. Sarcomere number, not length, determines relaxation velocity. Sarcomere number and relaxation time constant ($\tau$) varied inversely—as number increased, $\tau$ decreased. A. Muscle $\tau$s (from Fig. 11C) vs. sarcomere number. A power fit to the data was used because of the inverse relationship. A power fit of the theoretical equation, $\tau = k \times$ sarcomere number $^{-1}$ (line with long dashes), has an R value of 0.96. The best fit to my data (line with short dashes) has an R value of 0.96 and a similar exponent (-1.4). B. Hoyle proposed a logarithmic relationship between relaxation time and sarcomere length. My data showed no such relationship and a logarithmic fit had an R value of 0.54.