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RESEARCH ARTICLE

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Fascicle measurement methods do result in lengths that are significantly different

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Abstract

Fascicle length is a key anatomical variable used to estimate functional adaptations of muscles. Although a recent study concluded that the fascicles measured on different specimens using in situ measurement and chemical dissection yield results that are not significantly different, these two methods have not been systematically tested within individual specimens. To that end, we compare the two measurement techniques on contralateral sides of (1) carnivoran masticatory muscles and (2) rat leg muscles to evaluate their statistical comparability. Our results show that the two methods result in statistically significant fascicle length differences, with in situ measurements being significantly greater in architecturally simpler muscles across a weighted average of masticatory muscles and among individual muscles. This trend holds for the rat soleus muscles but is reversed for the architecturally more complex gastrocnemius, with chemical dissection resulting in greater fascicle lengths. This suggests that pennation may influence methodological compatibility, with more complex connective tissue potentially obscuring fascicle ends and architecturally simpler muscles being prone to stretching when measured in situ. Given the importance of this anatomical measure, these results have potentially wide implications. Although small muscles with complex connective tissue yield shorter fascicle lengths when measured in situ, in general, in situ measurements are significantly longer by a factor of 1.3. Comparing fascicles measured using these traditional methods and through imaging techniques like DiceCT and micro-MRI, which allow evaluation of intact specimens, may provide further insight into the true anatomical reliability of these methods that unambiguously produce significantly different measurements.

Katherine M. Clark and Reece A. Brown contributed equally to the study.

KEYWORDS
chemical dissection, fascicle length, fascicle measurement, fiber length, in situ measurement, PCSA, pennation

1 | INTRODUCTION

One of the key variables in muscle fiber architecture research is fascicle length, the measure of the length of bundles of muscle fibers. Fascicle length is directly related to a muscle's stretch and contractile velocity and can be used to calculate physiological cross-sectional area (PCSA) which relates directly to a muscle's intrinsic force production capability (Massey et al., 2015; Scott et al., 1996). There are two prevalent gross anatomical methods for taking this measure: (1) 'in situ' measurement (e.g., as used in Anapol & Jungers, 1986; Taylor & Vinyard, 2004; Payne et al., 2006; Rose et al., 2013; Taylor et al., 2015) in which muscles are sliced based on the orientation of their superficial fascicles and then representative fascicles are measured in the planes along which the muscle was sliced and (2) 'chemical dissection' measurement (e.g., as used in Gans, 1974; Antón, 2000; Herrel et al., 2008; Huq et al., 2015; Marchi et al., 2018; Martin et al., 2019; Martens et al., 2024) in which the connective tissues binding fascicles are dissolved in acid and then representative fascicles are measured from throughout the whole muscle. Although either method can be used to measure a small or large number of fascicles depending on the goals of the investigator, the extent of anatomical access differs: in situ measurements are necessarily restricted to the fascicles exposed in the available planes of section, whereas chemical dissection permits sampling from throughout the entire muscle belly. Although both techniques are commonly used, neither approach is regarded as the methodological standard for accuracy and the similarity of the values they produce has been disputed. Potential reasons for error introduced by both methods have been proposed (Hartstone-Rose et al., 2018; Taylor et al., 2024) though a recent paper by Taylor et al. (2024) reported no differences when using the two methods on different individuals of strepsirrhines and platyrrhines in a subset of masticatory muscles. As intraspecific variation in muscle architecture is widely acknowledged (e.g., Charles et al., 2022; Taylor et al., 2018), we seek to test this claim of methodological compatibility on a sample of carnivore masticatory muscles and rat limb muscles. Importantly, we do not seek to establish whether one method is more accurate than the other; rather, we seek to assess consistency (i.e., precision) between the two methods. This was done by statistically evaluating fascicle length measurements taken using the two methods applied to contralateral sides of the same individuals.

1.1 | The importance of fascicle length

Fascicles, the functional and structural units of skeletal muscles, are comprised of bundles of muscle fibers, the cellular units of muscle contraction (Gans, 1982). Their arrangement and length, or architecture, determine the functional capabilities of a muscle (Infantolino et al., 2012): whereas longer fascicles can stretch farther and contract over a longer distance in the same amount of time and therefore are faster than short fascicles, more short fascicles can be packed into a muscle of the same volume via pennation. In this paper, we use the term pennation to describe fascicles oriented obliquely to the line of action within a muscle plane. Because PCSA represents the sum of the cross-sectional areas of all muscle fibers within a muscle, and force production capabilities are related to the number of contractile elements in parallel, pennate muscles with more numerous short fascicles can produce greater force than muscles with fewer, longer, less pennate fibers (Lichtwark & Wilson, 2008). Thus, there is a fundamental tradeoff in muscle architecture: for a given size, a muscle can be maximally adapted for force production (with relatively short fascicle lengths) or stretch/speed (with relatively long fascicle lengths) or a compromise between the two, but if both force and stretch or speed are needed, then the muscle's volume must increase—that is, increasing the number of long fascicles (Gans & de Vree, 1987).

Fascicle length relates to PCSA via the following equation from Schumacher (1961): $q = m/lp$ where q is PCSA, m is muscle mass, l is fascicle length and p is the density constant of muscle. PCSA and fascicle length assessments are commonly used in research on correlates of muscle anatomy and function such as evaluations of masticatory force and gape (e.g., Davis et al., 2010; Deutsch et al., 2025; Dickinson et al., 2024; Ginot et al., 2018; Hartstone-Rose et al., 2012; Hartstone-Rose et al., 2022; Perry et al., 2011; Taylor et al., 2018; Taylor & Vinyard, 2009; Williams et al., 2009), digging (Martin et al., 2019), and locomotion and postural behavior (Boettcher et al., 2019; Huq et al., 2015; Leischner et al., 2018; Marchi et al., 2018; Organ et al., 2009). A more thorough understanding of how the methodological

approaches track each other can therefore allow for a more accurate assessment of muscular capabilities.

1.2 | Fascicular chemical dissection

The chemical dissection technique of separating muscle fascicles, refined by Gans (1982), consists of placing muscles in acid (usually sulfuric or nitric, e.g., Antón, 1999; Leischner et al., 2018; Boettcher et al., 2020) to dissolve connective tissue until individual fascicle bundles can be separated. The fascicles are then removed from the acid, placed in a solution to halt digestion, such as 50% glycerol, and measured with a protractor (e.g., as done by Rayne & Crawford, 1972; Gans, 1982; Sacks & Roy, 1982), calipers, (e.g., as done by Leischner et al., 2018) or photographed alongside a scale bar and measured digitally using, for instance, ImageJ (e.g., as done by Boettcher et al., 2020; Leonard et al., 2020).

1.3 | In situ fascicular measurement

The in situ approach to fascicular measurement was first described by Anapol and Jungers (1986). In the original derivation of this approach, muscles were sectioned at regular intervals approximately 1 cm apart, and at each section six neighboring fascicles—three proximal and three distal—were measured. The total number of sampling sites increases in wider muscles such that multiple sections are taken along the length of the muscle (Anapol & Jungers, 1986; Rose et al., 2013). Over the years, the method has gone through different iterations, typically involving changes to the number of sampling sites, planes, and fascicles measured (Moore et al., 2013; Payne et al., 2006; Rockenfeller et al., 2024; Rose et al., 2013). These adaptations seem to be made at the discretion of the investigator, as no particular number of planes or fascicles measured seems to represent a standard in the practice. Unlike the chemical approach, slicing muscles via the in situ method allows direct measurement of the angle of pennation of the fascicles relative to their tendon in whatever state they were dissected in (Organ et al., 2009). Although the resulting angle of pennation can be incorporated into the equation for PCSA to allow analysis of the component of PCSA that is orthogonal to the line of action of the muscle, Perry et al. (2011) point out that this 'reduction' only accounts for the orthogonal pull *within* muscle sections, but not the orthogonal angle of the entire muscle relative to the overarching direction of movement (see their Figure 5). This is why many studies of muscle architecture omit this reduction calculation.

The in situ method was further refined by Anapol and Barry (1996) to minimize variation due to muscle position at the time of measurement by factoring resting sarcomere length into the estimation of fiber length. This is done based on the theory that there is some ideal "resting length" at which a sarcomere can produce its maximum amount of tension. Muscles of specimens that are positioned with joints at different levels of excursion may be stretched beyond that ideal length or contracted to be shorter than that ideal length. Thus, this technique has been used to "correct" observed fiber length by the ratio of observed sarcomere length to ideal sarcomere resting length—an approach that has been applied for position-dependent variation in both limb muscles (Felder et al., 2005) and mammalian jaw adductors (Eng et al., 2009; Taylor et al., 2019). However, most studies of muscle architecture omit the incorporation of sarcomere measurements not only because it skips a step requiring either specialized equipment or microscopy generally considered unnecessary when measuring muscles at relatively consistent excursions, but also because adjusting fascicle lengths of individual muscles that are part of a cohesive system leads to a theoretical configuration of the system that is physiologically impossible (Hartstone-Rose et al., 2018; Perry & Prufrock, 2018). For instance, differentially changing the fascicle lengths of the superficial and deep portions of the masseter and temporalis based on their measured sarcomere lengths would result in a theoretically optimal length for each muscle portion that corresponds to a completely different amount of gape excursion angle for each muscle. That is, lengthening or shortening the superficial masseter by *X*% and the deep masseter by *Y*% based on their respective sarcomere lengths would yield different theoretical gape positions for each of these muscle portions. Doing this for all of the masticatory adductors compounds this chimeric condition. Thus, there seems to be growing consensus that the best way to minimize the effect of fascicles stretched to different amounts is not to correct for these differences after their measurement but to study specimens that are confined to similar joint angles when possible. For instance, rather than "correcting" masticatory fascicle lengths of specimens with different amounts of gape (resulting in individual muscle fascicle lengths that theoretically match no individual gape), we should strive to study specimens at the same gape (Perry, 2008). Thankfully, most specimens included in masticatory studies are frozen or fixed at near occlusion (e.g., as used in Perry et al., 2011; Hartstone-Rose et al., 2012; Dickinson et al., 2024; Taylor et al., 2024). Because there is greater variation in the postcranial configuration of preserved specimens (i.e., limb and tail postures of animals in museum storage tanks vary greatly), this is more of a

concern for analyses of limb and postural muscles (e.g., as corrected for in Organ et al., 2009) and greater consideration about the employment of corrections should be made to avoid chimeric theoretical muscle excursions across individual joints of interest. With that said, when specimens are compared with their joints at similar angles (as was employed in this study), fascicle measurement approaches can be compared without consideration of theoretically consistent sarcomere conditions.

1.4 | The compatibility of the two methodological approaches

While fascicle length is a key variable for anatomical study, there is no consensus among researchers as to the most accurate method for its measurement (Hartstone-Rose et al., 2018; Martin et al., 2020; Taylor et al., 2024; Taylor et al., 2025). Although chemical and in situ fascicular sectioning are sometimes both used within studies (Huq, 2013), few studies have directly compared the results obtained by each method.

A key issue with accurately assessing muscle architecture relates to accessing internal fibers without impacting positional relationships (Perry & Prufrock, 2018). Regarding this challenge, potential methodological issues concerning the use of the in situ method were described by Hartstone-Rose et al. (2018) in response to concerns voiced by colleagues who favor the chemical dissection approach. That paper argued that the physical slicing of the muscle based on the orientation of its superficial fascicles is problematic due to the incongruence of the orientations of fascicles from the superficial to deep portions of muscles, as illustrated in that paper showing divergent orientations of portions of the masseter muscles that are often sliced in situ together. In their examination of fascicle architecture in three-dimensional space using diffusible iodine-based contrast-enhanced computed tomography, Dickinson et al. (2018), Dickinson et al. (2020), and Dickinson et al. (2024) further demonstrated the incongruence of fascicle orientation not only *across* discrete layers of the muscles of the masticatory apparatus but within these individual muscle portions. These visualizations show that there is no single plane in any muscle represented with parallel enough fascicles from superficial to deep to bisect without slicing through fascicles. Differences in fascicle orientation between and within the superficial to deep portions of a single muscle make slicing between fascicular planes based solely on the visual differentiation of the superficial fascicles extremely difficult, if not impossible, as Hartstone-Rose et al. (2018) argues, potentially leading to cutting the deeper fascicles, and resulting in shorter fascicle length measurements. There are an increasing number of papers using diffusible, iodine-based contrast-enhanced computed tomography (DiceCT) to visualize individual fascicles digitally (e.g., Dickinson et al., 2024; Holliday et al., 2022; Sullivan et al., 2019). It is clear from all of these that the fascicles beneath the superficial-most have substantially different angles from those superficial fascicles and therefore, at least in every muscle that has been thus visualized so far, there is no plane through which a muscle can be bisected from superficial to deep without slicing through fascicles.

A recent paper by Taylor et al. (2024) disputes this criticism. Taylor et al. (2024) tested this assertion by comparing fascicle lengths obtained using chemical dissection (from Hartstone-Rose, unpublished data) with in situ measurements of the same muscle regions from different individuals. They found that the probability of obtaining longer fascicles using chemical dissection was no greater than chance and concluded that multiple sources of variation—including species-level differences, individual variation, measurement method, other unmeasured factors, and error—could all contribute to discrepancies among studies. Importantly, they emphasized that "within-species and within-individual sample variation is high, and more than sufficient to account for the observed differences" in fascicle length between datasets derived from different individuals.

The chemical dissection approach bears its own potential methodological issues as well. Processing a muscle in acid requires careful and consistent monitoring to avoid over or under-processing. Too much time spent in the acid, and fascicles can become weak and more easily prone to breakage or may dissolve entirely. On the other hand, if a muscle is not left in the acid long enough, some connective tissue will remain, making manual separation more difficult and potentially necessitating more force, possibly resulting in broken fascicles. Even when acid processing is done perfectly, separating fascicles is tedious, time-consuming, and requires significant practice to become adept.

While these methods have had scant direct comparisons done, both dissection approaches have been compared to their digital approach counterparts, such as DiceCT (Dickinson et al., 2018; Gignac et al., 2016). These studies find no statistically significant difference in fascicle lengths derived from anatomical and digital approaches; however, the corresponding PCSA values tend to be different. It is important to note however, that Dickinson et al. (2018) only compared chemically derived fascicle lengths among conspecifics, with no analysis of in situ sectioning or with respect to contralateral sides of individuals. As discussed in Taylor et al. (2025), these findings that suggest combining data from these approaches is ill advised and architectural differences

between them are likely due to the muscle shrinkage that occurs during the chemical processing required for DiceCT.

To date, no study has compared how fascicle lengths vary when chemical dissection is used on one side and in situ measurement is used on the other *within the same individual specimens*. Taylor et al. (2024) suggest that observed fascicle length may be more dependent on intraspecific variation than the method of measurement; however, without comparing truly matched samples (i.e., sampling contralateral sides of individuals using each method), any differences in fascicle length cannot be definitively attributed to the method of measurement as individual variation within the species remains a confounding variable. Although individuals are not perfectly bilaterally symmetrical, the magnitude of this bilateral asymmetry should be substantially less than that of intraspecific variation. We herein conduct a methodological comparison not only sampling contralateral muscles of relatively large samples of individuals but also across muscles of substantially different sizes that exhibit both relatively complex and simple architecture. This allows us to evaluate not only whether measurement methods result in significantly different fascicle measurements but also if there is are differential effects relating to specific muscle size and/or architecture.

1.5 | Hypotheses

We propose to evaluate the conclusions of Taylor et al. (2024), who found no significant differences between fascicle lengths derived from each method (i.e., both methods of obtaining fascicle lengths are comparable in the values they produce; H0) when comparing a subset of muscles between different individuals across species. We hypothesize that a more thorough methodological comparison using contralateral sides of the same specimens in large intraspecific samples will yield statistical differences in average fascicle length. We will test the following hypotheses.

H1. Following the predictions made by Hartstone-Rose et al. (2018), fascicle lengths obtained in situ are shorter than those derived from chemical dissection. As noted in the review by Martin et al. (2020), separating planes of muscles with varying pennation results in cut fascicles and shorter measurements.

H2. Contrary to Hartstone-Rose et al. (2018) and results published by Taylor et al. (2024), fascicle lengths obtained from the in situ method are longer than those derived from chemical dissection. We hypothesize that some of the length of some fascicles measured in situ is actually non-contractile tendons at either fascicular end—connective tissues that dissolve and are therefore not included in the fascicle length measurements based on the chemical dissection approach. Alternatively, it is possible that in situ measurement yields longer fascicle lengths because some "fascicles" are arranged in serial with tendinous connections in between (as shown for the semitendinosus of goats by Gans et al. (1989)) and thus these longer fascicle lengths are the functional length, while the chemically separated fascicle lengths represent only partial spans. Gans et al. (1989) predicted this to be the case because of a theoretical maximum length of muscle fibers, and thus we would expect relatively much longer in situ measurements in the longest muscles measured. Additionally, it is possible that errors in the application of the chemical dissection approach result in broken fascicles, or fascicles being shortened due to partial digestion by the acid while still appearing intact.

H3. The differences in fascicle length produced by the two methods vary based on the complexity of the muscle. Increased architectural complexity may make visualizations of whole fascicles difficult when measuring in situ. Because there is less variation in fascicular orientation of layers of relatively simple muscles, we expect the two approaches to yield more similar fascicle length measurements for relatively simple muscles when compared to those of complex muscles.

H4. The differences in fascicle length produced by the two methods vary based on the average fascicle length of the studied muscles. We hypothesize that this may be the case because smaller fascicle lengths are potentially harder to measure (especially using rulers or calipers) leading to greater variability in measurements, reducing the statistical ability to differentiate samples. Likewise, we may see significant differences between longer fascicles measured with the different methods because these tend to be less pennate muscles that are potentially more susceptible to stretch during the in situ measurement

approach—something that is not a concern for the chemical dissection approach.

H5. Fascicles measured through chemical dissection might be significantly different than those measured in situ for fixed or fresh muscles but not the other. Fascicle measurement methods have historically been applied to both fresh and fixed specimens, and we propose to evaluate the influence of preservation on the potential differential effects of the two methods.

2 | MATERIALS AND METHODS

To test these hypotheses, we use each of the two methods (described in detail below) to measure fascicle length on contralateral sides of the same specimens in two different anatomical systems (masticatory and crural) and lineages (carnivorans and rodents) of mammals.

2.1 | Sample

Each of the masticatory muscles was dissected bilaterally. These were taken from 23 wild, adult individuals (as determined by affirmation of dental maturity) of seven species of North American carnivorans (Table 1). Additionally, both hindlimbs of 40 adult (equal sex ratio) Wistar rats (*Rattus norvegicus*) were removed for dissection of the soleus and medial head of the gastrocnemius. Although Leonard et al. (2022) showed that formalin fixation does not have an effect on fascicle length, we confirmed this by structuring our rodent

TABLE 1 Sample.

Specimen ID	Species	Common name	Specific locality[a] (if known), state	Method applied on each side (left, right)
208095	*Canis lupus*	Gray wolf	DJ, AK	Chemical, in situ
208096	*Canis lupus*	Gray wolf	PW, AK	In situ, chemical
208097	*Canis lupus*	Gray wolf	PW, AK	In situ, chemical
208098	*Canis lupus*	Gray wolf	DJ, AK	Chemical, in situ
208100	*Canis lupus*	Gray wolf	ID	Chemical, in situ
208101	*Canis lupus*	Gray wolf	ID	Chemical, in situ
208102	*Canis lupus*	Gray wolf	PW, AK	In situ, chemical
208103	*Canis lupus*	Gray wolf	PW, AK	None[b], in situ
208104	*Canis lupus*	Gray wolf	PW, AK	In situ, chemical
208105	*Canis lupus*	Gray wolf	PW, AK	Chemical, in situ
208106	*Canis lupus*	Gray wolf	DJ, AK	Chemical, in situ
208107	*Canis lupus*	Gray wolf	DJ, AK	In situ, chemical
208108	*Canis lupus*	Gray wolf	DJ, AK	Chemical, in situ
208109	*Canis lupus*	Gray wolf	AK	In situ, chemical
208110	*Vulpes macrotis*	Kit fox	Wild	In situ, chemical
208111	*Vulpes macrotis*	Kit fox	Wild	In situ, chemical
208112	*Vulpes velox*	Swift fox	Wild	Chemical, in situ
208113	*Vulpes velox*	Swift fox	Wild	In situ, chemical
208114	*Canis latrans*	Coyote	DJ, AK	Chemical, in situ
208115	*Canis latrans*	Coyote	DJ, AK	Chemical, in situ
208116	*Urocyon cinereoargenteus*	Gray fox	R, NC	In situ, chemical
213102	*Lontra canadensis*	North American river otter	AK	Chemical, in situ
213103	*Martes americana*	American marten	AK	Chemical, in situ

[a]Delta Junction (DJ), AK; Prince of Wales (PW), AK; Raleigh (R), NC; wild *V. macrotis* from the Southwest of the United States; wild *V. velox* from the Midwest of the United States.

[b]The right side of this specimen was damaged prior to obtaining it, though data from its contralateral side were incorporated into the in situ fascicle length analyses.

sample as follows: half were dissected bilaterally and processed immediately, whereas hindlimbs of the other 20 rats were skinned and fixed in formalin prior to bilateral sharp dissection (see details below). No animal was killed for this research; all carnivore specimens were byproducts of the fur trade, and all rats were discarded control specimens from research not pertaining to this study. North Carolina State University does not require IACUC approval for use of specimens as cadavers.

2.2 | Carnivoran masticatory myology (including abbreviations)

The masticatory muscles of carnivorans, like those of most mammals, are characterized by multi-layered architecture and variable fascicle orientation (Hartstone-Rose et al., 2012, 2019, 2022). The spatial complexity of the fascicle orientation has only recently been documented in carnivorans in three-dimensional space (Davis et al., 2026); however, these methods follow those that have revealed fascicle orientation spatial complexity in primates (Dickinson et al., 2020, 2024). Those studies have shown that the directionality and depth of fascicle layers can vary considerably not only intraspecifically and *between* individual muscles but also *within* individual muscles. Following Hartstone-Rose et al. (2012) we separated the masticatory muscles into the following divisions: superficial, deep, and zygomatic temporalis (ST, DT, and ZT, respectively); superficial, deep, and zygomatic masseter, with the zygomatic portion being subdivided (following Brassard et al., 2020) into anterior and posterior portions (SM, DM, ZMA, and ZMP, respectively); medial and lateral pterygoids (MP and LP, respectively) and digastric (Dig), which in carnivorans is one body made up of the inseparable anterior and posterior bellies found in most mammals. For each specimen, the fascicles of the muscles of one side were measured using the in situ method, wherein muscles were sectioned along their lengths, and 3–5 representative fascicles were measured using calipers. Fascicles of the contralateral side were measured following the chemical dissection protocol.

All carnivore specimens were frozen fresh for storage and then thawed before each masticatory muscle was removed following standard sharp dissection protocol; immediately after its removal, morphometric data were collected including mass to the nearest 0.01 g, density, and length following Boettcher et al. (2019). Because all muscles could not be processed simultaneously, excised muscles from all but five carnivore specimens were fixed in 10% buffered formaldehyde (formalin) after dissection, but prior to chemical processing. Although fixing muscles after removal from bony attachments has been shown to cause moderate volumetric fascicle length shrinkage in some studies (e.g., Cutts, 1988), other studies have shown that although ethanol storage substantially affects muscle architecture muscles are not substantially influenced by freezing or formalin fixation (Leonard et al., 2022). All gross morphometrics and in situ fascicle measurements of the carnivoran masticatory muscles were taken on muscles unaltered by formalin fixation.

2.3 | Fixation

In order to confirm the effects of fixation on fascicle length (e.g., to determine the compatibility of the fascicle lengths derived from the fixed and unfixed carnivoran masticatory muscles), half of the rodent specimens were fixed prior to fascicle measurement. Following the method described in Hinks and Power (2024) for the fixation of rat hindlimbs prior to dissection, rat hindlimbs were skinned and submerged in 10% buffered formaldehyde (formalin) with their ankles and knees pinned at 90° for 2 weeks. After the fixation period, specimens were then dissected following the same procedures used for the fresh specimens.

2.4 | Rodent crural myology

To observe whether the two fascicle measurement methods vary in comparability relative to muscle architecture complexity, the medial head of gastrocnemius and the soleus (Figure 1a, left and right, respectively) were bilaterally dissected from our rodent sample. These muscles are both relatively simple compared to most of the masticatory muscles and functionally distinct hindlimb muscles in rats and were selected due to their well-defined fascicle orientation and consistent anatomical organization. This contrasts markedly with the layered and variable structure of masticatory muscles. While both muscles predominantly extend the ankle, the medial gastrocnemius is characterized by shorter fascicles arranged in a more pennate architecture at an angle to the tendon, and the soleus is comprised of longer fascicles running a longer span of the length of the muscle with a much lower degree of pennation (Figure 1b, right). These differences in architecture and function between the two muscles provide an additional opportunity to assess how dissection method affects fascicle length measurement across two simple fundamental muscle types.

2.5 | Rat leg dissection

The soleus and medial head of gastrocnemius were removed from the left and right sides of each rat

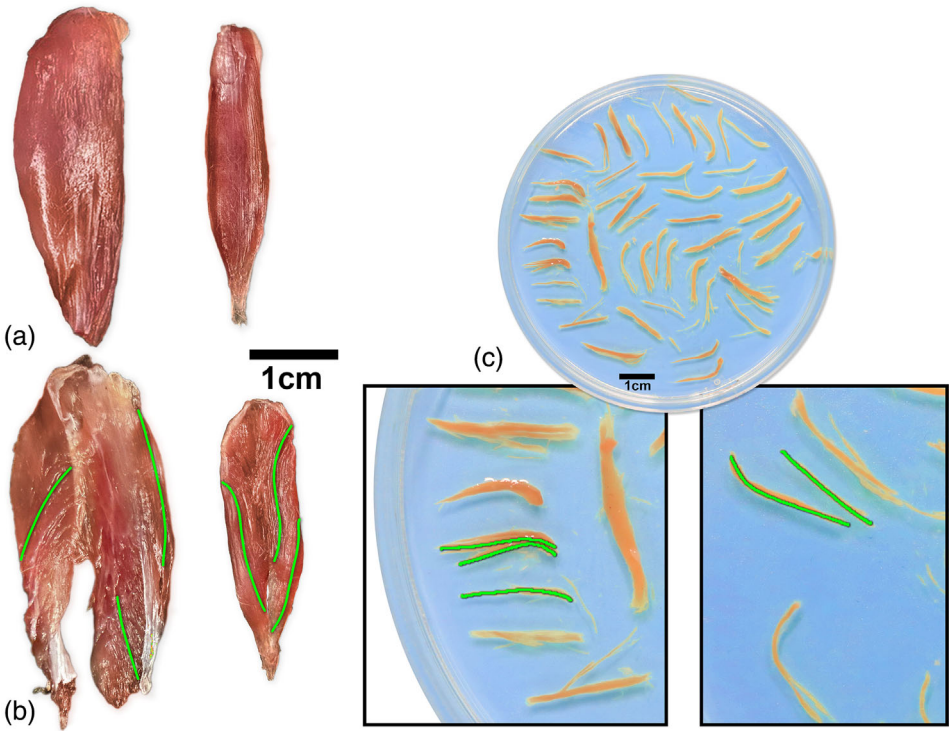

FIGURE 1 Crural muscle fascicle measurement. (a) Whole muscles. (b) Sliced and open along their long axes for in situ measurement. (c) Example petri dish (not to scale) and chemically disarticulated inset of example fascicles of each muscle. In all three, medial head of gastrocnemius is left; soleus is right. Green lines in (b) and (c) represent fascicles as they would be marked during digital measurement in ImageJ. For graphical clarity, only a few fascicles are marked, though many more unbroken fascicles (e.g., throughout the inset petri dish) would be measured for each muscle of each specimen.

following sharp dissection procedures and then morphometric data were collected including mass to the nearest 0.01 g and muscle length in mm. of each specimen. The fascicles of one randomly assigned side were measured using the in situ method, wherein the muscle was sectioned and three fascicles of various depths were sampled. Conversely, muscles of the other side were measured following chemical dissection.

2.6 | Chemical dissection

Following sharp dissection and morphometric measurement, both the rat leg and carnivoran masticatory muscles assigned to the chemical dissection protocol were placed in petri or larger glass dishes and submerged in 20%–30% nitric acid, as described by Martens et al. (2024), to dissolve connective tissue and allow for manual separation of fascicles. Although sulfuric acid has been used successfully in the past, it generally requires heat (e.g., to 70°C) to be effective and while nitric acid works well at room temperature. Using an acid that does not require an oven or incubator (which are at risk of corrosion) allows for more economical fume hood space use. Having extensive experience with both acids, it appears that they produce equivalently disarticulated fascicles. Time spent processing in acid varies for muscles based on acid choice and concentration, size and fixation with heated sulfuric being generally faster than room temperature nitric at the concentrations used. Smaller, unfixed muscles using 20%–30% nitric acid took between 2 and 5 days, whereas larger, formalin fixed muscles in the current study took 1–2 weeks to fully process. With regular monitoring and topping off of fluids when necessary, there does not seem to be any danger of drying. Once enough connective tissue had been dissolved so that fascicle bundles could be teased apart with minimal force, the nitric acid was removed and replaced with 50% glycerol. The fascicles of each individual muscle were then gently teased apart, and a subsample of ~40 intact fascicles (fewer in smaller muscles that contain fewer fascicles overall) from throughout the muscle were distributed evenly across the dish (Figure 1c). Each dish was then photographed with identifying information and a scale bar. Fascicles were then measured using ImageJ (Version 1.54). In this software, a straight line is stretched across the scale bar to "set the scale" and then the ratio of this known measurement to the pixel size is automatically used for all subsequent measurements of fascicles drawn along their curve (Figure 1, green lines). This scale is reset for each photograph.

Measurements were only taken of fascicles that could be fully visualized and deemed entirely intact. Broken fascicles often exhibit frayed ends and feathering of muscle fibers, so fascicles with these characteristics are easily excluded from measurement or removed from the dishes prior to photographing (Figure 2). Additionally, fascicles were only measured if the entire length was unobstructed. Overlapping or poorly visualized fascicles were not measured.

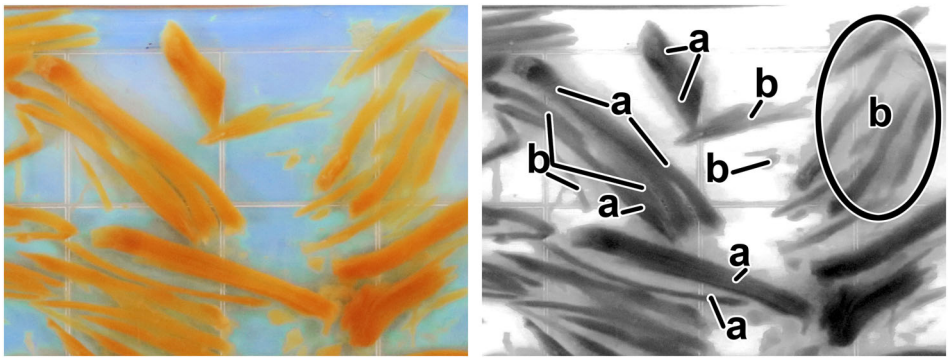

FIGURE 2 An example of a chemically dissected muscle used for measuring. Fascicles were only measured if ends were not obscured or broken (labeled 'a'), whereas fascicles whose ends were potentially damaged or middle sections were potentially stretched and broken were omitted (labeled 'b'). Version on right is contrast enhanced for explanative clarity, though well-lit, unmodified images (an excerpt of which is on the left) are clear enough for consistent measurement by practiced observers.

Prior to data collection, observers are trained on collections of photographs of previously established muscle fascicle lengths to ensure observer reliability. As in many studies using this method (e.g., Martens et al., 2024) measurements were taken on approximately 20–40 representative fascicles per rat leg muscle and 30–60 fascicles per carnivoran masticatory muscle, depending on the overall size of the muscle, to produce a single average fascicle length. Smaller muscles such as LP had fewer fascicles compared to others such as divisions of masseter and temporalis. Masticatory muscles were all measured by a single author, ESB. This was repeated by 1–2 additional experienced individuals on the same set of fascicles from the same dish photographs to ensure repeatability. Inter-rater reliability tests were conducted, and all achieved greater than 95% concordance. Rat leg fascicles were all measured by authors RAB and KMC. The average fascicle lengths for each muscle measured by each observer were averaged to produce a single value for each respective muscle.

2.7 | In situ sectioning

In situ sectioning measurements were taken following the procedures described by Taylor et al. (2015). While the majority of us have extensively and nearly exclusively used the chemical dissection approach (Martens et al., 2023, 2024), coauthor Herrel has used both procedures in many studies (e.g., in Ginot et al., 2018; Brassard et al., 2021, 2022, 2025; Herrel et al., 2008, 2024, 2025). Therefore, he collected all the in situ measurements for the more complex masticatory muscles. One of us (RAB) collected the in situ measurements for the simpler leg muscles. To do this, first, muscles were removed from their osteological origins and insertions. Next, an incision was made along the belly of the muscle following the orientation of the superficial-most fibers. Measurements were then taken of the exposed fibers using digital calipers, perpendicular to this cut, from the superficial muscle surface to their deep attachment (Figure 1b).

2.8 | Statistical analysis

All statistical analyses were conducted using a pooled *t*-test for each muscle to determine the comparability of methodological approach and impacts of fixation. For the carnivoran masticatory muscle dataset, weighted average fascicle length was calculated by taking the sum of each muscle's average fascicle length value multiplied by its mass obtained from both in situ measurements and chemical dissections and then dividing each sum by the total mass of each muscle set, allowing comparisons across a single representative masticatory muscle value (Figure 3). All analyses were performed using JMP Student Edition 19, with an a priori significance level of 0.05.

3 | RESULTS

There were no significant differences between the sides of any individuals for any of the muscles. The lowest *p*-value among the wolf masticatory adductors is $p > 0.68$, comparing the left and right ZMP masses. The left and right muscles for the all-carnivoran sample have even higher correlations, and the rodent gastrocnemius and soleus masses have values of $p > 0.92$ and $p > 0.84$, respectively. The differences between muscle architectural variables do not reflect side biases in the sample but, rather, relate to fascicle measurement method.

Comparing the two measurement methods—via pooled *t*-tests, the weighted average masticatory muscle

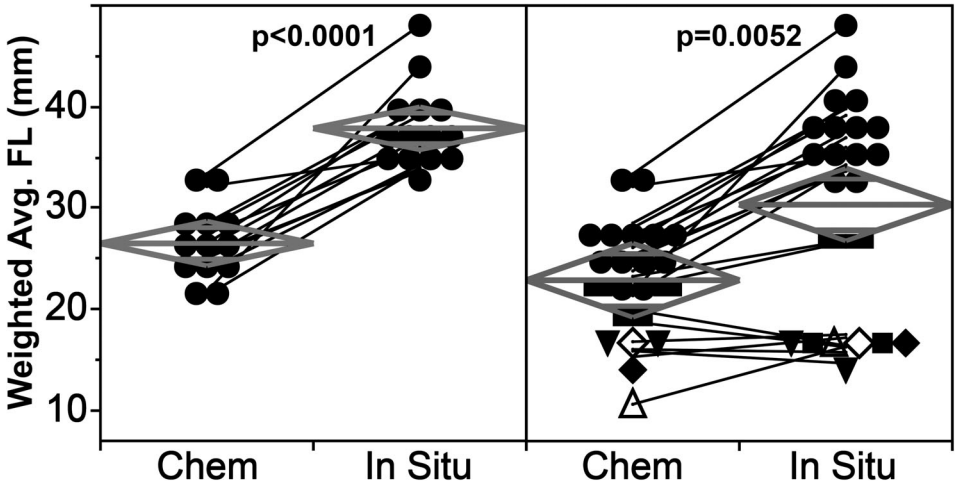

FIGURE 3 Comparison of weighted average masticatory muscle fascicle lengths in *Canis lupus* (circles; left) and across full carnivoran sample (right) measured using chemical dissection versus in situ measurement. Lines connect contralateral sides of the same specimen. Gray diamonds show means and 95% confidence intervals. Rectangles = *C. latrans*, squares = *Vulpes macrotis*, filled-in triangle = *V. velox*, open triangle = *Martes americana*, filled-in diamond = *Lontra canadensis*, open diamond = *Urocyon cinereoargenteus*.

fascicle length across the complete carnivoran sample ($n = 22$) is significantly different ($p = 0.005$), with the in situ measurements being significantly greater than those measured using the chemical dissection method (Figure 3, right). The methodological disparity is even more pronounced when restricting the analysis to the largest conspecific cohort in our carnivore sample, with the weighted average masticatory muscle fascicle lengths of the *Canis lupus* specimens being even more significantly longer when measured in situ relative to those measured after chemical dissection (p-value of <0.0001; Figure 4, left).

This trend is also observed when comparing the values of individual muscles. Of the 10 muscles sampled for the *C. lupus* sample, for the six muscles with the longest fascicles (ST, DT, ZT, SM, ZMA, and Dig), fascicle lengths are significantly longer when measured in situ (Figures 4 and 5 and Table 2). Although the variance is larger in the full carnivoran sample, nearly the same number of muscles show significant differences depending on the fascicle measurement method applied (Figures 4 and 5 and Table 2). In these significantly distinguishable muscles, the percent difference between the two methods varied from 17.7% to 59.0%, and in all cases, the significantly larger average fascicle lengths were measured using the in situ approach. Additionally, there was no statistical difference between the weighted average fascicle length of the carnivore specimens that were fixed in formalin prior to chemical dissection. When controlling for muscle mass, in situ dissection continued to produce consistently longer fascicle lengths for most muscles across the sample (ST: $p = 0.0028$, DT: $p = 0.0039$, ZT: $p < 0.0001$, SM: $p = 0.0370$, ZMA: $p = <0.001$, Dig: $p < 0.0001$). Notably, some muscles (DM, ZMP, MP, and LP) did not significantly differ in our analyses though, on average, fascicle lengths assessed through chemical analyses tended to be slightly longer in these muscles, with statistically insignificant percent differences ranging between 1.57% and 16.50%.

When assessing the *R. norvegicus* medial gastrocnemius and soleus, fascicle lengths derived from the two methods are also significantly different (Figure 6 and Table 3). However, while all of the masticatory muscle fascicles that significantly differ based on measurement methods were significantly longer when measured using the in situ method, this is not consistent between the crural muscles; that is, although the *R. norvegicus* soleus fascicle lengths are significantly greater when measured in situ (as was the case for the masticatory muscles), the in situ measurement method yielded highly significantly *smaller* fascicle measurements of gastrocnemius. This trend was true in both the formalin fixed and unfixed samples. However, the inverse of this trend was found in the medial gastrocnemius, where chemically derived fascicle lengths were significantly longer than their in situ counterparts. Importantly, fixation had no effect on fascicle lengths for either muscle (fixed versus fresh soleus: $p = 0.13$; fixed versus fresh gastrocnemius: $p = 0.49$; Table 4).

4 | DISCUSSION

Our findings demonstrate that, across our whole carnivoran sample, masticatory muscle fascicle lengths measured via in situ dissection were significantly greater than those obtained through chemical dissection, both in the

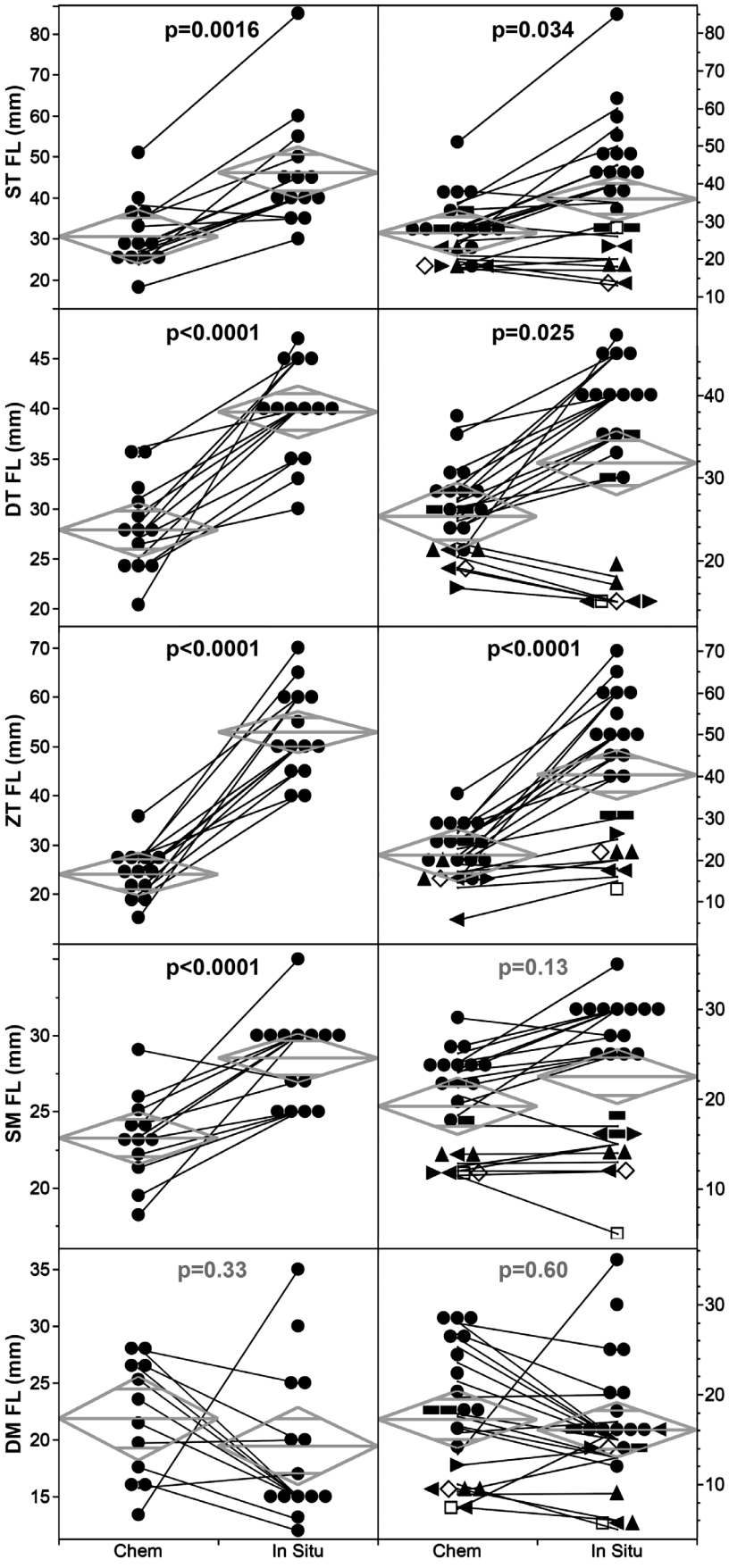

FIGURE 4 Comparison of average individual masticatory muscle fascicle lengths in *Canis lupus* (circles; left) and across full carnivoran sample (right) measured using chemical dissection versus in situ measurement. Lines connect contralateral sides of the same specimen. Points lacking a connecting line represent specimens for which one of the contralateral muscles was damaged and therefore could not be measured. Gray diamonds show means and 95% confidence intervals. Rectangles = *C. latrans*, squares = *Vulpes macrotis*, filled-in triangle = *V. velox*, open triangle = *Martes americana*, filled-in diamond = *Lontra canadensis*, open diamond = *Urocyon cinereoargenteus*.

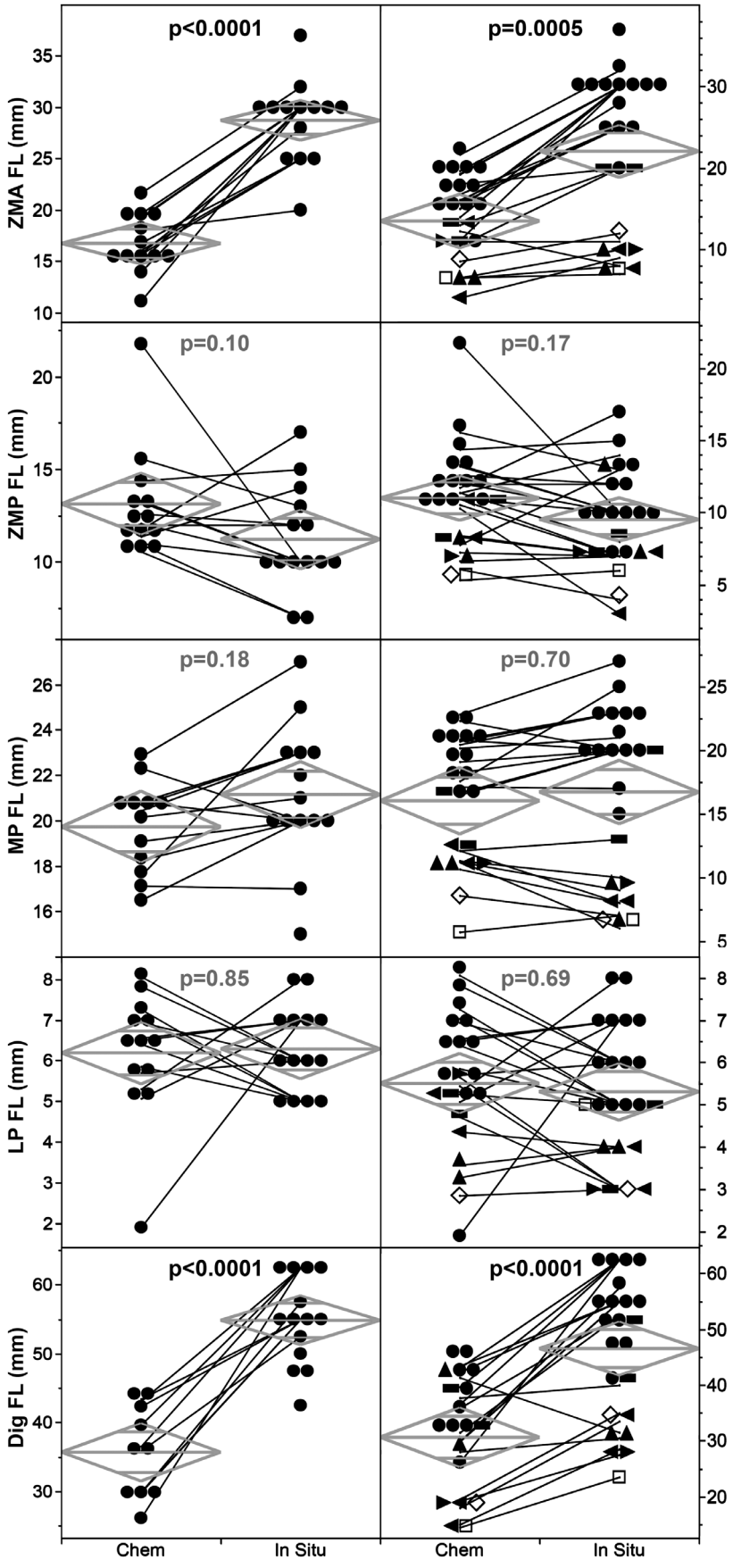

FIGURE 5 Comparison of average individual masticatory muscle fascicle lengths in *Canis lupus* (circles; left) and across full carnivoran sample (right) measured using chemical dissection versus in situ measurement. Lines connect contralateral sides of the same specimen. Points lacking a connecting line represent specimens for which one of the contralateral muscles was damaged and therefore could not be measured. Gray diamonds show means and 95% confidence intervals. Rectangles = *C. latrans*, squares = *Vulpes macrotis*, filled-in triangle = *V. velox*, open triangle = *Martes americana*, filled-in diamond = *Lontra canadensis*, open diamond = *Urocyon cinereoargenteus*.

TABLE 2 Carnivoran masticatory muscle average fascicle lengths (means and standard deviations in mm) for the full carnivoran and wolf samples with *p*-values comparing the two fascicle length measurement methods across the respective samples.

	Wolves only (mm)				All carnivorans (mm)			
Muscle	Chemical, mean (SD)	In situ, mean (SD)	*p*-Value	Difference (in situ–chemical)	Chemical, mean (SD)	In situ, mean (SD)	*p*-Value	Difference (in situ–chemical)
ST	30.47 (3.17)	46.07 (3.05)	0.0016[a]	15.60	26.84 (2.97)	35.87 (2.84)	0.034[a]	9.03
DT	27.84 (1.31)	39.64 (1.26)	<0.0001[a]	11.80	25.28 (2.00)	31.74 (1.91)	0.025[a]	6.46
ZT	24.03 (2.09)	52.86 (2.02)	<0.0001[a]	28.83	21.10 (3.10)	40.30 (2.89)	<0.0001[a]	19.21
SM	23.24 (0.84)	28.50 (0.77)	<0.0001[a]	5.25	19.17 (1.55)	22.48 (1.49)	0.13	3.31
DM	21.85 (1.78)	19.43 (1.65)	0.33	−2.423	17.19 (1.58)	16.04 (1.51)	0.60	−1.15
ZMA	16.73 (0.97)	28.71 (0.93)	<0.0001[a]	11.99	13.47 (10.19)	22.04 (1.59)	0.0005[a]	8.57
ZMP	13.12 (0.80)	11.21 (0.77)	0.10	−1.91	11.00 (0.76)	9.52 (0.74)	0.17	−1.48
MP	19.72 (0.76)	21.14 (0.70)	0.18	1.42	16.00 (1.30)	16.70 (1.24)	0.70	0.69
LP	6.19 (0.37)	6.29 (0.36)	0.85	0.10	5.50 (0.35)	5.30 (0.34)	0.69	−0.20
Dig	35.68 (2.02)	54.82 (1.71)	<0.0001[a]	19.14	30.60 (2.64)	46.52 (2.40)	<0.0001[a]	15.92

Note: We derived a composite mean by averaging the group-level means across individuals, and the SD reflects the variability among these grand means.
[a]Statistically significant differences.

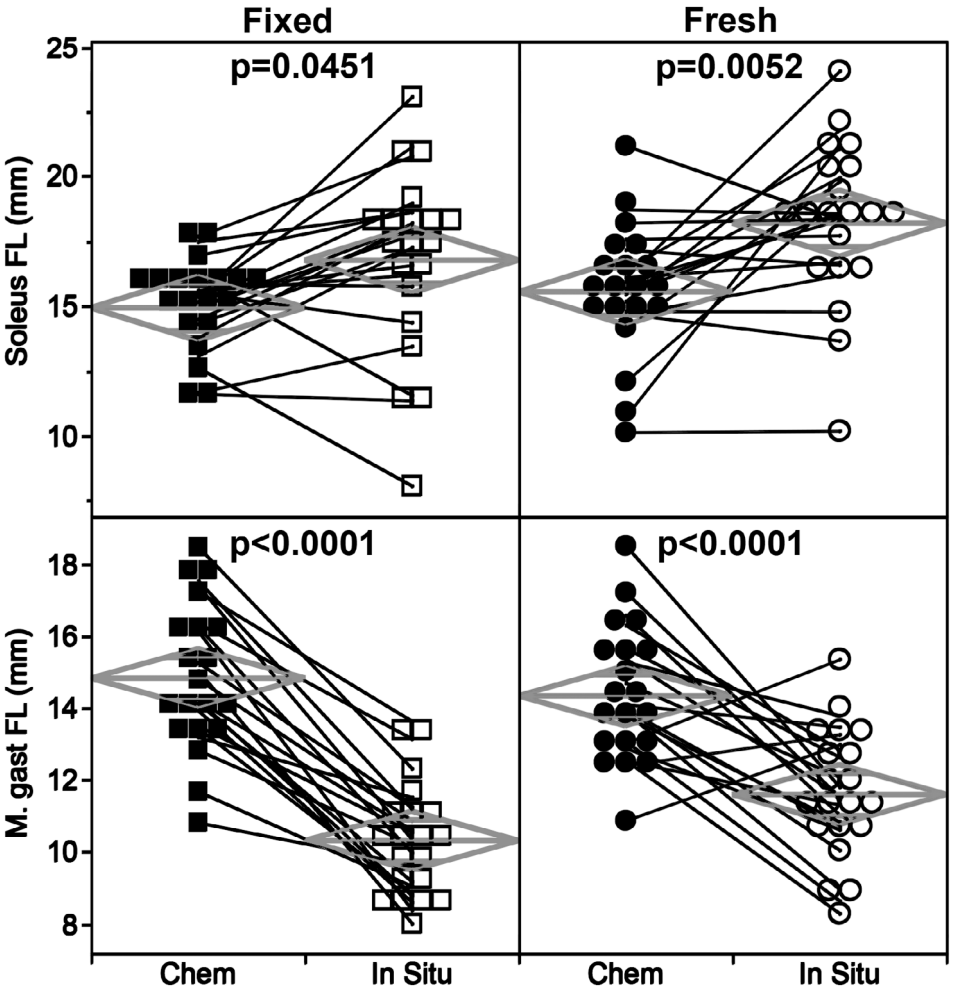

FIGURE 6 Comparison of fixed (square) and fresh (circles) Rattus norvegicus soleus and medical gastrocnemius fascicle lengths measured using the two methods. Gray diamonds show means and 95% confidence intervals. Filled in square/circle = chemically dissected, open square/circle = measured in-situ. Direct comparison of the muscles based on their fixation (e.g., comparing fixed and fresh soleus fascicles measured in situ) yields no significant differences for any of the four muscles by method comparisons.

TABLE 3 Rat crural muscle average fascicle lengths (in mm, including 95% confidence intervals) of each muscle, fixed and fresh, with *p*-values comparing the two fascicle length measurement methods.

	Mean			Chem		In situ	
	Chem (mm)	In situ (mm)	*p*-Value	Lower 95%	Upper 95%	Lower 95%	Upper 95%
Fresh soleus	15.54	18.21	0.0052	14.26	16.83	16.92	19.5
Fresh medial gastrocnemius	14.34	11.61	<0.0001	13.51	15.18	10.77	12.44
Fixed soleus	14.93	16.77	0.0451	13.67	16.2	15.5	18.03
Fixed medial gastrocnemius	14.83	10.32	<0.0001	14.01	15.65	9.5	11.14

TABLE 4 Average fascicle lengths (FL) of each *R. norvegicus* crural muscle and its corresponding difference, standard error difference, confidence interval, and *p*-value.

	Mean FL, fresh (mm)	Mean FL, fixed (mm)	Difference (fresh–fixed)	Standard error difference	Lower CL	Upper CL	*p*-Value
Soleus	16.88	15.85	1.03	0.64	−0.31	2.37	0.13
Medial gastrocnemius	12.98	12.58	0.40	0.58	−0.76	1.56	0.50

weighted average of all of the masticatory muscles and in each of the five individual muscles that have the longest fascicles (ST, DT, ZT, ZMA and Dig). This trend was even more pronounced in the focal intraspecific *Canis lupus* sample, where in situ measurements exceeded chemical measurements by substantial margins in these muscles as well as in superficial masseter, even after controlling for muscle mass. These results support our hypothesis that methodological differences in fascicle measurement influence outcomes and the extent to which the methods influence fascicle length varies based on muscle size. When focusing on the masticatory muscles from the *C. lupus* sample, a trend emerges between the average fascicle length of a muscle and whether the two methods yield significantly different measurements: The muscles with the longest fascicle are significantly different, whereas those with the shortest fascicles are statistically indistinguishable. Specifically, the threshold seems to be that, for fascicle lengths above ~21 mm, in situ measurement yields significantly longer measurements for these masticatory muscles. It is possible that this relates to the phenomenon described by Gans et al. (1989) who noted that one seemingly continuous muscle (the semitendinosus of the goat) actually separates into proximal and distal portions when the muscles are chemically broken down. Although some muscles appear to be single structures, some are combinations of embryologically distinct muscles that combine during development with a subtle, but notable raphe. For instance, the felid "clavobrachialis" has distinct trapezius and deltoid components, the only muscle in our sample that has this clear configuration is the carnivoran "digastric" muscle. Like the felid clavobrachialis, its two components (anterior and posterior) can be distinguished and measured separately in in situ measurement (as they were in our sample). Their relative proportions theoretically contribute appropriately to the weighted average single digastric value derived from the chemical approach in which these portions are generally left combined during sharp dissection to avoid fascicular damage. Thus, the highly significant difference between the in situ and chemical fascicle lengths of this combined muscle cannot be reasonably explained in this way. It is theoretically possible that some of the particularly long fascicle measurements are the result of measuring serially arranged fascicles; however, no previous author that we know of has suggested that what appear to be continuous fascicles in these muscles are actually multiple fascicles in series. Furthermore, when examining these muscles in specimens that have had their fascicles visualized digitally (e.g., Dickinson et al., 2024), the fascicles appear to be arranged relative to each other in layers (e.g., like the scales on a fish), attaching to tendons or aponeuroses rather than to each other serially. However, it is possible that some muscles do have this heretofore unrecognized condition and muscles for which the fascicle lengths are substantially different when measured using these two methods (e.g., the fascicles of zygomatic temporalis are essentially twice as long when measured in situ) should be studied further to evaluate whether they are configured in such a way to support the hypothesis of Gans et al. (1989).

With that said, we posit that the difference in fascicle lengths is better ascribed to differences in pennation. We hypothesize that muscles with relatively short, highly pennate fascicles possess dense connective tissue that

maintains structural integrity when the muscle is exposed during in situ sectioning, resulting in more consistent measurements between in situ and chemical approaches. In contrast, muscles with longer, less pennate fascicles have less internal connective support and appear more prone to stretching during in situ measurement. After chemical dissection, fascicles become more fragile, and any amount of force that would stretch the fibers actually causes them to break, which, in turn, causes them to be excluded from measurement. Therefore, we do not think that fascicles are stretched in ways that lead to overmeasurement by researchers during the chemical dissection process, though it is possible that the chemicals themselves cause relaxation or constriction at the cellular or protein level. We do not believe this to be the case and are not aware of any evidence that this occurs.

Another source of variation in this sample relates to the number of fascicles sampled. As discussed in Charles et al. (2022), although sampling less than 25 fibers is common in the literature, doing so may lead to mischaracterizations of muscle architecture properties.

To extend our analysis beyond the masticatory muscles to an even broader and more controlled sample, we examined the effects of fascicle length measurement method on the gastrocnemius and soleus hindlimb muscles of *Rattus norvegicus*. Although measurement method yielded significantly different results in *both* muscles, interestingly, fascicle lengths measured using the two methods followed opposing trends between the two muscles. As in the masticatory muscles, the fascicle lengths of the soleus were significantly longer when measured in situ than when chemically dissected. However, in the medial head of gastrocnemius, fascicle lengths were significantly longer when measured via chemical dissection.

Although both leg muscles had an average fascicle length under 21 mm (the approximate significance threshold found in our carnivoran masticatory sample), the finding that the soleus fascicles are longer when measured in situ seems to support our hypothesis that architectural complexity is driving the differences in fascicle length. Like the less complex masticatory muscles (especially the carnivoran digastric), the fascicles of the soleus are much more homogenous in their organization and the muscle, overall, has substantially less origin-to-insertion complexity and intramuscular connective tissue (Figure 1b). Why the fascicles of the medial head of gastrocnemius would be significantly longer when measured after chemical digestion is harder to determine. It is possible that the greater amount of connective tissue obscures some of the length of fascicles when measured in situ and dissolving that connective tissue in the acid phase of the chemical dissection process allows the fascicles to be measured more fully. Interestingly, although none of the fascicles measured via the chemical approach were significantly longer for any of the carnivoran masticatory muscles overall, on average the fascicles of the deep masseter and posterior portion of the even deeper zygomaticomandibularis were indeed slightly, though non-significantly, longer when measured after chemical digestion. Future analyses of these muscles should endeavor to assess whether the amount of internal connective tissue and architectural complexity influences the ease with which in situ fascicle measurements can be taken.

Although in situ and chemical approaches to measurement yielded significantly different fascicle lengths for the majority of both the masticatory and crural muscles examined, fixation did not play a significant role: samples of fresh and formalin-fixed specimens did not differ significantly in fascicle length when measured using the same method. The consistency of directional differences in both the fresh and fixed datasets strongly supports the conclusion that the observed variation is not an artifact of preservation state but rather reflects true methodological effects. The likelihood of this pattern arising by chance in two independent groups is statistically improbable.

4.1 | Implications

The observed inconsistencies in fascial length measured using different methods are functionally important, as fascicle length is a key variable in understanding muscle architectural adaptation and contraction (Gans, 1982; Huxley, 1957; Rayne & Crawford, 1972). Given that carnivorans rely on rapid, forceful jaw movements for prey capture and processing, overestimating fascicle lengths via in situ dissection or underestimating them via chemical dissection could lead to erroneous conclusions about muscle function and ecological adaptations. This has implications for papers that interpret feeding behaviors, such as rapid jaw movements, as well as those whose data set is compiled from researchers using different measurement techniques. This methodological insight calls for a re-evaluation of prior comparative studies—especially studies that come to conflicting conclusions relying on different methodological approaches, such as Hartstone-Rose et al. (2018) and Taylor et al. (2018).

4.2 | Limitations and future directions

While this study provides important insights into methodological discrepancies in fascicle length measurement, several limitations should be acknowledged. For

instance, while our carnivoran sample was relatively taxonomically diverse, it was limited in size for most species, potentially restricting our ability to detect subtle trends. This was particularly true for the smaller taxa in the sample that seemed to have less consistent results than the larger intraspecific sample of wolves (Figures 4 and 5, right). Additionally, while we controlled for muscle mass in our analyses, other factors such as individual variation in muscle architecture or post-mortem tissue changes could contribute to measurement variability. It would be valuable for future studies to incorporate a broader range of taxa with varying functional demands to determine whether our findings generalize across other taxonomic groups and feeding ecologies. Furthermore, expanding this research question to other functional groups of muscles would provide a more comprehensive understanding of the muscle structure–function relationship.

Most importantly, this research conclusively documents that, contrary to the assertions of recent studies, fascicles measured via in situ and chemical approaches are indeed significantly different for the majority of muscles. However, because this study compared only two methods, it is not possible to determine which is inherently more accurate. To make this assessment, future studies should compare measurements of fascicles derived from other approaches. For instance, fascicles are being measured via iodine-based contrast-enhanced computed tomography (e.g., Dickinson et al., 2024). As methodological approaches advance, micro-MRI may become a better alternative to iodine-based approaches as it does not induce volumetric shrinkage as is common in some forms of unbuffered iodine preparation. These are *truly* in situ in that they are being measured in muscles that are still fixed to their osteological attachments and maintain their three-dimensional relationships to each other. As long as care is taken to avoid myological shrinkage (Dawood et al., 2021), then this might be a contender for an additional comparative test of fascicle lengths. Likewise, as magnetic resonance imaging increases in resolution (Oudeman et al., 2016), it is possible that truly in situ muscle fascicles (potentially in living organisms) can be measured, yielding an even more accurate benchmark upon which to calibrate these cadaver-based approaches. Importantly, because of intraspecific variation, any comparison should use contralateral sides of individual specimens to most convincingly demonstrate methodological compatibility.

5 | CONCLUSIONS

Our results demonstrate that fascicle length measurements and thus PCSA calculations are sensitive to measurement methods, with in situ techniques yielding significantly longer values than chemical dissection in larger muscles. We propose that this discrepancy arises from overestimation due to differences in muscle architecture, with fascicles in less complex muscles being prone to stretching during in situ measurement and fascicle ends being more difficult to distinguish with the naked eye during in situ dissection in more complex muscles with greater amounts of connective tissue. Muscles that did not differ between methods were typically smaller and contained a higher degree of pennation, suggesting that architectural complexity may mitigate and potentially even reverse these methodological effects. These findings underscore the need to acknowledge the influence of artifacts introduced during different methodological approaches in muscle architecture studies, especially when combining datasets, and highlight the importance of considering methodological biases when interpreting functional morphology. In general, m. fascicle lengths measured using the in situ approach are significantly longer than those measured using chemical dissection when measuring the contralateral side of the same individual. Our numbers indicate that when combining samples that used both methods, in situ values may be greater by a factor of 1.33, or inversely, chemical values may be smaller by a factor of 0.75. This is driven by relatively simple muscle divisions (e.g., the superficial masseter), longer muscle fascicle lengths, and less pennation, which allows the potential of too easily stretching the fascicles beyond their resting length during their in situ measurement and thus resulting in longer measurements. However, in those same large animals, highly complex mm. that tend to have relatively small fascicle lengths (under ~22 mm in these carnivoran masticatory mm.) are not significantly different when measured using these different methods and therefore do not require correction. This is potentially due to their higher connective tissue content, making it less likely to stretch their fascicles when measured in situ. Relatively simple mm. from small animals (e.g., rat leg mm with fascicles running all the way from origin to insertion) are also significantly longer when measured using the in situ approach by a factor of 1.15 (inversely, chemical data are shorter by a factor of 0.87). However, fascicles of more complex bipennate mm. measured using the in situ approach are significantly *shorter* by a factor of 0.75 (inversely, chemical data are greater by a factor of 1.33). This inverse pattern is likely due to the difficulty in measuring fascicle lengths in muscles that have high connective tissue content—making it difficult to both see the full length of fascicles with complex attachments that may dive below the plane in which the muscle is split and to measure fascicles that are substantially curved using calipers, reticles, or rulers as is common in the in situ measurement approach (Figure 1).

Further analysis should be conducted to expand the comparison of fascicle lengths to those evaluated through other methods including imaging techniques like DiceCT and micro-MRI, which allow for measurement of fascicles within completely intact specimens, likely providing a greater degree of accuracy.

AUTHOR CONTRIBUTIONS

Adam Hartstone-Rose: Conceptualization; methodology; investigation; formal analysis; supervision; funding acquisition; visualization; project administration; resources; writing – original draft; writing – review and editing; data curation. **Reece A. Brown:** Conceptualization; investigation; writing – original draft; methodology; visualization; writing – review and editing; formal analysis; project administration; data curation; supervision. **Maggie Funderburk:** Investigation; writing – review and editing; writing – original draft; formal analysis; data curation. **Anthony Herrel:** Methodology; investigation; writing – review and editing; writing – original draft; conceptualization; supervision. **Katherine M. Clark:** Conceptualization; investigation; writing – original draft; methodology; visualization; writing – review and editing; formal analysis; project administration; data curation; supervision. **Elise Bush:** Investigation; formal analysis; writing – original draft; writing – review and editing; data curation.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study will be publicly available once the paper is accepted.

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