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Sarcopenia of the longitudinal tongue muscles in rats

Gary C. Sieck,

Genesis A. Hernandez-Vizcarrondo,

Alyssa D. Brown,

Matthew J. Fogarty*

Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, MN 55905, USA

Abstract

The tongue is a muscular hydrostat, with lingual movements occurring during breathing, chewing, swallowing, vocalization, vomiting, coughing and grooming/sexual activities. In the elderly, reduced lingual dysfunction and weakness contribute to increased risks of obstructive sleep apnea and aspiration pneumonia. In Fischer 344 (F344) rats, a validated model of aging, hypoglossal motor neuron death is apparent, although there is no information regarding tongue strength. The intrinsic tongue muscles, the superior and inferior longitudinal, transversalis and verticalis exist in an interdigitated state. Recently, we established a method to measure the specific force of individual intrinsic tongue muscle, accounting for the tissue bulk that is not in the direction of uniaxial force. In the longitudinal muscles of 6- (n = 10), 18- (n = 9) and 24-month-old (n = 12) female and male F344 rats, we assessed specific force, fatigability, fiber type dependent cross-sectional area (CSA) and overall CSA. Muscle force and fatigue was assessed *ex vivo* using platinum plate simulation electrodes. Tongue muscles were frozen in melting isopentane, and transverse sections cut at 10 μm. Muscle fiber type was classified based on immunoreactivity to myosin heavy chain (MyHC) isoform antibodies. In H&E stained muscle, CSA and uniaxial muscle contributions to total tongue bulk was assessed. We observed a robust ~30% loss of longitudinal specific force, with reductions in overall longitudinal muscle fiber CSA and specific atrophy of type IIx/IIb fibers. It will be important to investigate the mechanistic underpinnings of hypoglossal motor neuron death and tongue muscle weakness to eventually provide therapies for age-associated lingual dysfunctions.

Keywords

Muscle specific force; Muscle fiber type; Aging

*Correspondence to: Department of Physiology & Biomedical Engineering, Mayo Clinic, 200 1st St SW, Rochester, MN 55905, USA. fogarty.matthew@mayo.edu (M.J. Fogarty).

Author contributions

Conception and design of work (MJF). Acquisition of data (MJF, GAH-V and ADB). Analysis and interpretation of data (MJF and GCS). Drafted manuscript and figures (MJF). Revision of manuscript and figures (GCS, GAH-V and ADB). All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Conflict of Interest

The authors have no conflict, real or perceived, to declare.

1. Introduction

Among co-morbidities of aging, the incidence of airway infections such as pneumonia is 3-fold higher in older individuals, accounting for > 60% of healthcare expenditures in the USA (Chong and Street, 2008). Such respiratory infections are significant contributors to decreased quality of life and are a common cause of death among the elderly, who are at greater risk of hospitalization for respiratory failure and frequently require prolonged mechanical ventilation (Enright et al., 1994; Polkey et al., 1997; Tolep et al., 1995). Age is also a major risk factor for obstructive sleep apnea (OSA) (Gaspar et al., 2017), with OSA implicated in increasing the morbidity and mortality of airway infections (Andrade et al., 2018; Gale et al., 2019; Langmore et al., 1998), exacerbating the effects of sarcopenia. In sarcopenia and OSA, the increased risks of respiratory infections and aspiration pneumonias (Chong and Street, 2008) are likely due to impaired airway clearance behaviors (e.g. cough/sneeze) (Enright et al., 1994; Khurram et al., 2018; Polkey et al., 1997; Tolep et al., 1995) resulting from respiratory muscle weakness (Crow and Ship, 1996; Enright et al., 1994; Polkey et al., 1997; Tolep et al., 1995; Youmans et al., 2009) and/or dis-coordination of respiratory muscles (including the tongue muscles) (Langmore et al., 1998).

In both female and male Fischer 344 (F344), rats, validated for aging research, we have extensively characterized ventilatory and non-ventilatory (maximum transdiaphragmatic pressure, Pdi_{max}) behaviors via bilateral phrenic nerve stimulation, with ~30% reductions in old compared to young rats (Khurram et al., 2018) and ~25% reduction in diaphragm muscle (DIAm) maximum specific force (Po, normalized for muscle cross sectional area - CSA) (Fogarty et al., 2018a; Fogarty et al., 2020b; Khurram et al., 2018). Remarkably, no age-associated dysfunction was observed in ventilatory Pdi, consistent with conserved DIAm endurance and the resilience of type I and IIa fibers to atrophy (Fogarty et al., 2019b; Khurram et al., 2018). These results are entirely consistent with the loss of larger phrenic motor neurons and the degeneration of larger phrenic motor axons (Fogarty et al., 2018b; Fogarty and Sieck, 2023). Similar losses of hypoglossal motor neurons are also reported in old F344 rats (Fogarty, 2023). Although sarcopenia is not fully described in the tongue, there are reports in old F344/Brown-Norway (F344/BN) hybrid rats of reduced protrusion force (Nagai et al., 2008) and fiber atrophy (Kletzien et al., 2018). However, specific details on individual intrinsic and extrinsic tongue muscles are lacking and only males were studied.

The primary goal of this study was to measure the mechanical, fatigue and fiber type-specific properties of the intrinsic longitudinal (superior and inferior) tongue muscles in 6-, 18- and 24-month-old F344 rats. In addition to their role in the muscular hydrostat functions of the tongue, the intrinsic superior and inferior longitudinal muscles are involved in the propulsion of the food bolus during swallowing and ventilatory response to occlusion (Bailey and Fregosi, 2004; Gilbert et al., 2007; Sanders and Mu, 2013). In order to disambiguate the contribution of the superior and inferior longitudinal muscles to tongue forces from the rest of the heavily interdigitated intrinsic tongue muscles (i.e., the transversalis and verticalis muscles) (Bailey et al., 2006; Fogarty and Sieck, 2021c; McClung and Goldberg, 2000; Sanders and Mu, 2013), we recently established a manner to evaluate uniaxial forces and fatigability in individual tongue muscles (Fogarty and Sieck, 2021c) (Fig. 1A and B). We hypothesized that by 24-months of age, specific force, the

relative contribution of type IIx/IIb fibers to longitudinal muscle bulk and the CSA of type IIx/IIb fibers will be reduced compared to 6- and 18-month-old F344 rats.

2. Methods

2.1. Ethical approval

All animal use and protocols were approved by the internal Mayo Clinic Institutional Animal Care and Use Committee (IACUC, approval #00003068–17) and complied with State and Federal laws and NIH guidelines. All animal experiments herein complied with the ethical principles outlines by the Journal.

2.2. Experimental animals and anesthesia

We used 31 pathogen-free 6-months (5 females and 5 males), 18-months (4 females and 5 males) and 24-months (5 females and 7 males) old F344 rats, obtained from Charles River Laboratories and the NIA. Rats were maintained on an alternating 12:12 h light-dark cycle with ad libitum access to fresh water and chow. An acclimation period of at least 48 h was provided before conducting any experiments. Animals were deeply anaesthetized (indicated by absence of both deep pain and palpebral reflexes) with intramuscular ketamine (80 mg/kg) and xylazine (10 mg/kg) and euthanized by exsanguination.

2.3. Muscle isometric force and fatigue assessment

For assessment of the superior and inferior longitudinal muscles, the entire intrinsic tongue was isolated and excised, placed in a sylgard-bottomed chamber of Rees-Simpson's solution (pH 7.4) at 26 °C and bubbled with carbogen gas (95% O_2/5% CO_2), in a manner identical to our past report (Fogarty and Sieck, 2021c) (Fig. 1B). A suture (5–0 silk, Ethicon) was placed in the midline of the tongue, ~0.5 mm from the tip, in a manner identical to previous studies (Fogarty and Sieck, 2021c; Fuller et al., 1998; Gilliam and Goldberg, 1995). The base of the tongue was securely clamped and pinned to the chamber bottom and the suture tied to a force transducer (Aurora Scientific, 6350, Cambridge Technology, MA). The output of the force transducer data was digitized (1 kHz sampling rate) and recorded in LabChart software (ADInstuments, Dunedin, New Zealand). Electrical field stimulation was achieved via platinum plate electrodes placed on either side of the muscle, with stimulation current provided by a 701 C stimulator (Aurora Scientific, ON, Canada). Optimal length (*Lo*) and supramaximal stimulus settings (~150 mA) were established in a manner identical to prior studies (Fogarty and Sieck, 2021c). To quantify tetanic forces, stimulus trains (500 ms duration) with 0.5 ms current pulses, delivered at 5, 10, 20, 40, 50, 75 and 100 Hz were used in a manner identical to past *ex vivo* studies in rat tongue (Fogarty and Sieck, 2021c).

Fatigue was assessed using a pattern of direct muscle stimulation where supramaximal (~150 mA) stimulus pulses (0.08 ms duration) were delivered at 40 Hz in 330 ms duration trains repeated each s (33% duty cycle) for 120 s (Fogarty et al., 2019b; Fogarty and Sieck, 2021c), consistent with previous 40 Hz fatigue tests conducted in the tongue (Fogarty and Sieck, 2021c; Fuller and Fregosi, 2000; Nagai et al., 2008; van Lunteren and Manubay, 1992). A fatigue index was calculated as a ratio of the residual force after 120 s to the initial force. Fatigue was characterized by a decrease in specific and relative force with time.

In all force and fatigue experiments, specific force was calculated by normalizing force to the estimated CSA of the muscle (muscle CSA = muscle strip weight (g)/(Lo (cm) × 1.056 g/cm^3) and expressed as N/cm^2, in a manner identical to past reports in tongue (Fogarty and Sieck, 2021c). Due to the structural complexity and intimate interdigitation of the intrinsic tongue muscles (Abe et al., 2002; Bailey et al., 2006; Fogarty and Sieck, 2021c; Saito and Itoh, 2003; Sanders and Mu, 2013), these specific force results were normalized to the area fraction of the tongue musculature (parallel to the longitudinal force vector) compared to the total area of the tongue tissue bulk, in a manner identical to our past study (Fogarty and Sieck, 2021c).

2.4. Muscle histology and fiber type specific proportions and CSA

The entire blade of the tongue (for longitudinal muscle assessment) was stretched to L_o and fresh-frozen on cork in melting isopentane, in a manner identical to past reports (Fogarty et al., 2019b; Fogarty et al., 2020b; Fogarty and Sieck, 2021c) and stored at − 80 °C until processed. Serial cryo-sections (10 μm) were cut using a cryostat (Reichert Microscope Services, Depew, NY, USA) such that longitudinal or genioglossus tongue muscles were sectioned transverse. The middle part of each muscle belly was assessed for CSA (Fig. 1A).

Brightfield mosaic images of muscle fibers stained for hematoxylin and eosin, were acquired using a 20x objective (Olympus IX71) in a manner previously established (Fogarty et al., 2020a; Fogarty et al., 2021a; Fogarty and Sieck, 2021c; Fogarty et al., 2020d). In ImageJ, the circumference of individual tongue muscle fibers was circumscribed using the freehand draw tool and noted, with fibers excluded if they had a circularity characteristic of < 0.5, indicating that they were oblique to the orientation uniaxial force. The entire tongue tissue in the section was then circumscribed, and the relative contribution of the superior and inferior longitudinal muscles to the total tongue CSA was calculated. The proportion of muscle fiber CSA that was perpendicular to longitudinal uniaxial force, relative to the total CSA of tongue tissue bulk (that comprised muscle fibers, interstitial space and oblique/longitudinal muscle fibers from other muscles) was reported for each muscle assessed and defined as the muscle fiber contribution to total tongue bulk.

Immunoreactivity of tongue muscle fibers for antibodies specific to different MyHC isoforms was used to classify fiber types, as previously described (Abe et al., 2002; Cullins and Connor, 2017; Fogarty and Sieck, 2021c; Glass et al., 2019). MyHC$_{slow}$ (BA-F8, 1:3 dilution; Developmental Studies Hybridoma Bank [DSHB], Iowa City, IA) and MyHC$_{2A}$ (SC-71, 1:3 dilution; DSHB) antibodies were incubated together in one section, while immunoreactivity for MyHC$_{2x}$ (6H1, 1:1 dilution; DSHB) was assessed in an adjacent section. These MyHC isoform antibodies have been extensively used and validated in previous studies (Abe et al., 2002; Cullins and Connor, 2017; Glass et al., 2019). Immunolabeled tongue tissue cross-sections were imaged using a 20x oil-immersion objective (NA 1.0) on an Olympus FV2000 (Olympus USA) laser confocal microscope with a 1024 × 1024 pixel array, with similar acquisition parameters across preparations. A non-biased stereological sampling approach was used to represent the different tongue muscle compartments. The mean fiber type specific CSA of longitudinal tongue muscles was determined using morphometric tools in ImageJ, in a manner identical to past reports

(Fogarty et al., 2019b; Fogarty et al., 2020b; Fogarty and Sieck, 2021c). Note that these measurements do not include any interstitial space or muscle fibers that are not in the plane perpendicular to uniaxial force (see above).

2.5. Statistical methods

All statistical analyses were performed using PRISM software (GraphPad PRISM Version 9.4.0, La Jolla, CA). Statistical significance was established at the $P < 0.05$ level. All data are presented as mean ± 95% confidence intervals, unless otherwise specified and assessed for normality with Kolmogorov-Smirnov tests. For comparisons between three groups, one-way ANOVAs with Tukey's post hoc tests were used. Where both muscles and stimulation or time or fiber type were factors, two-way ANOVAs with repeated measures were used to compare ages and stimulation frequency or time, with Bonferroni post hoc tests when warranted (i.e., when the effect of muscle, time/stimulation/fiber type or their interaction was $P < 0.05$). The experimental n is an individual muscle with no separation between left and right sides, with mean values comprising ~30 replicates per fiber type per muscle for the fiber type specific CSA and ~125 individual fibers for overall longitudinal muscle fiber CSA. We had a priori exclusion criteria for replicate data, namely, all data greater than 2.5 standard deviations from the mean were removed, consistent with our past reports (Fogarty et al., 2021a; Fogarty et al., 2019b; Fogarty et al., 2020b; Fogarty and Sieck, 2021c) and with expert statistical guidance (Van Selst and Jolicoeur, 1994), with > 96% of all data being within our exclusion range. In the present study, no exclusion was required for force, fatigue, or muscle contribution to tissue bulk data. Although sex as a biological variable cannot be excluded, we and others have previously observed no sex differences in the number, size and distribution hypoglossal motor neurons or tongue muscle properties (Fogarty et al., 2020c; Fogarty and Sieck, 2021c; Glass et al., 2019; Kanjhan et al., 2016; Kidder et al., 2014). Nonetheless, values for the major outcome measures in the present study were stratified by sex (Table 1).

3. Results

3.1. Animal characteristics

In female and male F344 rats, body mass tended to increase between 6-months (388 ± 55 g; $n = 10$) to 18-months old (456 ± 74 g; $n = 9$), before falling significantly by ~24% between 18- and 24-months (346 ± 30 g; $P = 0.0055$, Tukey's post test, $n = 12$) in the present study ($F_{(2, 28)} = 5.8$; $P = 0.0077$, One-way ANOVA). Consistent with this, we did observe a significant effect of sex ($F_{(1, 25)} = 13.5$; $P = 0.0011$) and age ($F_{(2, 25)} = 8.0$; $P = 0.0021$), but no effect of sex-age interaction ($F_{(2, 25)} = 0.3$; $P = 0.774$) on body mass of F344 rats (two-way ANOVA; Table 1).

3.2. Tongue muscle fiber contribution to total tongue tissue bulk in longitudinal muscles

The proportion of tongue tissue bulk comprising either the superior and inferior longitudinal muscle fibers and transversalis muscle fibers in the proximal region of the tongue blade was quantified (Fig. 1C). We did not observe any differences in the contribution of longitudinal muscle fibers with age ($F_{(2, 28)} = 0.8$; $P = 0.460$, One-way ANOVA), with the % of uniaxial fibers ~18–20% in 6- (19.6 ± 3.0%; $n = 10$), 18- (20.9 ± 3.6%; $n = 9$) and 24-month-old

(18.6 ± 2.2%; $n = 12$) rats (Fig. 1D). When sex was taken into consideration, neither sex ($F_{(1, 25)} = 1.0$; $P = 0.974$), age ($F_{(2, 25)} = 0.7$; $P = 0.488$), nor a sex-age interaction ($F_{(2, 25)} = 0.4$; $P = 0.705$) had an effect on the longitudinal muscle fiber contribution to total tongue bulk (two-way ANOVA; Table 1).

3.3. Aging reduced isometric specific force production of the longitudinal muscles

Isometric forces generated across a range of stimulation frequencies by the superior and inferior longitudinal muscles at 6-, 18- and 24-months old, with the maximum tetanic force represented (Fig. 2A). Twitch specific forces of the superior and inferior longitudinal muscles at 6- (1.6 ± 0.4 N/Cm^2, $n = 10$), 18- (1.7 ± 0.6 N/Cm^2, $n = 9$) and 24-months-old (1.0 ± 0.4 N/Cm^2, $n = 12$) was affected by age ($F_{(2, 28)} = 3.5$; $P = 0.0446$, One-way ANOVA; Fig. 2B), although Tukey's post-tests did not reveal any specific changes between individual age groups. Although the effect of age held ($F_{(2, 25)} = 3.6$; $P = 0.0425$), we did not observe a significant effect of sex ($F_{(1, 25)} = 0.1$; $P = 0.836$) or sex-age interaction ($F_{(2, 25)} = 0.7$; $P = 0.519$) on twitch specific forces (two-way ANOVA; Table 1).

The maximum tetanic specific force of the superior and inferior longitudinal muscles was reduced in 24-month-old rats (3.3 ± 0.6 N/Cm^2, $n = 12$), by ~33% compared to 6-month (4.9 ± 1.0 N/Cm^2, $n = 10$; $P = 0.0349$, Tukey's post-test) and ~38% in 18-month-old (5.3 ± 1.5 N/Cm^2, $n = 9$; $P = 0.0073$, Tukey's post-test) rats ($F_{(2, 28)} = 6.3$; $P = 0.0055$, One-way ANOVA; Fig. 2C). There were no differences between 6- and 18-month-old rats ($P = 0.783$, Tukey's post-test). Although the effect of age held ($F_{(2, 25)} = 5.8$; $P = 0.0083$), we did not observe a significant effect of sex ($F_{(1, 25)} = 0.01$; $P = 0.908$) or sex-age interaction ($F_{(1, 25)} = 0.3$; $P = 0.739$) on maximum specific forces (two-way ANOVA; Table 1). Overall force (regardless of muscle mass) followed a similar trend, with force (N) in 24-month-old rats (0.15 ± 0.02 N, $n = 12$) reduced by 21% compared to 6-month-old rats (0.19 ± 0.03 N, $n = 10$ $P = 0.037$, Tukey's post-test) and 25% compared to 18-month-old (0.20 ± 0.04 N, $n = 9$; $P = 0.0035$, Tukey's post-test) rats ($F_{(2, 28)} = 7.1$; $P = 0.0008$, One-way ANOVA). There were no differences between 6- and 18-month-old rats ($P = 0.58$, Tukey's post-test).

There was a marked relationship on longitudinal specific force with frequency of stimulation ($F_{(1.7, 48.2)} = 151.1$; $P < 0.0001$), age ($F_{(2,28)} = 9.1$; $P = 0.0009$), and the stimulation frequency-age interaction between ($F_{(12, 168)} = 7.3$; $P < 0.0001$; two-way ANOVA; Fig. 3A). From 40 Hz and greater stimulations, longitudinal muscle specific forces were reduced between 6- and 24-month-old rats ($P < 0.05$, for each comparison; Bonferroni post-tests; Fig. 3A). In the case of 18-month-old rats, there were no differences compared to 6-month-olds ($P > 0.99$ for all comparisons), with differences compared to 24-month-old rats with stimulation frequencies of 75 and 100 Hz ($P < 0.0474$ in both comparisons; Bonferroni post-tests; Fig. 3A). We observed no differences between 6- and 18-month-old rats ($P > 0.99$ in all comparisons; Bonferroni post-tests; Fig. 3A).

When expressed as a % of maximum specific forces at each stimulation frequency, the stimulation frequency had a significant effect on longitudinal muscle force ($F_{(2.6, 73.8)} = 243.4$; $P < 0.0001$), but not age ($F_{(2,28)} = 1.0$; $P = 0.394$) not the stimulation frequency-age interaction ($F_{(12, 168)} = 1.4$; $P < 0.168$; two-way ANOVA; Fig. 3B).

3.4. Aging influences fatigue index, but not residual forces of the longitudinal tongue muscles

Fatigue was assessed in the superior and inferior longitudinal muscles with specific forces declining during 120 s of repetitive activation (Fig. 4). The overall fatigue index was significantly increased in 24-month-old rats (0.206 ± 0.044, $n = 12$; $F_{(2, 28)} = 6.5$; $P = 0.0049$, One-way ANOVA) by ~56% compared to 6- (0.132 ± 0.020, $n = 10$; $P = 0.0059$, Tukey's post-test) and ~39% 18-month-olds (0.148 ± 0.030, $n = 9$; $P = 0.0385$, Tukey's post-test), respectively (Fig. 4B). There were no differences between 6- and 18-month-old rats ($P = 0.787$, Tukey's post-test; Fig. 4B). Although the effect of age held ($F_{(2, 25)} = 5.7$; $P = 0.0095$), we did not observe a significant effect of sex ($F_{(1, 25)} = 1.4$; $P = 0.246$) or sex-age interaction ($F_{(2, 25)} = 0.1$; $P = 0.948$) on the fatigue index (two-way ANOVA; Table 1).

Following the 120 s of repetitive activation, the residual force generated by the superior and inferior longitudinal muscles was unchanged between 6- (0.57 ± 0.19 N/Cm^2, $n = 10$), 18- (0.65 ± 0.20 N/Cm^2, $n = 9$) and 24-month-old rats (0.49 ± 0.13 N/Cm^2, $n = 12$; $F_{(2, 28)} = 1.1$; $P = 0.335$, One-way ANOVA; Fig. 4C). We did not observe a significant effect of age ($F_{(2, 25)} = 1.4$; $P = 0.270$), sex ($F_{(1, 25)} = 1.6$; $P = 0.218$) or sex-age interaction ($F_{(2, 25)} = 0.8$; $P = 0.476$) on the residual force following fatigue (two-way ANOVA; Table 1).

3.5. Longitudinal muscle fiber type properties in aging

A mixture of muscle fiber types, type I, type IIa and type IIx/IIb, was observed in the longitudinal muscles of 6-month-old F344 rats (Fig. 5A), whilst the expression of type I fibers was very rare to absent at 18- and 24-months-old. Notably, this was not due to the ineffectiveness of immunolabelling, as 24-month-old genioglossus muscle exhibited type I fibers (Fig. 5B). Accordingly, we excluded type I fibers from our fiber type specific assessments.

The mean longitudinal muscle fiber CSA was influenced by age ($F_{(2,23)} = 5.8$; $P = 0.0089$), fiber type ($F_{(1,23)} = 192.3$; $P < 0.0001$) and the interaction between age and fiber type ($F_{(2,23)} = 7.8$; $P = 0.0026$; two-way ANOVA; Fig. 5C). For type IIa fibers, there was no difference in CSA between 6- (486 ± 54 μm^2), 18- (454 ± 115 μm^2) and 24-month-old (448 ± 35 μm^2) F344 rats ($P > 0.99$ in all age comparisons; Bonferroni post test; Fig. 5E). By contrast, by 24-month-old (738 ± 94 μm^2) F344 rats had an ~27% and ~30% reduction in mean type IIx/IIb fiber CSA compared to 6- (1009 ± 168 μm^2; $P = 0.0005$) and 18-months-old (1051 ± 171 μm^2; $P = 0.0006$), respectively (Bonferroni post-tests; Fig. 5E). There were no differences in type IIx/IIb longitudinal muscle fiber CSA between 6 and 18 months old ($P > 0.99$; Fig. 5C).

There was no sex ($F_{(1, 20)} = 0.9$; $P = 0.351$), age ($F_{(2, 20)} = 0.7$; $P = 0.526$) or sex-age interaction ($F_{(2, 20)} = 0.1$; $P = 0.863$) effect on type I longitudinal muscle fiber CSA (two-way ANOVA, Table 1). For type IIx/IIb fiber CSA, the effect of age held ($F_{(2, 20)} = 8.1$; $P = 0.0027$), though we did not observe a significant effect of sex ($F_{(1, 20)} = 1.9$; $P = 0.187$) or sex-age interaction ($F_{(2, 20)} = 1.1$; $P = 0.367$; two-way ANOVA; Table 1).

3.6. Longitudinal muscle CSA was reduced in aging

As there was some variability in the MyHC expression and the disambiguation of type I fibers in the longitudinal muscles, we assessed whether there was any difference with age in overall longitudinal muscle fiber CSAs (Fig. 6). The overall CSA was significantly reduced in 24-month-old rats (509 ± 47 μm^2, $n = 12$; $F_{(2, 28)} = 14.1$; $P < 0.0001$, One-way ANOVA) by ~26% compared to 6- (691 ± 91 μm^2, $n = 10$; $P = 0.006$, Tukey's post-test) and ~29% 18-month-olds (718 ± 74, $n = 9$; $P = 0.002$, Tukey's post-test), respectively (Fig. 6B). There were no differences between 6- and 18-month-old rats ($P = 0.824$, Tukey's post-test; Fig. 6B). Although the effect of age held ($F_{(2, 25)} = 13.8$; $P < 0.0001$), we did not observe a significant effect of sex ($F_{(1, 25)} = 1.3$; $P = 0.259$) or sex-age interaction ($F_{(2, 25)} = 0.2$; $P = 0.835$) on the overall longitudinal muscle CSA (two-way ANOVA; Table 1).

The size distributions of longitudinal muscle fiber CSA was also assessed, with a shift to smaller CSAs in 24-month-old rats compared to 6- ($P < 0.001$) and 18-month-old rats ($P < 0.001$; Kolmogorov-Smirnov test; Fig. 6C).

4. Discussion

The present study is the first to catalogue the age-associated changes in specific force and fatigue properties as well as fiber type-specific morphology of the intrinsic superior and inferior longitudinal muscles in the rat tongue. There were five main findings: i) There were no systematic changes to longitudinal tongue muscle contributions to total tongue tissue bulk with aging; ii) When normalized for muscle fiber contribution to total tongue tissue bulk, the maximum specific force generated by the longitudinal muscles was reduced in old age; iii) The fatigue characteristics of longitudinal tongue muscles differed with age, however, the residual specific force following fatigue was unchanged; iv) The CSAs of type IIx/IIb longitudinal tongue fibers was reduced by 24-months-old, with type I and IIa fibers relatively unaffected; and v) Consistent with fiber-type specific results, overall longitudinal muscle fiber CSA was also reduced at 24-months-old. Taken together, our results suggest that longitudinal tongue muscle sarcopenia is concomitant with the onset of mortality in Fischer 344 rats (i.e., occurs between 18- and 24-months old) and with the loss of hypoglossal motor neurons (Fogarty, 2023). The pattern of fatigability and the disproportionate effects of aging on type IIx/IIb fibers is consistent with sarcopenia being selective to type FF motor units.

In situ, the individual intrinsic and extrinsic tongue muscles exist in a complex interdigitated state, with the genioglossus insertion ingratiated with the intrinsic muscles. These tongue muscles must coordinate their movements together, in addition to orchestrating activations with other muscles of the oropharynx and the DIAm. These tongue movements contribute to many behaviors, including ventilation, chewing, swallowing, grooming (erotic behaviors in humans), vocalization (speech in humans) and expulsive manoeuvres (coughing, sneezing and vomiting) (Bailey and Fregosi, 2004; Bailey et al., 2006; Doty and Bosma, 1956; Eckstein and Hart, 2000; Lowe, 1980; Macneilage and Sholes, 1964; Mathew et al., 1982; Mitchinson and Yoffey, 1947; Sachs, 1988; Satoh et al., 1998; Spruijt et al., 1992). In humans there are many lingual behaviors that may be impaired in the elderly or in age-associated disorders. These coalesce around reduced oral motor function (Baum and Bodner,

1983), maximal tongue strength (Crow and Ship, 1996; Youmans et al., 2009) and increased risk of OSA and aspiration pneumonia (Chong et al., 2008; Gaspar et al., 2017; Langmore et al., 1998) in the elderly.

The intense investigation of the effects of aging on tongue muscle function have to date centred on the F344/BN hybrid rat model. In these rats, reduced protrusion force and some indication of tongue muscle fiber atrophy is apparent (Kletzien et al., 2018; Nagai et al., 2008). One complication with this approach is that retraction and protrusion forces generated by hypoglossal nerve stimulation are a summation of the activity of multiple tongue muscles that may not be activated in a strictly synergistic manner (Bailey et al., 2006; Lowe, 1980; Setzke et al., 2020). Furthermore, by contrast to the loss of hypoglossal motor neurons in 24-month-old F344 rats (Fogarty, 2023), and the robust observations of motor neuron death in aging F344 rats (Fogarty et al., 2018b; Fogarty and Sieck, 2023; Hashizume et al., 1988; Jacob, 1998; Johnson and Duberley, 1998; Kanda and Hashizume, 1998) there is no loss of tongue-innervating hypoglossal motor neurons in the aged F344/BN hybrids (Schwarz et al., 2009). Motor neuron loss is also evident in the cervical and lumbar spinal cord of aged humans (Cruz-Sanchez et al., 1998; Kawamura et al., 1977; Tomlinson and Irving, 1977; Zhang et al., 1996). Thus, this study provides an important contribution by disambiguating the impact of other tongue muscles by using our uniaxial force evaluation approach in females and males (Fogarty and Sieck, 2021c), in a model of aging where motor neuron death is present, similar to elderly humans.

In longitudinal muscle tongue muscle cross-sections, there were no differences in non-uniaxial muscle fiber contributions to tongue blade muscle bulk, which also comprises the vasculature, interdigitated muscle fibers in different orientations and the interstitium. This is similar to aging DIAm of F344 rats, which does not experience changes in the interstitium with age (Elliott et al., 2016; Khurram et al., 2018). Thus, it is likely that any reduction in specific force with age was not due to fatty or connective tissue infiltrations into the muscle.

Lack of information on the force properties of individual tongue muscles has been identified as a major interference to the understanding of tongue muscle function for a number of years, having been identified as a startling gap in the literature for some decades (Lowe, 1980; Sokoloff, 2000; van Lunteren and Manubay, 1992). In the present study, the observation of reduced specific force (normalized to muscle cross sectional areas) of the longitudinal intrinsic muscles of the tongue is entirely novel. Notably, the force loss without normalisation for muscle cross sectional area is ~20–25%, compared to the ~33% when factoring specific force. As these rats grow throughout the lifespan, slightly larger tongue muscles and differing growth rates (i.e., 6 vs 18 and 18 vs 24 months) may confound and/or mitigate some of the decrement in “absolute” force generation. Beyond atrophy (i.e., when accounting for differences in muscle mass/CSA), we have three possibilities for reduction in intrinsic muscle force generation: i) the fraction of cross-bridges in the strongly bound force generating state, ii) the average force produced per cross-bridge, and/or iii) the number of myosin heads in parallel per half-sarcomere (Fogarty and Sieck, 2021c). These factors are dependent on fiber type (Geiger et al., 2000; Geiger et al., 1999; Han et al., 2003) and, with direct relevance to hypoglossal motor neuron death in aged F344 rats (Fogarty, 2023), denervation (Geiger et al., 2003; Geiger et al., 2001). With denervation

(and likely aging), the fraction of bound cross-bridges at given calcium concentrations are unchanged, along with the average force per cross-bridge. By contrast reduction in myosin heads per half-sarcomere (i.e., myosin concentration) reduces specific force directly (Geiger et al., 2000) and indirectly (as cross-bridges of IIx/IIb fibers produce more force than I and IIa fibers) (Geiger et al., 2000). In denervated (Geiger et al., 2003; Geiger et al., 2001) and aging (Elliott et al., 2016) muscle, myosin heavy chain concentration is reduced. In other limb muscles, aging-associated conversion of type IIx/IIb fibers to type IIa fibers is evident (Rowan et al., 2012), reducing forces per cross bridge (Fogarty and Sieck, 2021c). It is unknown the reduction in myosin heavy chain concentration or the degree of fiber type conversion (as IIx/b fibers were not directly labeled) in the tongue throughout age. Regardless, both of these possibilities are consistent with denervation of longitudinal tongue muscle fibers (and potential fiber-type conversion) due to hypoglossal motor neuron death (Fogarty, 2023), and are entirely congruent with our observation of reduced longitudinal specific force in aging.

The reduction in the maximum specific force of the longitudinal muscles in old F344 rats is entirely consistent with a loss of hypoglossal motor neurons (Fogarty, 2023), similar to our observations in F344 rats of phrenic motor neurons (Fogarty et al., 2018b; Fogarty and Sieck, 2023) and DIAm (Fogarty et al., 2019b; Fogarty et al., 2020b; Khurram et al., 2018), with sarcopenia occurring concomitant with motor neuron loss and denervation of muscle fibers (Fogarty et al., 2019a). Consistent with past results in the DIAm, there were no aging effects on longitudinal tongue muscle specific force at 18-months-old (Fogarty et al., 2020b). The magnitude of force loss in 24- compared to 6-month-old female and male F344 rats was ~33%, with hypoglossal motor neuron loss at the same age of ~15% (Fogarty, 2023). At the same ages, DIAm specific force was reduced by ~30% (Brown et al., 2021; Fogarty et al., 2019b; Fogarty et al., 2020b; Khurram et al., 2018) and phrenic motor neuron loss of ~20% (Fogarty et al., 2018b) in female and male F344 rats. In both cases, though overall motor neuron loss is moderate, the loss of larger motor neurons approaches ~30% of hypoglossal and ~60% of phrenic motor neurons (Fogarty, 2023; Fogarty et al., 2018b), with these neurons innervating the type IIx/IIb fibers that contribute the greatest to the overall maximum specific force (Fogarty and Sieck, 2021c; Geiger et al., 2000). Despite relatively less motor neuron loss of overall and larger hypoglossal motor neurons compared to phrenic motor neurons, the tongue's action as a muscular hydrostat (Gilbert et al., 2007; Kier and Smith, 1985; Smith and Kier, 1989) means that each motion of the tongue involves the movement of a lot of tissue "dead weight", with ~80% of the longitudinal tongue tissue not containing fibers contracting parallel to the axis of shortening (Fogarty and Sieck, 2021c). By contrast, the DIAm, although also unconventional in the case of skeletal muscle by producing volume and pressure changes (Fogarty and Sieck, 2019c), rather than torque has muscle fibers arranged in series, with only ~15% of the tissue not containing fibers contracting in the axis of shortening (Elliott et al., 2016; Khurram et al., 2018). This accounts for the age-associated denervation of tongue muscle fibers (~15% denervation) having similar force decrements to DIAm (~30% loss of specific force) despite greater levels of denervated DIAm fibers (~30% phrenic axon loss). Alternatively, the innervation ratio of type FF motor units in the longitudinal muscles may be greater than type S and FR units, similar to type-dependent limb muscle innervation ratios (Rafuse et al., 1997), as opposed

to DIAm motor units, which do not have type-dependent differences in innervation ratio (Fogarty and Sieck, 2021c). Both of these alternate explanations are consistent with our observations in F344 rats, and in the SOD1 amyotrophic lateral sclerosis rat model, where there is a marked loss of tongue function (Smittkamp et al., 2010; Smittkamp et al., 2014) without hypoglossal motor neuron death (Llado et al., 2006).

The longitudinal muscle fibers of the tongue contain a relatively high proportion of fatigable type IIx/IIb fibers (Cullins and Connor, 2017; Fogarty and Sieck, 2021c), so our relatively low fatigue index ~0.13 is not unexpected. Despite the increased fatigue index in old F344 rats, commonly interpreted as reduced fatigability, there was no change in the residual longitudinal muscle specific force following the 120 s of repeated contractions. This changed fatigue index is a result of the greater initial forces present in younger rats, which we have previously shown is a result of the reduction in the contribution of IIx/IIb fibers in DIAm, not a result of intrinsic changes in fatigability per se (Fogarty et al., 2022; Fogarty et al., 2021b; Fogarty et al., 2019b).

We assessed the superior and inferior longitudinal muscles for fiber type specific CSA. Consistent with past efforts in rat (Cullins and Connor, 2017; Fogarty and Sieck, 2021c), the vast majority of superior and inferior longitudinal muscle fibers were identified as type IIx/IIb, with the remainder type IIa, with an almost negligible amount of type I fibers being present in the superior longitudinal muscle of young rats. As we had difficulty in sampling sufficient type I longitudinal muscles in 18- and 24-month-old rats, fiber type specific comparisons were limited to type IIa and type IIx/IIb fibers. Notably, type I fibers were still present at these later ages, being readily observed in the genioglossus. Our observation of selective reduction of type IIx/IIb CSA in the longitudinal fibers of 24-month-old F344 rats is consistent with the reduced maximum specific force and maintenance of force following fatigue observed in this study, and with the loss of larger hypoglossal motor neurons (Fogarty, 2023) (likely innervating IIx/IIb fibers (Fogarty and Sieck, 2021c)) in old F344 rats. Previously, the overall proportion of $MyHC_{2X}$-expressing (ie., type IIx) and $MyHC_{2B}$-expressing (i.e., type IIb) superior and inferior longitudinal fibers was shown to be increased and decreased, respectively, in the anterior region of the tongue blade in old F344/BN hybrids (Cullins and Connor, 2017), with the fiber diameter unchanged (Cullins and Connor, 2017). In the present study, we assessed the middle portion of the tongue, where reports in F344/BN hybrids show no difference with age in fiber proportions or fiber diameters (Cullins and Connor, 2017) of superior and inferior longitudinal muscles. Notably, as the longitudinal fibers were not uniform, CSA, used in the present study, rather than mean diameter best describes fiber morphology. Our CSA assessment shows IIx/IIb fibers to be larger than IIa longitudinal tongue muscle fibers, with diameters in sufficient to provide this distinction in F344/BN hybrids (Cullins and Connor, 2017). To further characterise aging effects, we assessed the mean and distribution of longitudinal muscle fiber CSA, regardless of type, by using H & E staining. We show reduced longitudinal muscle fiber CSA, with a shift in the frequency distribution to smaller fibers in old age.

Our observations of longitudinal muscle weakness and atrophy (general and specific to IIx/IIb fibers) in females and males is entirely consistent with loss of hypoglossal motor neurons in F344 rats (Fogarty, 2023) and tongue force dysfunction previously observed in

the male F344/BN hybrids (Nagai et al., 2008). Clinically, the denervation, weakness and atrophy of tongue muscles may underpin age-associated dysphagia, aspiration pneumonia and OSA, which are strong predictors of human morbidity and mortality in aging (Baum and Bodner, 1983; Crow and Ship, 1996; Gaspar et al., 2017; Youmans et al., 2009). It will be important to investigate the temporal and mechanistic underpinnings of hypoglossal motor neuron death and tongue muscle sarcopenia to eventually provide therapies for age-associated lingual dysfunctions.

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References

Abe S, Maejima M, Watanabe H, Shibahara T, Agematsu H, Doi T, Sakiyama K, Usami A, Gojyo K, Hashimoto M, Yoshinari M, Ide Y, 2002. Muscle-fiber characteristics in adult mouse-tongue muscles. Anat. Sci. Int 77, 145–148. [PubMed: 12418096]

Andrade AG, Bubu OM, Varga AW, Osorio RS, 2018. The relationship between obstructive sleep apnea and Alzheimer's disease. J. Alzheimers Dis 64, S255–S270. [PubMed: 29782319]

Bailey EF, Fregosi RF, 2004. Coordination of intrinsic and extrinsic tongue muscles during spontaneous breathing in the rat. J. Appl. Physiol 96 (1985), 440–449. [PubMed: 14527967]

Bailey EF, Huang YH, Fregosi RF, 2006. Anatomic consequences of intrinsic tongue muscle activation. J. Appl. Physiol 101 (1985), 1377–1385. [PubMed: 16825524]

Baum BJ, Bodner L, 1983. Aging and oral motor function: evidence for altered performance among older persons. J. Dent. Res 62, 2–6. [PubMed: 6600463]

Brown AD, Davis LA, Fogarty MJ, Sieck GC, 2021. Mitochondrial fragmentation and dysfunction in type IIx/IIb diaphragm muscle fibers in 24-month old fischer 344 rats. Front. Physiol 12, 727585. [PubMed: 34650442]

Chong CP, Street PR, 2008. Pneumonia in the elderly: a review of the epidemiology, pathogenesis, microbiology, and clinical features. South Med. J 101, 1141–1145. [PubMed: 19088525]

Crow HC, Ship JA, 1996. Tongue strength and endurance in different aged individuals. J. Gerontol. A Biol. Sci. Med. Sci 51, M247–250. [PubMed: 8808997]

Cruz-Sanchez FF, Moral A, Tolosa E, de Belleroche J, Rossi ML, 1998. Evaluation of neuronal loss, astrocytosis and abnormalities of cytoskeletal components of large motor neurons in the human anterior horn in aging. J. Neural Transm. (Vienna) 105, 689–701. [PubMed: 9826111]

Cullins MJ, Connor NP, 2017. Alterations of intrinsic tongue muscle properties with aging. Muscle Nerve 56, E119–E125. [PubMed: 28181263]

Doty RW, Bosma JF, 1956. An electromyographic analysis of reflex deglutition. J. Neurophysiol 19, 44–60. [PubMed: 13286721]

Eckstein RA, Hart BL, 2000. The organization and control of grooming in cats. Appl. Anim. Behav. Sci 68, 131–140. [PubMed: 10771321]

Elliott JE, Omar TS, Mantilla CB, Sieck GC, 2016. Diaphragm muscle sarcopenia in Fischer 344 and Brown Norway rats. Exp. Physiol 101, 883–894. [PubMed: 27126607]

Enright PL, Kronmal RA, Manolio TA, Schenker MB, Hyatt RE, 1994. Respiratory muscle strength in the elderly. Correlates and reference values. Cardiovascular Health Study Research Group. Am. J. Respir. Crit. Care Med 149, 430–438. [PubMed: 8306041]

Fogarty MJ, 2023. Loss of larger hypoglossal motor neurons in aged Fischer 344 rats. Respir. Physiol. Neurobiol 314, 104092. [PubMed: 37331418]

Fogarty MJ, Sieck GC, 2019c. Evolution and functional differentiation of the diaphragm muscle of mammals. Compr. Physiol 9, 715–766. [PubMed: 30873594]

Fogarty MJ, Sieck GC, 2021c. Tongue muscle contractile, fatigue, and fiber type properties in rats. J. Appl. Physiol 131 (1985), 1043–1055. [PubMed: 34323593]

Fogarty MJ, Sieck GC, 2023. Aging affects the number and morphological heterogeneity of rat phrenic motor neurons and phrenic motor axons. Physiol. Rep

Fogarty MJ, Mantilla CB, Sieck GC, 2018a. Breathing: motor control of diaphragm muscle. Physiol. (Bethesda) 33, 113–126.

Fogarty MJ, Omar TS, Zhan WZ, Mantilla CB, Sieck GC, 2018b. Phrenic motor neuron loss in aged rats. J. Neurophysiol 119, 1852–1862. [PubMed: 29412773]

Fogarty MJ, Gonzalez Porras MA, Mantilla CB, Sieck GC, 2019a. Diaphragm neuromuscular transmission failure in aged rats. J. Neurophysiol 122, 93–104. [PubMed: 31042426]

Fogarty MJ, Mantilla CB, Sieck GC, 2019b. Impact of sarcopenia on diaphragm muscle fatigue. Exp. Physiol 104, 1090–1099. [PubMed: 30924589]

Fogarty MJ, Brandenburg JE, Sieck GC, 2020a. Diaphragm neuromuscular transmission failure in a mouse model of an early-onset neuromotor disorder. J. Appl. Physiol (1985).

Fogarty MJ, Marin Mathieu N, Mantilla CB, Sieck GC, 2020b. Aging reduces succinate dehydrogenase activity in rat type IIx/IIb Diaphragm Muscle Fibers. J. Appl. Physiol (1985).

Fogarty MJ, Mu EWH, Lavidis NA, Noakes PG, Bellingham MC, 2020c. Size-dependent dendritic maladaptations of hypoglossal motor neurons in SOD1(G93A) mice. Anat. Rec. (Hoboken)

Fogarty MJ, Sieck GC, Brandenburg JE, 2020d. Impaired neuromuscular transmission of the tibialis anterior in a rodent model of hypertonia. J. Neurophysiol 123, 1864–1869. [PubMed: 32292122]

Fogarty MJ, Enninga EAL, Ibirogba ER, Ruano R, Sieck GC, 2021a. Impact of congenital diaphragmatic hernia on diaphragm muscle function in neonatal rats. J. Appl. Physiol (1985).

Fogarty MJ, Losbanos LL, Craig TA, Reynolds CJ, Brown AD, Kumar R, Sieck GC, 2021b. Muscle specific deletion of the vitamin-D receptor in mice is associated with diaphragm muscle weakness. J. Appl. Physiol (1985).

Fogarty MJ, Brandenburg JE, Zhan WZ, Sieck GC, 2022. Diaphragm muscle function in a mouse model of early onset spasticity. J. Appl. Physiol (1985).

Fuller D, Mateika JH, Fregosi RF, 1998. Co-activation of tongue protrudor and retractor muscles during chemoreceptor stimulation in the rat. J. Physiol 507 (Pt 1), 265–276. [PubMed: 9490849]

Fuller DD, Fregosi RF, 2000. Fatiguing contractions of tongue protrudor and retractor muscles: influence of systemic hypoxia. J. Appl. Physiol 88, 2123–2130. [PubMed: 10846026]

Gale CR, Deary IJ, Batty GD, 2019. Cognitive ability and risk of death from lower respiratory tract infection: findings from UK Biobank. Sci. Rep 9, 1342. [PubMed: 30718728]

Gaspar LS, Alvaro AR, Moita J, Cavadas C, 2017. Obstructive sleep apnea and hallmarks of aging. Trends Mol. Med 23, 675–692. [PubMed: 28739207]

Geiger PC, Cody MJ, Sieck GC, 1999. Force-calcium relationship depends on myosin heavy chain and troponin isoforms in rat diaphragm muscle fibers. J. Appl. Physiol 87, 1894–1900. [PubMed: 10562634]

Geiger PC, Cody MJ, Macken RL, Sieck GC, 2000. Maximum specific force depends on myosin heavy chain content in rat diaphragm muscle fibers. J. Appl. Physiol 89, 695–703. [PubMed: 10926656]

Geiger PC, Cody MJ, Macken RL, Bayrd ME, Sieck GC, 2001. Effect of unilateral denervation on maximum specific force in rat diaphragm muscle fibers. J. Appl. Physiol 90, 1196–1204. [PubMed: 11247914]

Geiger PC, Bailey JP, Zhan WZ, Mantilla CB, Sieck GC, 2003. Denervation-induced changes in myosin heavy chain expression in the rat diaphragm muscle. J. Appl. Physiol 95, 611–619. [PubMed: 12704093]

Gilbert RJ, Napadow VJ, Gaige TA, Wedeen VJ, 2007. Anatomical basis of lingual hydrostatic deformation. J. Exp. Biol 210, 4069–4082. [PubMed: 18025008]

Gilliam EE, Goldberg SJ, 1995. Contractile properties of the tongue muscles: effects of hypoglossal nerve and extracellular motoneuron stimulation in rat. J. Neurophysiol 74, 547–555. [PubMed: 7472362]

Glass TJ, Valmadrid LCV, Connor NP, 2019. The adult Ts65Dn mouse model of down syndrome shows altered swallow function. Front. Neurosci 13, 906. [PubMed: 31555077]

Han YS, Geiger PC, Cody MJ, Macken RL, Sieck GC, 2003. ATP consumption rate per cross bridge depends on myosin heavy chain isoform. J. Appl. Physiol 94, 2188–2196. [PubMed: 12588786]

Hashizume K, Kanda K, Burke RE, 1988. Medial gastrocnemius motor nucleus in the rat: age-related changes in the number and size of motoneurons. J. Comp. Neurol 269, 425–430. [PubMed: 3372722]

Jacob JM, 1998. Lumbar motor neuron size and number is affected by age in male F344 rats. Mech. Ageing Dev 106, 205–216. [PubMed: 9883984]

Johnson IP, Duberley RM, 1998. Motoneuron survival and expression of neuropeptides and neurotrophic factor receptors following axotomy in adult and ageing rats. Neuroscience 84, 141–150. [PubMed: 9522369]

Kanda K, Hashizume K, 1998. Effects of long-term physical exercise on age-related changes of spinal motoneurons and peripheral nerves in rats. Neurosci. Res 31, 69–75. [PubMed: 9704980]

Kanjhan R, Fogarty MJ, Noakes PG, Bellingham MC, 2016. Developmental changes in the morphology of mouse hypoglossal motor neurons. Brain Struct. Funct 221, 3755–3786. [PubMed: 26476929]

Kawamura Y, O'Brien P, Okazaki H, Dyck PJ, 1977. Lumbar motoneurons of man II: the number and diameter distribution of large- and intermediate-diameter cytons in "motoneuron columns" of spinal cord of man. J. Neuropathol. Exp. Neurol 36, 861–870. [PubMed: 894329]

Khurram OU, Fogarty MJ, Sarrafian TL, Bhatt A, Mantilla CB, Sieck GC, 2018. Impact of aging on diaphragm muscle function in male and female Fischer 344 rats. Physiol. Rep 6, e13786. [PubMed: 29981218]

Kidder IJ, Mudery JA, Bailey EF, 2014. Neural drive to respiratory muscles in the spontaneously breathing rat pup. Respir. Physiol. Neurobiol 202, 64–70. [PubMed: 25125330]

Kier WM, Smith KK, 1985. Tongues, tentacles and trunks - the biomechanics of movement in muscular-hydrostats. Zool. J. Linn. Soc 83, 307–324.

Kletzien H, Hare AJ, Leverson G, Connor NP, 2018. Age-related effect of cell death on fiber morphology and number in tongue muscle. Muscle Nerve 57, E29–E37. [PubMed: 28440544]

Langmore SE, Terpenning MS, Schork A, Chen Y, Murray JT, Lopatin D, Loesche WJ, 1998. Predictors of aspiration pneumonia: how important is dysphagia? Dysphagia 13, 69–81. [PubMed: 9513300]

Llado J, Haenggeli C, Pardo A, Wong V, Benson L, Coccia C, Rothstein JD, Shefner JM, Maragakis NJ, 2006. Degeneration of respiratory motor neurons in the SOD1 G93A transgenic rat model of ALS. Neurobiol. Dis 21, 110–118. [PubMed: 16084734]

Lowe AA, 1980. The neural regulation of tongue movements. Prog. Neurobiol 15, 295–344. [PubMed: 7244250]

van Lunteren E, Manubay P, 1992. Contractile properties of feline genioglossus, sternohyoid, and sternothyroid muscles. J. Appl. Physiol 72 (1985), 1010–1015. [PubMed: 1568954]

Macneilage PF, Sholes GN, 1964. An electromyographic study of the tongue during vowel production. J. Speech Hear Res 7, 209–232. [PubMed: 14213498]

Mathew OP, Abu-Osba YK, Thach BT, 1982. Influence of upper airway pressure changes on genioglossus muscle respiratory activity. J. Appl. Physiol. Respir. Environ. Exerc Physiol 52, 438–444. [PubMed: 7037716]

McClung JR, Goldberg SJ, 2000. Functional anatomy of the hypoglossal innervated muscles of the rat tongue: a model for elongation and protrusion of the mammalian tongue. Anat. Rec 260, 378–386. [PubMed: 11074403]

Mitchinson AG, Yoffey JM, 1947. Respiratory displacement of larynx, hyoid bone and tongue. J. Anat 81, 118–120.

Nagai H, Russell JA, Jackson MA, Connor NP, 2008. Effect of aging on tongue protrusion forces in rats. Dysphagia 23, 116–121. [PubMed: 17694408]

Polkey MI, Harris ML, Hughes PD, Hamnegard CH, Lyons D, Green M, Moxham J, 1997. The contractile properties of the elderly human diaphragm. Am. J. Respir. Crit. Care Med 155, 1560–1564. [PubMed: 9154857]

Rafuse VF, Pattullo MC, Gordon T, 1997. Innervation ratio and motor unit force in large muscles: a study of chronically stimulated cat medial gastrocnemius. J. Physiol 499 (Pt 3), 809–823. [PubMed: 9130174]

Rowan SL, Rygiel K, Purves-Smith FM, Solbak NM, Turnbull DM, Hepple RT, 2012. Denervation causes fiber atrophy and myosin heavy chain co-expression in senescent skeletal muscle. PloS One 7, e29082. [PubMed: 22235261]

Sachs BD, 1988. The development of grooming and its expression in adult animals. Ann. N. Y. Acad. Sci 525, 1–17.

Saito H, Itoh I, 2003. Three-dimensional architecture of the intrinsic tongue muscles, particularly the longitudinal muscle, by the chemical-maceration method. Anat. Sci. Int 78, 168–176. [PubMed: 14527131]

Sanders I, Mu L, 2013. A three-dimensional atlas of human tongue muscles. Anat. Rec. (Hoboken) 296, 1102–1114. [PubMed: 23650264]

Satoh I, Shiba K, Kobayashi N, Nakajima Y, Konno A, 1998. Upper airway motor outputs during sneezing and coughing in decerebrate cats. Neurosci. Res 32, 131–135. [PubMed: 9858020]

Schwarz EC, Thompson JM, Connor NP, Behan M, 2009. The effects of aging on hypoglossal motoneurons in rats. Dysphagia 24, 40–48. [PubMed: 18716837]

Setzke C, Broytman O, Russell JA, Morel N, Sonsalla M, Lamming DW, Connor NP, Teodorescu M, 2020. Effects of inhaled fluticasone propionate on extrinsic tongue muscles in rats. J. Appl. Physiol 128 (1985), 576–585. [PubMed: 31944881]

Smith KK, Kier WM, 1989. Trunks, tongues, and tentacles - moving with skeletons of muscle. Am. Sci 77, 29–35.

Smittkamp SE, Spalding HN, Brown JW, Gupte AA, Chen J, Nishimune H, Geiger PC, Stanford JA, 2010. Measures of bulbar and spinal motor function, muscle innervation, and mitochondrial function in ALS rats. Behav. Brain Res 211, 48–57. [PubMed: 20211206]

Smittkamp SE, Spalding HN, Brown JW, Yeh HW, Stanford JA, 2014. Relationships between tongue motility, grip force, and survival in SOD1-G93A rats. Physiol. Behav 125, 17–20. [PubMed: 24291387]

Sokoloff AJ, 2000. Localization and contractile properties of intrinsic longitudinal motor units of the rat tongue. J. Neurophysiol 84, 827–835. [PubMed: 10938309]

Spruijt BM, van Hooff JA, Gispen WH, 1992. Ethology and neurobiology of grooming behavior. Physiol. Rev 72, 825–852. [PubMed: 1320764]

Tolep K, Higgins N, Muza S, Criner G, Kelsen SG, 1995. Comparison of diaphragm strength between healthy adult elderly and young men. Am. J. Respir. Crit. Care Med 152, 677–682. [PubMed: 7633725]

Tomlinson BE, Irving D, 1977. The numbers of limb motor neurons in the human lumbosacral cord throughout life. J. Neurol. Sci 34, 213–219. [PubMed: 925710]

Van Selst M, Jolicoeur P, 1994. A solution to the effect of sample size on outlier elimination. Q. J. Exp. Psychol. A: Hum. Exp. Psychol 47A, 631–650.

Youmans SR, Youmans GL, Stierwalt JA, 2009. Differences in tongue strength across age and gender: is there a diminished strength reserve? Dysphagia 24, 57–65. [PubMed: 18690406]

Zhang C, Goto N, Suzuki M, Ke M, 1996. Age-related reductions in number and size of anterior horn cells at C6 level of the human spinal cord. Okajimas Folia Anat. Jpn 73, 171–177. [PubMed: 8942193]

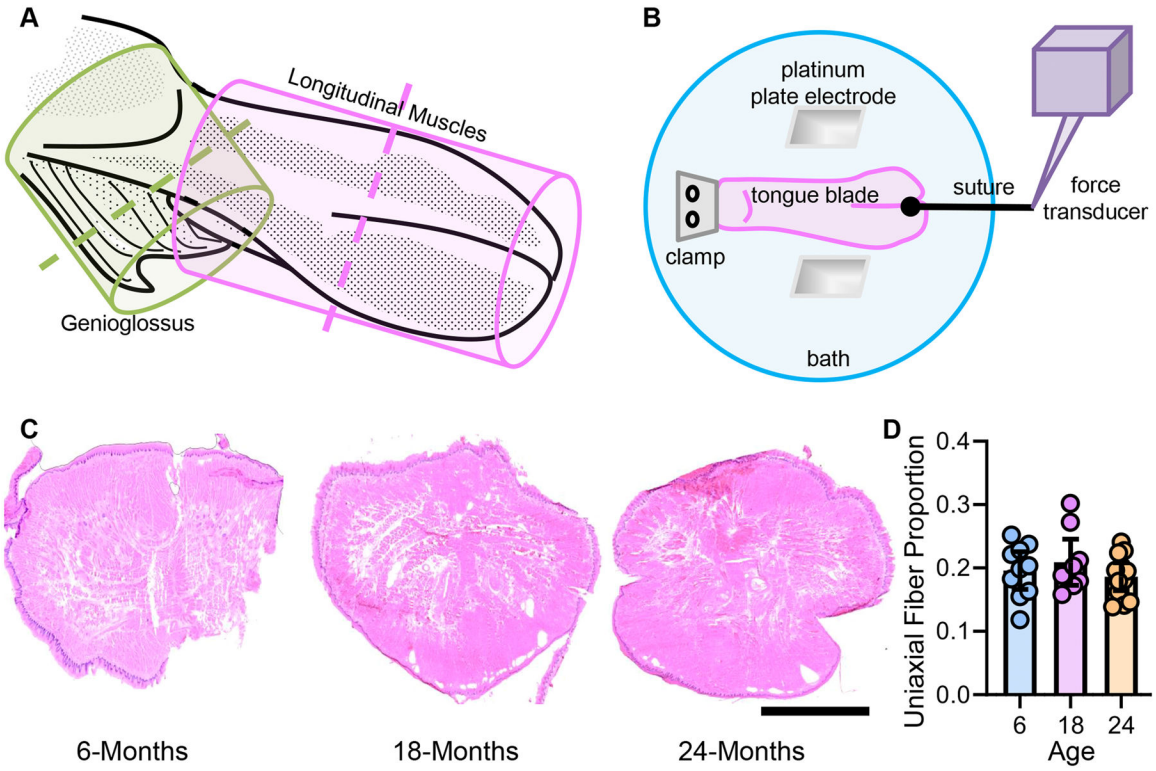

Fig. 1. Experimental design and evaluation of muscle fibre contributions to total tongue tissue bulk.

A shows a schematic of the longitudinal (lavender) and genioglossus (green) muscles. The plane of sectioning for CSA evaluations is shown by the dashed line. The blade of the tongue is excised from the base to exclude as much of the genioglossus as possible for assessing the uniaxial longitudinal muscle force. **B** shows the experimental apparatus for assessing longitudinal tongue muscle (lavender) uniaxial force, via stimulation with platinum plate electrodes in a tissue bath. **C** shows H & E staining of a transverse section of the proximal region of the tongue blade, with the superior and inferior longitudinal muscles in cross-section. **D** scatterplot (each symbol represents data from one rat) shows the proportion of muscle fibers in the uniaxial direction of longitudinal muscle force is not altered with age ($P = 0.46$; One-way ANOVA). All data mean ± 95% CI, $n = 10$ 6-month, $n = 9$ 18 month and $n = 12$ 24-month. Scale bar: 2500 μm.

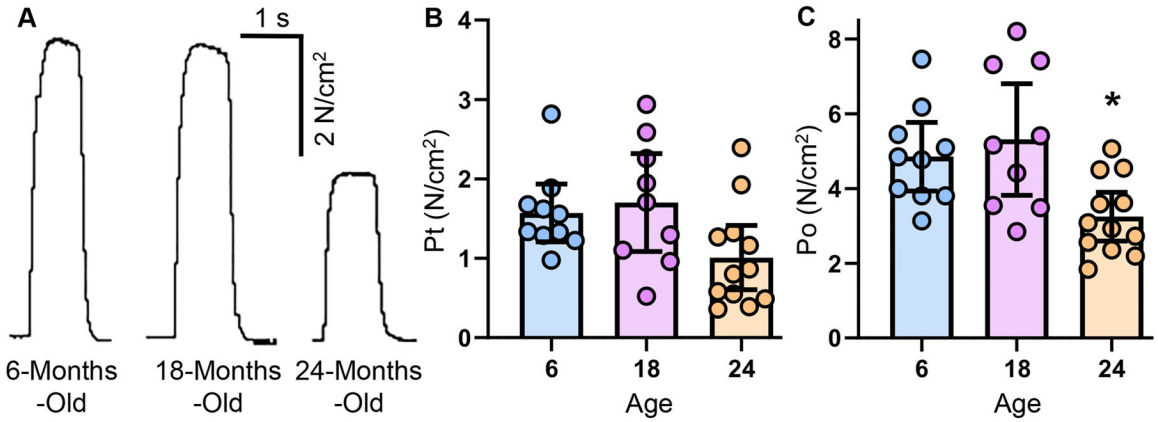

Fig. 2. Reduced longitudinal muscle force in old rats.

A shows representative traces of maximum longitudinal muscle specific force at 6-, 18- and 24-months old. **B** scatterplot of the twitch specific force (Pt, N/cm^2) showing an age dependent reduction in force (One-way ANOVA, $P = 0.045$). **C** Scatterplot of the maximum tetanic specific force (Po, N/cm^2) showing an age dependent reduction in force (One-wat ANOVA, $P = 0.004$), with Tukey's post-tests showing specific reductions between 24-months-old and 6- and 18-month-old rats (* Indicated $P < 0.05$ compared to all other groups). All data mean ± 95% CI, $n = 10$ 6-month, $n = 9$ 18 month and $n = 12$ 24-month.

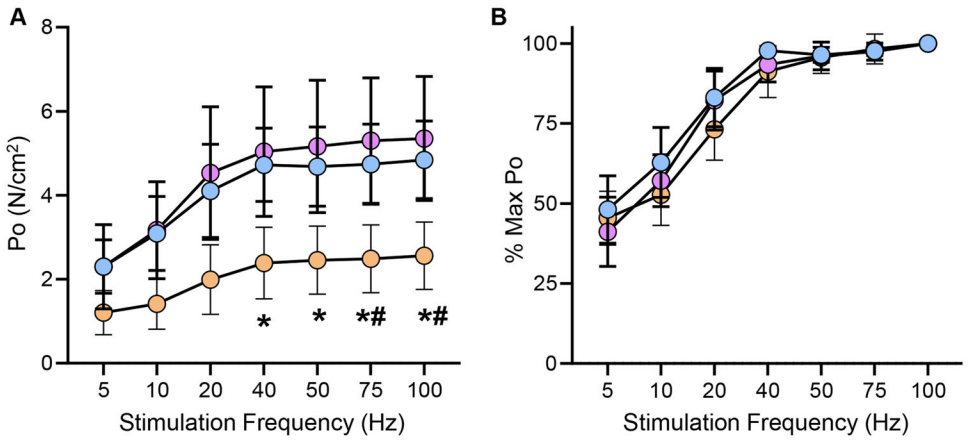

Fig. 3. Stimulation frequency and differences in aging longitudinal muscle force.

A Plot shows specific tetanic forces (Po, N/Cm^2) for the longitudinal muscles across a range of simulation frequencies was reduced with age (two-way ANOVA, $P < 0.0009$). At stimulation frequencies of 40 Hz and greater, forces at 24-months were reduced compared to 6-months-old (indicated by *; Bonferroni post-tests). At stimulation frequencies of 75 Hz and greater, forces at 24-months were reduced compared to 18-months-old (indicated by $^{\#}$; Bonferroni post-tests). **B** Plot shows specific tetanic forces relative to maximum tetanic force (%) were dependent on frequency, but not age for the longitudinal muscles (two-way ANOVA, $P = 0.394$). All data mean ± 95% CI, $n = 10$ 6-month, $n = 9$ 18 month and $n = 12$ 24-month.

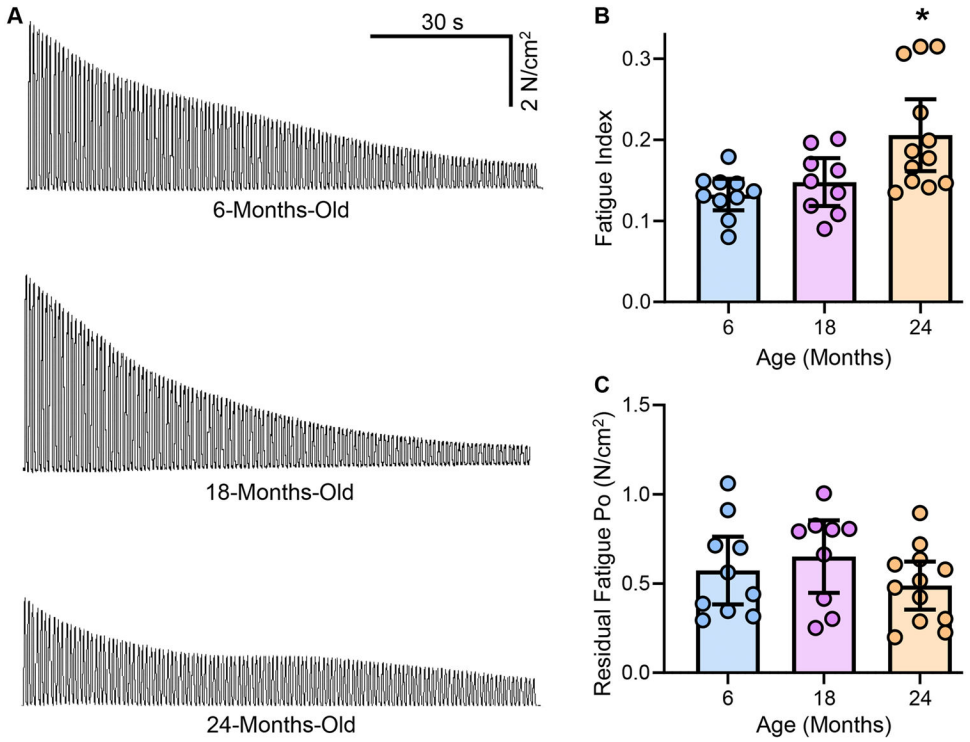

Fig. 4. Fatigue properties of differs between longitudinal muscles with aging.

A shows representative traces of specific force during 120 s of repeated contractions of longitudinal muscle in 6-, 18- and 24-month-old rats. **B** Scatterplot shows an increased fatigue index for the longitudinal muscle of 24-month-old rats compared to 6- and 18-month-old rats. $P < 0.0001$; One-way ANOVA with Tukey's post-tests, * indicates difference between 6- and 24-month-old and 18- and 24-month-old rats. indicates post hoc tests), transversalis and genioglossus muscles across a range of simulation frequencies. * . between genioglossus and longitudinal muscles ($P < 0.0001$) and the genioglossus and transversalis muscles. **C** Scatterplot of residual specific force (N/Cm^2) for the longitudinal muscles was not altered by age ($P = 0.335$; One-way ANOVA). All data mean ± 95% CI, $n = 10$ 6-month, $n = 9$ 18 mo*n*th and $n = 12$ 24-month.

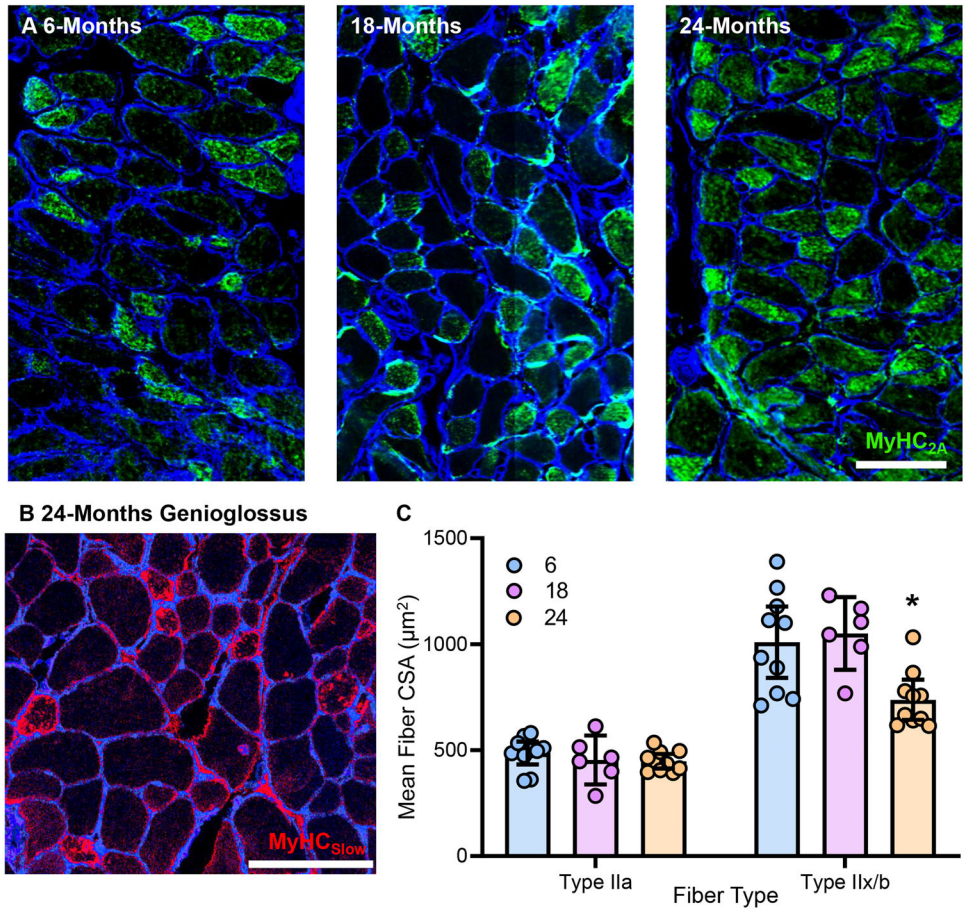

Fig. 5. Reduced CSA of type IIx/IIb longitudinal tongue muscle fibers in 24-month-old rats.

A Pictomicrographs of 6-, 18- and 24-month-old longitudinal muscles immunolabelled with laminin (blue) and myosin heavy chain 2 A ($MyHC_{2A}$ - green) antibodies. **B** Pictomicrograph of 24-month-old genioglossus showing robust expression of $MyHC_{slow}$ (red). **C** shows scatterplot (mean ± 95% CI) of the mean fiber CSA (μm^2) of type IIa and IIx/IIb longitudinal muscle fibers in 6-, 18- and 24-month-old rats, with reduced CSA of type IIx/IIb fibers of 24-month-old rats. $P = 0.0089$, Two-way ANOVA with Bonferroni post-tests, * indicates difference between 6- and 24-month-old and 18- and 24-month-old rats. $n = 10$ 6-month, $n = 6$ 18 month and $n = 10$ 24-month. Scalebar: 75 µm.

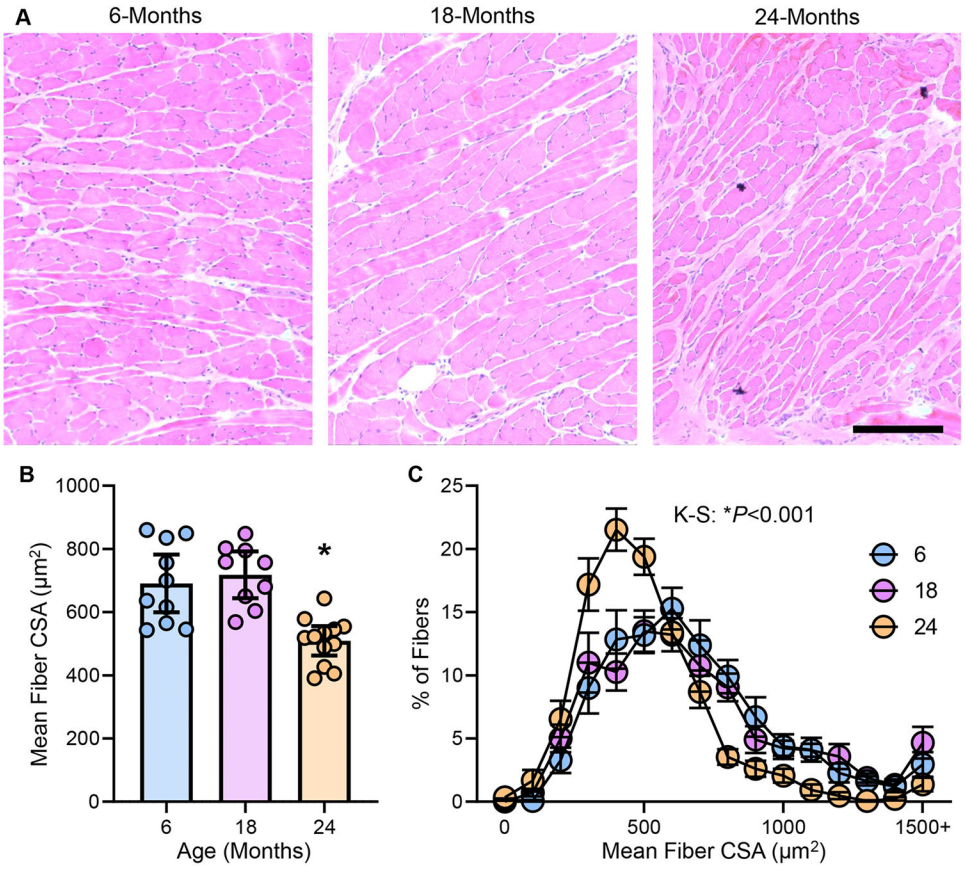

Fig. 6. Longitudinal muscle fiber CSA is reduced in aging.

A Photomicrographs show representative images of longitudinal tongue muscle fibres at 6-, 18- and 24-months-old. **B** Shows a scatterplot (mean ± 95% CI) of the mean longitudinal muscle fiber CSAs with marked reductions at 24- compared to 6- and 18-months-old ($P < 0.0001$; One-way ANOVA). * indicates $P < 0.05$ compared to all other muscles (Tukey's post-test). **C** Shows a frequency histogram (mean ± SEM) of the % of longitudinal fiber size distributions, with Kolmogorov-Smirnov tests showing a distribution favouring smaller fibers in 24-month old rats compared to younger ages. $n = 10$ 6-month, $n = 9$ 18 month and $n = 12$ 24-month. Scalebar: 100 μm.

Table 1

Sex Stratified Outcome Measures.

Parameter	Female (*n*)	Male (*n*)	Δ Change (% Female)	Sex Effect
Body Mass (g)	6: 336 ± 29 (*5*)	6: 440 ± 23 (*5*)	31%	**P* = 0.0005
	18: 389 ± 27 (*4*)	18: 491 ± 54 (*5*)	26%	
	24: 309 ± 10 (*5*)	24: 375 ± 12 (*7*)	21%	
Fiber Contribution (%)	6: 18.7 ± 2.2 (*5*)	6: 20.5 ± 1.6 (*5*)	10%	*P* = 0.974
	18: 21.7 ± 2.0 (*4*)	18: 20.3 ± 2.6 (*5*)	7%	
	24: 18.9 ± 1.4 (*5*)	24: 18.4 ± 1.5 (*7*)	3%	
Twitch Force (N/cm^2)	6: 1.5 ± 0.1 (*5*)	6: 1.7 ± 0.3 (*5*)	13%	*P* = 0.519
	18: 1.9 ± 0.5 (*4*)	18: 1.6 ± 0.3 (*5*)	16%	
	24: 0.8 ± 0.2 (*5*)	24: 1.1 ± 0.3 (*7*)	38%	
Maximum Tetanic Force (N/cm^2)	6: 4.6 ± 0.5 (*5*)	6: 5.1 ± 0.7 (*5*)	11%	*P* = 0.908
	18: 5.6 ± 0.6 (*4*)	18: 5.1 ± 1.1 (*5*)	9%	
	24: 3.1 ± 0.4 (*5*)	24: 3.3 ± 0.4 (*7*)	6%	
Fatigue Index	6: 0.123 ± 0.011 (*5*)	6: 0.142 ± 0.013 (*5*)	15%	*P* = 0.246
	18: 0.138 ± 0.023 (*4*)	18: 0.155 ± 0.016 (*5*)	12%	
	24: 0.188 ± 0.034 (*5*)	24: 0.219 ± 0.026 (*7*)	16%	
Residual Fatigue Force (N/cm^2)	6: 0.47 ± 0.08 (*5*)	6: 0.68 ± 0.14 (*5*)	45%	*P* = 0.270
	18: 0.68 ± 0.10 (*4*)	18: 0.63 ± 0.15 (*5*)	7%	
	24: 0.39 ± 0.09 (*5*)	24: 0.56 ± 0.08 (*7*)	44%	
Type IIa Fiber CSA (μm^2)	6: 491 ± 35 (*5*)	6: 481 ± 36 (*5*)	2%	*P* = 0.351
	18: 470 ± 97 (3)	18: 437 ± 20 (3)	7%	
	24: 472 ± 25 (*5*)	24: 423 ± 12 (*5*)	10%	
Type IIx/IIb Fiber CSA (μm^2)	6: 889 ± 67 (*5*)	6: 1129 ± 115 (*5*)	27%	*P* = 0.187
	18: 1046 ± 34 (*3*)	18: 1056 ± 145 (*3*)	1%	
	24: 714 ± 31 (*5*)	24: 761 ± 81 (*5*)	7%	
Overall Fiber CSA (μm^2)	6: 682 ± 56 (*5*)	6: 699 ± 64 (*5*)	3%	*P* = 0.259
	18: 694 ± 65 (*4*)	18: 737 ± 31 (*5*)	6%	
	24: 468 ± 30 (*5*)	24: 538 ± 25 (*7*)	15%	

All values mean ± SEM, with percentage changes (Δ Change) relative to female values. All tests Two-Way ANOVAs