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TITLE

Motor unit behavior adaptations across lifespan: sex differences in young, middle-aged and old adults

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ABSTRACT

Aging is associated with neuromuscular decline, but how sex modulates motor unit adaptations across adulthood remains unclear. This study examined age- and sex-related differences in motor unit firing behavior in young (YG), middle-aged (MA), and older (OLD) adults by integrating high-density surface EMG decomposition with assessments of muscle morphology and daily physical activity. Linear mixed-effects models revealed significant effects of age and sex on mean firing rate: females showed higher rates than males at 30% maximal voluntary contraction (MVC) in YG and MA groups, but not in OLD, and no sex differences were observed at 50% MVC. Firing-rate variability was consistently higher in females. During force-increasing contractions, OLD adults showed reduced motor unit discharge modulation; in early-recruited units, reductions were significant in OLD females relative to both YG and MA females, while males showed reductions across both early- and late-recruited units. Males exhibited greater muscle thickness, cross-sectional area, and maximal torque, and daily physical activity was lower in OLD participants. These findings indicate that neuromuscular aging is associated with reduced discharge-rate modulation and a convergence of motor unit behavior between sexes in older age. Physical activity may contribute, underscoring the importance of sex-sensitive strategies to preserve neuromuscular function.

Keywords

Motor unit, Aging, Sex differences, Neuromuscular adaptations, HDEMG decomposition

INTRODUCTION

Aging is a physiological process characterized by a progressive loss of muscle mass[1], strength[2] and power[3], accompanied by a decline in neuromuscular function[4]. Extensive evidence indicates that the nervous and musculoskeletal systems deteriorate in older adults, leading to several dysfunctions in motor coordination

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and of the excitation–contraction coupling[5]. Aging is linked to an adaptive remodeling of the neuromuscular system[6], which, however, still deteriorates from structural integrity and functional capacity perspectives[7,8] with significant consequences for motor performance[9,10].

Relevant components of the neuromuscular system, such as motor neurons, neuromuscular junctions, and muscle fibers, undergo significant changes with advancing age, leading to modifications in motor unit behavior across the lifespan[11–13]. One of the primary neurophysiological mechanisms associated with aging is a progressive loss of motor neurons, often accompanied by instability at the neuromuscular junction[7,14]. Between ~50 and 60 years of age, the body attempts to counteract the denervation of muscle fibers through reinnervation by surviving motor neurons, producing larger motor units and clustering of fibers[15,16]. As aging progresses, these denervation-reinnervation cycles become increasingly inefficient[8,17,18]. Around the eighth decade of life, denervation is thought to increasingly outpace reinnervation in many individuals, although the timing and magnitude of this process vary considerably between people leading to a net reduction in muscle fibers, especially type II fibers[15,19,20]. Neural changes therefore manifest not only as fewer motor units but also as reduced motor unit firing rates and increased synchronization, which could be seen as an effect of a reduced capacity of differentiating firing coding between motor units[12,21–24]. In addition, early-recruited motor units increase their firing rate to a lesser extent during rising-force contractions in older individuals[25,26]. Collectively, these modifications contribute to the decline in muscle force observed in older adults[27] and may also be associated with greater fatigability[28].

In this context, sex differences in motor unit characteristics, such as firing rate, recruitment threshold and discharge variability, have already been documented[29–37]. Young women tend to discharge low-threshold motor units at higher rates during steady, moderate, isometric contractions, whereas men may exceed women firing rates at maximal intensities[29,30,33,35]. Women also show larger estimates of persistent inward currents, hinting at intrinsic motoneuronal differences[31]. Interestingly, in highly active masters athletes, while motor unit remodeling occurs similarly in both sexes only women exhibit progressive slowing of motor unit discharge with advancing age[38]. Conversely, in less-active older cohorts, females maintain higher motor unit firing rates than age-matched males at a given relative torque yet still present smaller muscle cross-sectional area and poorer force steadiness[39]. Taken together, these findings may suggest that differences in muscle phenotype and lifelong physical-activity patterns interact to produce sex-specific adaptations of motor unit behavior across the adult lifespan.

In this scenario, much of our current understanding of motor unit firing properties in aging has been derived from studies that either exclusively sampled men or pooled the sexes[32]. Given the growing

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evidence that males and females display distinct neuromuscular aging trajectories—whether in terms of muscle-mass loss, firing-rate slowing or hormone-related modulation[39–41]—it has become more and more important to differentiate the age stratification by sex. Accordingly, the present study aims to characterize sex differences in motor unit recruitment and firing properties at two critical stages of later life: middle-aged and older adulthood. These cohorts are compared against young adults to determine how motor unit behavior diverges before overt neuromuscular decline sets in. Clarifying these variations is essential for designing interventions that preserve muscle function and physical performance in both women and men as the population ages[42]. Expanding motor unit research to include balanced male–female samples is therefore not only scientifically rigorous[32] but also central to developing effective, sex-sensitive health strategies[43].

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RESULTS

Muscle morphology

Subject-level analysis of muscle morphology revealed significant group differences across groups (Table 1). Muscle thickness showed significant overall effects of age ($p < 0.001$, $\eta p^2 = 0.173$) and sex ($p < 0.001$, $\eta p^2 = 0.195$), with males exhibiting greater values than females. CSA, assessed only in the MA and OLD groups, showed a strong sex-related effect ($p < 0.001$, $\eta p^2 = 0.402$), with greater values in males than females. Subcutaneous adipose tissue thickness showed the strongest sex effect among the morphological variables ($p < 0.001$, $\eta p^2 = 0.603$), being greater in females than males, together with an age effect ($p < 0.001$, $\eta p^2 = 0.180$).

Physical activity

Both total activity counts and active time differed significantly across groups (Table 1). Total activity counts showed significant effects of age ($p < 0.001$, $\eta p^2 = 0.178$) and sex ($p < 0.001$, $\eta p^2 = 0.189$), with a smaller Age × Sex interaction effect ($\eta p^2 = 0.033$). Active time showed a similar pattern, with significant effects of age ($p < 0.001$, $\eta p^2 = 0.197$) and sex ($p < 0.001$, $\eta p^2 = 0.222$), and a smaller Age × Sex interaction ($\eta p^2 = 0.052$). In line with the descriptive summaries, females tended to show higher activity levels than males in middle adulthood, whereas activity was lower in the OLD group.

Torque measures

MVC torque was lower in MA (estimated difference = -40.3 Nm, $p = 0.038$) and OLD (estimated difference = -97.4 Nm, $p < 0.001$) than in the YG group and was higher in males than females (estimated difference = 90.6 Nm, $p < 0.001$). The CoV of the torque (torque steadiness) at 30% and 50% MVC contractions is shown in Figure 1a. The linear mixed-effects model revealed a significant main effect of Age ($F(2,198) = 5.37$, $p = 0.005$, $\eta p^2 = 0.051$), whereas Sex, Intensity, and all interaction terms were not significant (all $p \geq 0.245$). Post hoc contrasts indicated that females had higher torque CoV than males in the MA and OLD groups at both intensities; for example, at 30% MVC the female–male difference was 1.79% in the MA group (95% CI 0.40 to 3.19, $p = 0.013$) and 2.33% in the OLD group (95% CI 0.77 to 3.88, $p = 0.004$). Age-related increases in torque CoV were most evident in females, who showed higher values in MA and OLD than in YG participants at both 30% and 50% MVC; for example, at 30% MVC the OLD–YG difference in females was 3.41% (95% CI 1.37 to 5.45, $p = 0.001$). In males, only the comparison between OLD and YG participants at 30% MVC was statistically significant (estimated difference = 2.14%,

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95% CI 0.16 to 4.12, p = 0.036).

Motor unit firing properties

Table 2 shows that motor unit yield was generally higher in males than females, both in terms of the total number of analyzed motor units and the average number of motor units identified per contraction and per participant. This sex difference was particularly evident in the YG group, in which males showed the highest overall yield. Across age groups, motor unit yield tended to be lower in females and somewhat reduced in OLD participants, although decomposition quality, as reflected by SIL values, was comparable across groups.

Figure 1b shows the average firing rate of all the decomposed motor units during the plateau phase of the contraction at 30% (left panel) and 50% (right panel) MVC. The linear mixed-effects model revealed significant main effects of Age ($F(2, 3069) = 3.41$, $p = 0.033$, $\eta p^2 = 0.002$) and Sex ($F(1, 3069) = 4.48$, $p = 0.034$, $\eta p^2 = 0.001$), as well as a significant Intensity × Sex interaction ($F(1, 3069) = 5.74$, $p = 0.017$, $\eta p^2 = 0.002$). All effect sizes were small. No main effect of Intensity was found ($F(1, 3069) = 0.61$, $p = 0.434$), and the remaining interactions were not significant (all $p \geq 0.237$). Post hoc contrasts indicated that at 30% MVC, females showed higher mean firing rates than males in the YG and MA groups (YG: estimated difference = 1.40 pps, 95% CI 0.10 to 2.70, $p = 0.034$; MA: estimated difference = 0.98 pps, 95% CI 0.20 to 1.77, $p = 0.014$), whereas no sex differences were observed in the OLD group ($p = 0.267$). At 50% MVC, no significant sex differences were detected in any age group (all $p \geq 0.351$). Regarding age-related comparisons, in females, mean firing rate was lower in OLD than YG participants at 30% MVC (estimated difference = -1.21 pps, 95% CI -2.39 to -0.02, $p = 0.047$) and lower in OLD than MA participants at both 30% MVC (estimated difference = -1.11 pps, 95% CI -1.98 to -0.24, $p = 0.013$) and 50% MVC (estimated difference = -1.45 pps, 95% CI -2.73 to -0.18, $p = 0.026$). No significant age-related differences were observed in males (all $p \geq 0.117$).

Figure 1c shows the CoV of the firing rate during the plateau for both 30% and 50% MVC. The linear mixed-effects model revealed a significant main effect of Sex ($F(1, 3069) = 7.22$, $p = 0.007$, $\eta p^2 = 0.002$), whereas the main effects of Age ($F(2, 3069) = 1.67$, $p = 0.189$, $\eta p^2 = 0.001$) and Intensity ($F(1, 3069) = 2.19$, $p = 0.139$, $\eta p^2 = 0.001$) were not significant. None of the interaction terms reached significance (all $p \geq 0.321$). Post hoc contrasts showed that females had higher firing-rate CoV than males at 30% MVC in the YG (estimated difference = 5.51%, 95% CI 1.49 to 9.53, $p = 0.007$), MA (estimated difference = 4.70%, 95% CI 2.21 to 7.18, $p < 0.001$), and OLD groups (estimated difference = 2.97%, 95% CI 0.10 to 5.84, $p = 0.043$). At 50% MVC, females also showed higher firing-rate CoV than males in the YG (estimated difference = 4.12%, 95% CI 0.22 to 8.01, $p = 0.038$) and OLD groups (estimated difference =

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3.17%, 95% CI 0.32 to 6.03, p = 0.029), whereas no sex difference was detected in the MA group (p = 0.105). Regarding age-related comparisons, in males, firing-rate CoV at 50% MVC was lower in the OLD than in the MA group (estimated difference = -3.67%, 95% CI -6.14 to -1.20, p = 0.003), whereas no other age contrasts reached significance.

Figure 2a shows the modulation of firing rates during the ramp of the five groups of motor unit recruited at progressively increasing torques (10% MVC intervals) for the 50% MVC contractions. The firing rates of motor units increased with the force level in all groups of participants, but this modulation was less prominent in the OLD than in the YG participants, particularly for females. Figure 2b summarizes this behavior through Δ firing rate, analyzed as a function of Age, Sex, and Recruitment Group (Early vs Late). The linear mixed-effects model revealed significant main effects of Age ($F(2,1175) = 7.51$, $p = 0.00057$, $\eta p^2 = 0.013$) and Recruitment Group ($F(1,1175) = 4.14$, $p = 0.042$, $\eta p^2 = 0.004$), as well as a significant Age × Sex × Recruitment Group interaction ($F(2,1175) = 4.17$, $p = 0.016$, $\eta p^2 = 0.007$). The main effect of Sex was not significant ($F(1,1175) = 1.83$, $p = 0.176$), and none of the two-way interactions reached significance (Age × Sex: $p = 0.062$; Age × Recruitment Group: $p = 0.067$; Sex × Recruitment Group: $p = 0.076$). Post hoc contrasts did not reveal significant sex differences in Δ firing rate within any Age × Recruitment Group combination (all $p \geq 0.149$). In early-recruited motor units, OLD females showed lower Δ firing rate than YG females (estimated difference = -1.63 pps, 95% CI -3.20 to -0.07, p = 0.041) and than MA females (estimated difference = -2.29 pps, 95% CI -3.45 to -1.13, $p < 0.001$). In males, early-recruited motor units also showed lower Δ firing rate in the OLD than YG (estimated difference = -1.97 pps, 95% CI -3.22 to -0.60, p = 0.004). For late-recruited motor units, the age-related reduction was evident in males, with lower Δ firing rate in the OLD than in the YG group (estimated difference = -1.47 pps, 95% CI -2.74 to -0.20, p = 0.024) and in the OLD than in the MA group (estimated difference = -1.36 pps, 95% CI -2.42 to -0.31, p = 0.012). No significant age-related contrasts were observed in late-recruited motor units in females.

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DISCUSSION

This study was designed to investigate motor unit behavior across different stages of adulthood, with a particular focus on sex-related differences in neuromuscular aging. The rationale stems from growing evidence that aging affects neuromuscular system differently in males and females, yet few studies have examined these changes in parallel across multiple age groups. By integrating HDEMG-based motor unit analysis with the assessment of muscle morphology and daily physical activity, we aimed to provide an integrated characterization of age- and sex-related differences across neural, muscular, and behavioral domains.

Overall, the results indicate that aging is associated with alterations in motor unit firing behavior, particularly a lower capacity to modulate discharge rate during force-increasing contractions. The reduction in discharge-rate modulation was evident in OLD adults, consistent with a decline in rate-coding capacity. In contrast, sex differences in motor unit firing properties were more dependent on contraction intensity and age group: females exhibited higher firing rates at lower contraction intensities in YG and MA adults, whereas these differences were attenuated in older age and at higher force levels. Notably, while sex differences in muscle morphology and maximal torque were consistently observed across groups, motor unit differences appeared more dependent on contraction conditions and age group. Together, these findings indicate that neuromuscular changes in motor unit discharge behavior are more strongly modulated by the combined effects of age and sex than muscle structural or mechanical features, suggesting that alterations in neural control may represent a more significant indicator of neuromuscular aging — one that manifests differently between sexes.

Our findings indicate significant differences in mean motor unit firing rate across age groups and between sexes, with a significant Sex × Intensity interaction. Sex differences in motor unit firing rates were evident only at 30% MVC, with females exhibiting higher firing rates than males in the YG and MA groups, whereas no sex-based difference was observed in the OLD group. Notably, this convergence of MUs behavior in older age was accompanied by a significant reduction in firing rate from MA to OLD in females, which a) was not observed in males, and b) was evident at both contraction intensities. Therefore, similar firing rates were observed between sexes in the OLD group at both 30% and 50% MVC.

These findings partly align with previous reports showing higher discharge rates in females during submaximal contractions[29], and may reflect differences in neural strategies employed to generate force, possibly compensating for the smaller muscle size and lower force-generating capacity that can be found for female volunteers [12,33,35]. However, the reduction in firing rate observed in females with advancing age

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may indicate a reduced ability to sustain such strategies, contributing to the attenuation of sex differences in OLD adults. While the underlying mechanisms cannot be determined from the present data, these patterns may reflect age-related changes in motor unit properties, such as altered motoneuron excitability or contractile function[24,57]. In addition, differences in physical activity across groups may also contribute to these patterns, although this aspect is considered in more detail below.

The modulation of firing rates during force ramps appeared less pronounced in the OLD group, consistent with an age-related reduction in the ability to increase firing rates with increasing force demand[26]. This pattern was reflected in the flattening of the firing rate profiles in OLD adults, particularly in the lower threshold motor unit groups (Figure 2), and was captured by the Δ firing rate analysis. Overall, aging was associated with a reduced capacity to modulate motor unit discharge, although this pattern varied across recruitment groups and sexes.

Age-related reductions in Δ firing rate were most evident in early-recruited motor units in females, where OLD participants showed lower values than both YG and MA participants. In males, Δ firing rate reductions were observed in OLD in both early-recruited motor units relative to YG and in late-recruited motor units relative to both YG and MA. Notably, no clear sex differences in Δ firing rate emerged within specific age or motor unit recruitment groups (early- vs. late- recruited), indicating that the modulation capacity of the motoneuron pool is altered with aging but does not consistently affect one sex over the other. These findings are consistent with previous evidence showing that aging is associated with a reduced ability to modulate motor unit discharge according to force demands, reflecting a compromised rate-coding capacity[14,25,26], also linked to reductions in both muscle quantity and quality[58]. However, the reduced Δ firing rate observed in OLD adults should be interpreted cautiously, as this metric reflects the net modulation of motor unit discharge during voluntary contractions rather than a single physiological mechanism. Changes in this measure may arise from alterations in descending drive, intrinsic motoneuron excitability, or both[59], which cannot be disentangled in the present dataset.

The different behavior observed between early- and late-recruited motor units further suggests that aging may not affect the full motor unit pool uniformly. Early-recruited units showed clearer age-related reductions in females, whereas in males changes were observed across a broader range of recruitment thresholds. In fact, the reduction in Δ firing rate from MA to OLD adults appeared more pronounced in females for early-recruited motor units, whereas this transition was less evident in males, contributing to a convergence of modulation capacity in older age. Given the role of early-recruited motor units in the control of low-force contractions[60], these subgroup-specific patterns may have functional implications, as knee extensor torque values between 10 and 30% of MVC are similar values to the force expressed during

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the stance phase in walking[61].

Another possible explanation for the observed subgroup-specific aging patterns is that endocrine changes may interact with neuromuscular adaptations across the adult lifespan. In particular, menopause-related hormonal changes[62,63] have been proposed as a factor influencing neuromuscular function[41,64], and fluctuations in estrogens appear to modulate motor unit behaviour[31,65]. In our cohort, all MA female participants were postmenopausal, with an average age at menopause of 51 ± 4 years and an average of 7 ± 5 years since menopause at the time of enrolment. However, because circulating hormone levels were not measured in the present study, this interpretation remains hypothetical and should be addressed directly in future work.

Another interpretive lens relates to fiber-type composition and its remodeling with aging. Males typically have a higher proportion of type II fibers, which are considered more susceptible to age-related deterioration, including denervation[20]. Evidence suggests fiber type-specific atrophy[19], with type II (fast-twitch) fibers showing a greater reduction from young to old age, while type I (slow-twitch) fibers appear to be relatively better preserved[66]. In principle, these processes could contribute both to the general morphological decline observed with aging and to recruitment-dependent changes in motor unit firing modulation. Motor unit loss cannot be excluded as a contributing factor, as previous work has linked lower motoneuron number estimates with sarcopenia in older adults after adjustment for sex[6]. In the present study, the segmentation of motor units by recruitment threshold and the analysis of firing rate modulation revealed that age-related differences in Δ firing rate were not uniform across early- and late-recruited units, suggesting that aging may affect different portions of the motor unit pool in different ways. However, because muscle fiber type, denervation status, and reinnervation processes were not measured directly, any link between the present motor unit results and fiber-type-specific remodeling remains speculative.

In terms of mechanical output, torque variability increased with age, where females demonstrated higher torque variability than males in the MA and OLD groups, whereas no sex difference was detected in the YG group. The observed decline in torque steadiness in females may be influenced by multiple factors including musculotendinous properties (e.g., reduced tendon elasticity[67]) and lower absolute strength[30]. In fact, MA and OLD females showed higher torque variability than YG females, whereas age-related differences in males were less consistent. These findings are in line with previous work showing reduced steadiness of force production with aging [50,51,68]. Females also exhibited greater variability in motor unit firing rates (CoV of firing rates), which may contribute to their poorer torque steadiness and could reflect differences in the neural drive to the active motor neuron pool[23]. However, despite poorer torque

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steadiness, the OLD group showed lower firing-rate variability in some conditions, particularly at higher contraction intensity. This may reflect reduced flexibility in motor output and lower discharge modulation capacity in older adults, such that even relatively small fluctuations in neural drive may have a proportionally larger effect on force output when muscle strength is reduced[50]. In addition, recent evidence suggests that force steadiness during steady contractions is modulated not only by single motor unit discharge variability, but also by the variance of the common synaptic input to the active motor neuron pool[69]. Accordingly, the higher torque variability observed in some of our groups may reflect differences in the structure of neural drive during the plateau phase, including the balance between common input and independent noise, rather than changes in average motor unit firing variability alone. Because common synaptic input was not directly quantified in the present study, this interpretation should be considered mechanistic context rather than a direct inference from our data.

In contrast to the motor unit results, morphological variables showed relatively preserved sex-related differences across the studied groups. Males exhibited greater muscle thickness than females across all ages. In addition, CSA, which was available only in the MA and OLD groups, was greater in males than females. Together with the marked sex difference in MVC torque, these findings indicate that sex-related differences in muscle size and strength remain evident with aging. Aging was nonetheless accompanied by lower MVC and modest reductions in some morphological measures, indicating that structural decline still contributes to the overall phenotype in both sexes [46,70,71].

Finally, physical activity also differed across groups in line with age-matched data in literature [72]. Daily physical activity (TAC and Active Time) was similar between YG and MA but reduced in OLD. Interestingly, sex differences were most evident in middle adulthood, where females showed a more active behavior compared to males, partially in accordance with Belcher et al., 2021[73]. Because physical activity is known to influence neuromuscular function[74], it represents a relevant contextual factor when interpreting the present motor unit findings.

Exploratory covariate-adjusted analyses indicated that the principal findings for mean firing rate were unchanged after accounting for TAC and muscle thickness, and that neither covariate was itself a significant predictor. For firing-rate variability, the main effect of sex remained robust across adjusted models, whereas age-related effects were weaker and less consistent. Accordingly, these results suggest that physical activity and muscle morphology may contribute to the observed variability, but, within our cohorts, do not fully explain the main age- and sex-related patterns in motor unit behavior observed.

Some limitations should be considered when interpreting these findings. First, the cross-sectional design does not allow direct inference on within-person neuromuscular trajectories with aging, and therefore

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the observed differences between age groups should not be interpreted as definitive longitudinal changes. In addition, the dataset shows an imbalanced sex distribution across some age groups, particularly in the older cohort, where the number of females was lower than the number of males. This reflects, in part, the fact that the present dataset was drawn from the broader TRAJECTOR-AGE cohort under predefined eligibility criteria, but it nonetheless reduces the precision of sex-specific estimates in older females and limits the strength of inference regarding sex-by-age patterns.

A second limitation concerns the identification of motor units from HDEMG signals. Decomposition yield can be influenced by anatomical and recording-related factors, including subcutaneous tissue thickness, muscle size, and muscle–electrode distance, all of which affect the detectability of surface motor unit action potentials. In our cohort, females showed greater subcutaneous tissue thickness, which may have attenuated surface motor unit action potential amplitude and contributed to the lower number of reliably identified motor units. Therefore, the sex-related difference in motor unit yield may reflect both physiological differences and reduced decomposition detectability. Although the use of linear mixed-effects models allowed us to account for the hierarchical structure of the data, residual methodological bias in motor unit detection cannot be fully excluded. Future studies should combine repeated longitudinal assessments with protocols specifically designed to quantify decomposition reliability and potential sex-related detection bias.

In conclusion, this study provides new insights into the sex- and age-related differences in neuromuscular function by simultaneously assessing muscle morphology, motor unit behavior, and physical activity across adulthood. The results highlight that while males and females may adopt distinct neuromuscular strategies during early and middle adulthood, advanced age was associated with a convergence in motor unit behavior between sexes. The greater torque variability and higher motor unit firing rates observed in females may reflect compensatory neural strategies to offset smaller muscle size and lower force-generating capacity. On the other hand, the diminished modulation of firing rates and reduced discharge rates in older adults indicate a decline in motor unit control with aging. Taken together, these results point to a multifactorial deterioration in motor unit control with aging, influenced by neural, muscular, and biomechanical factors. These findings emphasize the importance of considering sex in the study of neuromuscular aging and suggest that preserving motor unit recruitment and discharge properties may be critical targets for interventions. Future studies should better understand the mechanisms (such as cumulative motor neuron loss, fiber-type homogenization, and impaired rate-coding mechanisms) driving these age- and sex-related differences in neuromuscular function particularly exploring the interplay between muscle morphology, endocrine changes, motor unit behavior, and the role of exercise training in mitigating their decline, with the goal of developing sex-specific strategies to preserve neuromuscular

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function in aging populations. These data will also help to improve physical activity guidelines and pose the basis for more tailored approaches. Finally, longitudinal assessment of these factors over two or more years are warranted to better understand their actual trajectories across life span.

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METHODS

This study is part of a longitudinal project named TRAJECTOR-AGE[44] aiming to investigate the 2-year time course of neuromuscular, structural, and metabolic changes and their interplay occurring with aging. All the procedures used in this study comply with the Declaration of Helsinki and are registered at http://clinicaltrials.gov (NCT06168591) and were approved by the AVEN Ethical Committee (Emilia Romagna region, Italy) on July 5, 2022 (protocol #28022; study ID 283/2022/SPER/UNIPR).

Subjects

Sixteen young (YG) adults, forty-six middle-aged (MA) adults, and thirty-five old (OLD) adults were enrolled in this study. The anthropometric characteristics of the participants are reported in Table 3. The YG group was composed of healthy participants from 20 to 32 years old with no neurological and musculoskeletal impairments or disease. The MA and OLD community-dwelling, mobile, and cognitively unimpaired participants were enrolled based on the following criteria: Mini-Mental State Examination score ≥ 24, gait speed > 0.8 m/s (assessed over 4 meters), Short Physical Performance Battery score > 9, Timed Up-and-Go test < 20 seconds, and grip strength > 27 kg for males or > 16 kg for females. Exclusion criteria comprise neurological disorders (including stroke), diabetes, late-stage cancer, severe chronic kidney disease, severe liver insufficiency, severe cardiac disorders, major recent heart surgery, and severe osteoarthritis. All participants signed written informed consent for the study after a detailed explanation of the purposes and procedures.

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Experimental protocol

High-density electromyographic (HDEMG) signals and force profiles were detected from the vastus lateralis (VL) during varying-force, isometric contractions (Figure 3). At the beginning of the experimental procedure, the muscle architecture of all participants was assessed to document the anatomical and morphological characteristics of the analyzed muscle. Ultrasound images of VL muscle were acquired with participants lying relaxed supine, maintaining a hip angle of 160° (see *Ultrasound images recordings* for detailed procedures).

Following ultrasound imaging recordings the neuromuscular evaluation was conducted. Participants were seated on a leg extension machine (Figure 3a, BH L010 Leg Extension, BH Fitness-EXERCYCLE, S.L., Vitoria-Gasteiz, Spain) with the right leg secured to the pushing arm at a knee angle of 120° (where 180° is the full extension). The knee extension machine was instrumented to measure force through a load cell under isometric conditions (see *To* improve consistency of ultrasound measurements, acquisitions

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were performed by trained operators using standardized anatomical landmarks and acquisition procedures. However, repeated measurements were not collected in the present dataset, and therefore intra- and inter-rater reliability indices could not be quantified directly.

Force recordings for details). The time course of the recorded torque and the target torque level were displayed to the participants on a monitor as visual feedback. During the warm-up phase, participants were instructed on the use of the visual feedback by performing contractions of varying intensities, ranging from low to high. Subsequently, each participant performed three maximal voluntary contractions (MVCs), each sustained for 5 seconds. The highest recorded torque value from these trials was designated as the MVC torque and served as a reference for subsequent contractions. Isometric knee extensions of the VL muscle were then performed while force and HDEMG signals were recorded (see *HDEMG recordings* for detailed methodology). Participants were required to match a trapezoidal force profile displayed on the feedback screen. The trapezoidal profile consisted of three phases: (i) a ramp phase from 0% to 30% or 50% of MVC with a slow force increase (2% MVC/s); (ii) a plateau phase lasting 15 seconds at either 30% or 50% of MVC; and (iii) a descent phase from 30% or 50% MVC back to 0% at the same rate of force change as the initial ramp phase. Participants performed one trapezoidal contraction at 30% MVC during the training phase. Following this, four contractions were recorded: two trapezoidal contractions with a plateau at 30% MVC, two with a plateau at 50% MVC. All contractions were separated by two minutes of rest.

At the end of the experimental protocol, the participants were also monitored during one week with an actigraphy to measure the level of general physical activity during normal life (see *Physical activity* assessment paragraph for detailed methodology).

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Ultrasound images recordings

Ultrasound imaging (Esaote, Genoa, Italy) was performed by two expert operators (CMB, MVF) using a linear probe (LA523, frequency range 6-12 MHz) to measure VL cross-sectional area (CSA), muscle thickness, and subcutaneous adipose tissue thickness. The assessments were performed with the probe placed at 50% between the greater trochanter and the distal boundary of the lateral femoral condyle[45,46] using water-based conductive gel applied to the surface of the ultrasound probe, improving the acoustic coupling while minimizing contact pressure on the skin. CSA was assessed with the probe transversally aligned to the muscle using the extended-field-of-view. After identification of the border where the VL meets the rectus femoris, the probe was aligned and moved slowly and continuously in the medio-lateral direction to construct the image of the whole muscle[47]. Images were also acquired with the probe placed

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longitudinal to the muscle to standardize the point in which muscle thickness and subcutaneous adipose tissue thickness were assessed. A snapshot was performed at 50% of the femur length and at 50% of the distance between the mid-and lateral ends of the VL. For the YG individuals, only the snapshot of the VL (ArtUs, Telemed) was acquired with a 38.4 mm linear transducer (L12-5N40-A4 - frequency range 5-12 MHz) using the same procedure described above. To improve consistency of ultrasound measurements, acquisitions were performed by trained operators using standardized anatomical landmarks and acquisition procedures. However, repeated measurements were not collected in the present dataset, and therefore intra- and inter-rater reliability indices could not be quantified directly.

Force recordings

The force exerted during knee extension contractions was measured with a load cell (MODEL TF 031, full scale 100 kg, sensitivity 2 mV/V, CCT Transducers, Torino, Italy) placed in series with the cable of the leg extension machine. The load cell output was amplified with a general-purpose acquisition system (GAM, ReC Bioengineering and LISiN, Politecnico di Torino, Turin, Italy) synchronized with the HDEMG amplifier. To provide real-time feedback on the force generated by the participant, a custom script developed in MATLAB (R2022b, The MathWorks Inc., MA, USA) was used to display the force levels on a monitor during the experiment.

HDEMG recordings

Muscle activity of the VL was recorded using a 64-electrode grid (arranged in 8 columns with a 10 mm inter-electrode distance and 8 rows with a 20 mm inter-electrode distance), covering an area of 70 × 140 mm (Figure 3b) specifically designed for the study. Ultrasound imaging was employed to identify the direction of the muscle fascicles at 50% of the femur length. After skin preparation, the electrode grid was securely positioned over the belly of the VL muscle (Figure 3b), ensuring that 50% of the femur length was aligned between the second and third columns (from proximal to distal), with the rows aligned to the fascicles' direction previously identified.

Monopolar HDEMG signals were detected and conditioned (bandwidth 10–500 Hz, gain 192), then sampled at 2048 Hz with 16-bit resolution using a wireless high-density electromyography acquisition system (MEACS, ReC Bioengineering and LISiN, Politecnico di Torino, Turin, Italy)[48]. Electrophysiological signals and torque data were synchronized during post-processing using common external pulses that were simultaneously recorded by both the HDEMG system and the torque measurement system[49].

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Physical activity assessment

Physical activity was assessed using the triaxial wrist-worn Actigraph GT9X Link (Actigraph, Pensacola, FL, USA) accelerometer on the non-dominant wrist with a sampling frequency of 60 Hz. Participants were fitted with the accelerometer and instructed to wear it for the next 7 days, 24 h/day, in the free-living environment. After completing the 7-day data collection, participants returned the accelerometer to the clinical research center.

Measurements of the VL muscle morphology

The captured images were subsequently analyzed using ImageJ software (National Institutes of Health, USA). CSA was analyzed using the freehand tool and determined as the maximum muscle area excluding aponeurosis; muscle thickness was determined as the vertical distance between superficial and deep aponeurosis; and subcutaneous adipose tissue thickness was determined as the vertical distance between the skin and the superficial aponeurosis. For the YG participants, only the longitudinal snapshot of the vastus lateralis was acquired using a different ultrasound system (ArtUs, Telemed), allowing the assessment of muscle thickness but not CSA. Accordingly, CSA was available only for the MA and OLD groups, whereas muscle and subcutaneous thicknesses were compared across all age groups.

Torque signal

Force signals were converted into torque by multiplying by the constant lever arm of the leg extension and then low-pass filtered at 100 Hz (4th-order Butterworth). Plateau onset was identified automatically from the torque trace after low-pass filtering at 2 Hz, as the point at which torque reached the target level and stabilized. Torque steadiness and motor unit firing properties (see *Analysis of motor unit firing*) were quantified over a fixed 10-second window within this steady segment, excluding ramp-up and ramp-down phases. Torque steadiness was defined as the coefficient of variation (CoV) as the percentage ratio between the standard deviation and the mean value[50,51].

HDEMG signals decomposition and editing

The HDEMG signals were bandpass filtered within the 20–400 Hz range using a 4^{th} order, bidirectional Butterworth filter. Following this preprocessing, the signals were decomposed into individual motor unit firing patterns using a validated method based on convolution kernel compensation (CKC) (Holobar et al., 2010; Holobar & Zazula, 2007). This decomposition was performed with a MATLAB-based software (DEMUSE, version 4.0, System Software Laboratory, Faculty of Electrical Engineering and Computer

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Science, University of Maribor, Slovenia). Candidate motor units were first screened using the decomposition quality threshold (SIL > 0.8) adopted in our workflow before manual inspection. Thereafter, all discharge trains were visually inspected and, when necessary, edited by two experienced operators (MC and MB) according to established recommendations for motor unit analysis[52,53]. During this procedure, motor units were retained only when discharge patterns were judged physiologically plausible and sufficiently stable over time. Inspection included evaluation of discharge frequency and regularity (acceptable firing rate in the range 5-30 Hz and coefficient of variation < 50%), and the presence of missed or spurious discharges. Operators were blinded to participant age group and sex during editing, and uncertain cases were resolved by consensus. We report silhouette values (SIL) for the retained motor units as an index of source separation quality[54].

Analysis of motor unit firing

Motor unit behavior was assessed by computing the following variables of interest: (i) the mean and the CoV of the firing rate (pulses per seconds, pps) of all the decomposed motor units during the constant-torque phase (plateau) at 30% and 50% of the MVC, (ii) the difference between the firing rates of motor units grouped for recruitment torque intervals (from lower to higher threshold motor units), (iii) the firing rate modulation as a function of the exerted torque.

The ramp-up phases of the torque were segmented into intervals corresponding to 10% of the MVC to group the decomposed motor units based on their recruiting threshold (Figure 4a). This segmentation resulted in three motor unit groups for the 30% MVC trials (Figure 4a) and five motor unit groups for the 50% MVC trials. For each motor unit group, the mean firing rates were computed for the torque interval in which motor units were recruited and for the following ones (Watanabe et al., 2016). Figure 4b shows an example for the 30% MVC contraction: the mean firing rate for first group (i.e. motor unit recruited within the 0-10% MVC interval) was evaluated across all three contraction intervals (0-10%, 10%-20%, and 20%-30% MVC). For the group of motor units recruited within the second interval (i.e. 10-20% MVC), the mean firing rate was computed only for the 10-20% and 20-30% MVC intervals. Similarly, this approach was applied to the 50% MVC contractions considering five motor unit groups and five contraction intervals [26].

The modulation of motor unit firing rates was quantified for each motor unit as the difference between the mean firing rate at the maximum torque and the mean firing rate at the recruitment interval (Δ firing rate). This analysis was performed for the 50% ramp, and the motor units were split into two groups, those recruited between 0 and 20% MVC (early-recruited motor units) and those recruited between 20

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and 40% MVC (late-recruited motor units).

Measurements of physical activity

Accelerometer data were downloaded and pre-processed using the ActiLife software (version 6.13.4) to derive 1-min epoch activity counts. Only participants with ≥4 valid days of accelerometer data were included in the analysis. A valid day was defined as ≤10% missing data; non-valid days were excluded. We calculated the total activity counts (TAC) per day and the time spent in active state. TAC was derived from the sum of activity counts for each minute during a valid day. Because the accelerometer was worn on the non-dominant wrist, sedentary and active time were classified using the cut-point validated for this specific placement [55]: minutes with <1853 counts were classified as "sedentary", whereas minutes with ≥1853 counts were classified as "active"[55]. Data were processed using the *R arctools package*[56].

Statistics

All statistical analyses were performed in MATLAB (v. R2025b, MathWorks, Natick, MA).

MVC torque, VL muscle thickness, subcutaneous adipose tissue thickness, CSA, physical activity metrics (total activity counts and active time), and torque steadiness (coefficient of variation of torque during the plateau phase) were analyzed at the subject level. MVC torque, CSA, muscle thickness, and subcutaneous adipose tissue thickness were modelled using linear models with Age (YG, MA, OLD), Sex (F, M), and their interaction as fixed factors and BMI as a covariate. Physical activity metrics were analyzed using linear models with Age, Sex, and their interaction.

Torque steadiness was analyzed using a linear mixed-effects (LME) model with Age, Sex, and contraction Intensity (30% MVC, 50% MVC) as fixed factors, including all two-way and three-way interactions, and Subject as a random intercept (i.e., 1 | Subject) to account for repeated measurements across intensities. Planned post-hoc contrasts were computed to evaluate: (i) sex differences within each Age × Intensity combination, and (ii) pairwise age group differences within each Sex × Intensity combination.

For each motor unit firing property (mean firing rate and coefficient of variation of the firing rate), an LME model was fitted, with Age, Sex, and Intensity as fixed factors, including all two-way and three-way interactions. To account for the repeated-measures structure of the data — multiple motor units recorded from the same subject across intensities — Subject was included as a random effect, with Intensity as a random slope (i.e., Intensity | Subject). Following the omnibus model, post hoc contrasts were used to evaluate sex differences within each Age × Intensity combination and age-group differences within each Sex × Intensity combination.

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For the analysis of Δ firing rate, restricted to the 50% MVC contractions, a separate LME was fitted including Age, Sex, and Recruitment Group (Early: groups 1–2; Late: groups 3–4) as fixed factors, with Subject as a random intercept (i.e., 1 | Subject).

For the mixed-effects models, fixed effects were summarized using F statistics, exact p-values, and approximate partial η (equation 1).

$$\eta_p^2 = (F \times df_1) / (F \times df_1 + df_2) \quad (1)$$

The significance threshold was set at α = 0.05, and results are reported as F(df1, df2) = value, p = value. Significance levels in figures are denoted as: * p < 0.05, ** p < 0.01, *** p < 0.001.

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DATA AVAILABILITY

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

CODE AVAILABILITY

Custom analysis code used in this study is available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

F.L., R.R, S.P., M.V.F., and A.B. conceptualized the project. M.C. and A.B. conceived the study and designed the experiments. M.C., M.B., M.V.F., A.P. and C.M.B. performed the experiments and collected the data. M.C., M.B., A.P. and C.M.B. analyzed the data. M.C., M.B., C.M.B., M.V.F., and A.B interpreted the data results. M.C., and A.B. wrote the original draft. All authors reviewed, contributed, approved the final manuscript, and are accountable for all aspects of the work. All people designated as authors qualify for authorship, and all those who qualify for authorship are listed.

COMPETING INTERESTS

The Authors declare that the research was conducted in the absence of any conflict of interest.

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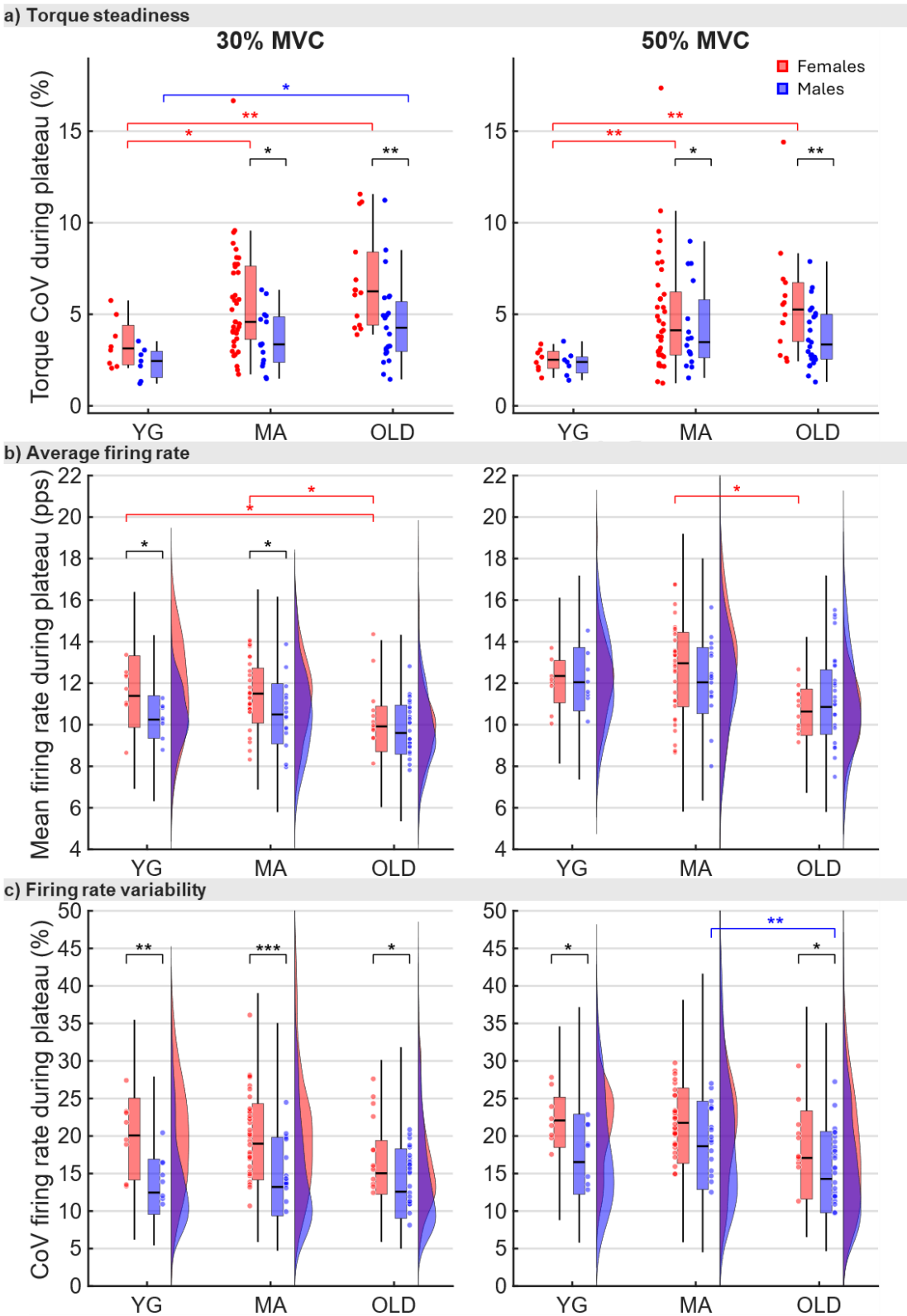


Figure 1 Results of torque steadiness, average firing rate, and firing rate variability. a) Boxplots and jitter plots of the coefficient of variation of the torque during the plateau phase of both 30 and 50% MVC

contractions for all the subjects divided by age group and sex. b) Boxplots and probability density functions of mean firing rate during plateau, and c) coefficient of variation of the firing rate for all the motor units divided by age group and sex. Plotted points represent participant-level summaries (i.e., each point corresponds to a subject's mean). Statistical significances are reported in black for comparison between sex and with the respective color for comparison among age groups.

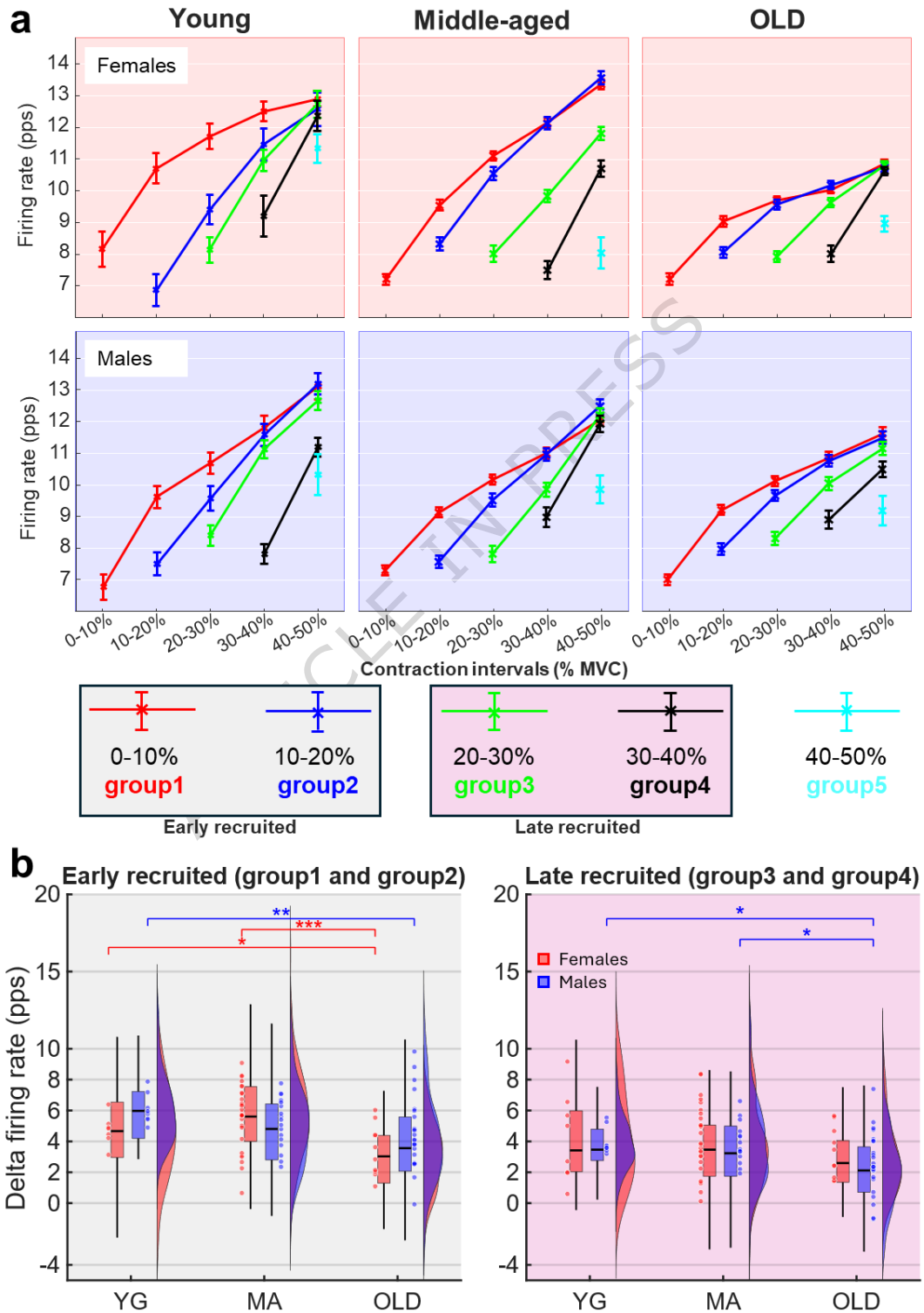
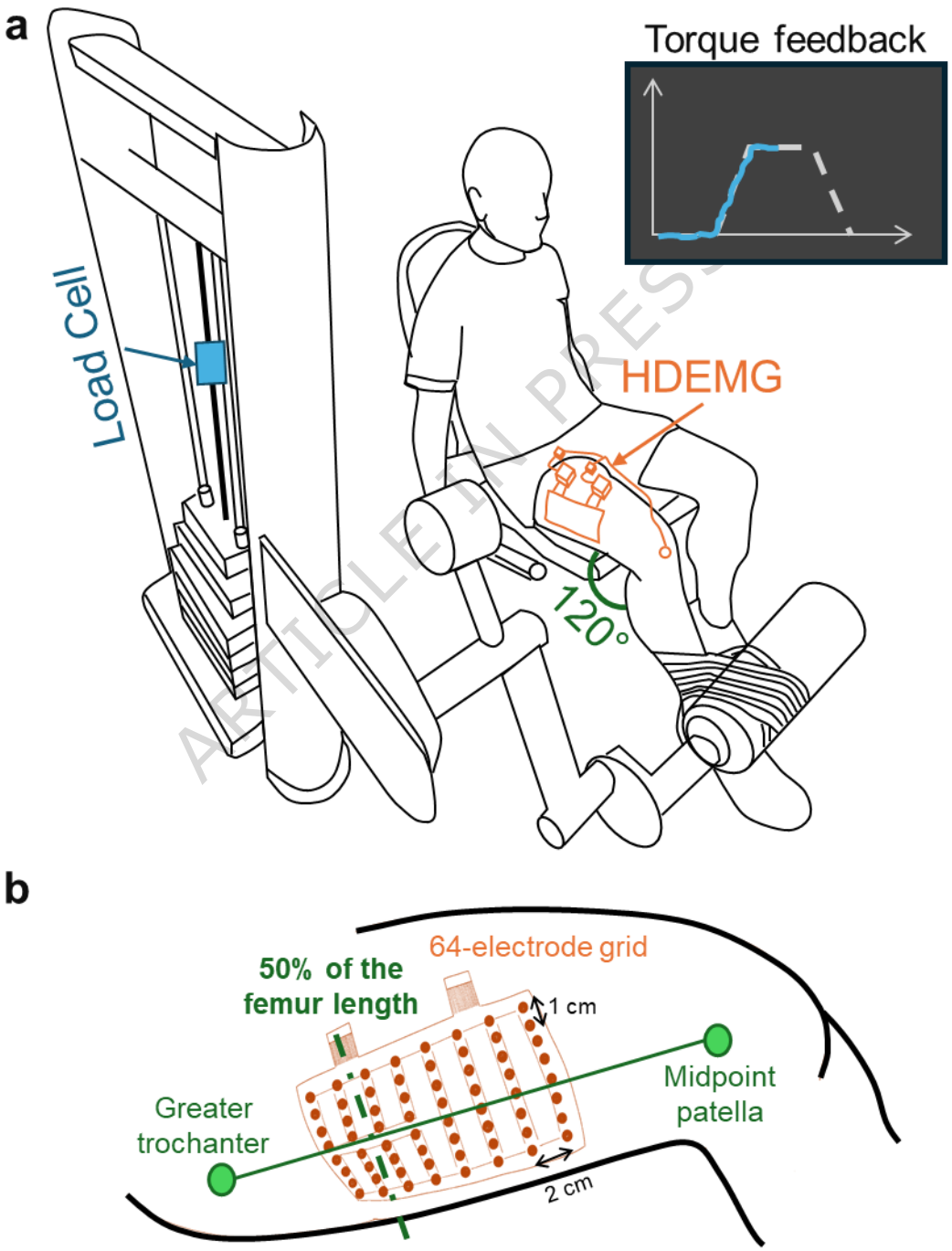


Figure 2 Firing rate modulation with increasing force. a) Modulation of firing rates during the ramp of the five motor unit groups recruited at different thresholds for the 50% MVC contractions. The upper

panels belong to female divided among age groups, while the lower panels refer to male. b) Boxplots and probability density functions of the Δ firing rate (difference between the average firing rates at the recruitment interval and at the maximum force) of motor units divided in two major group (early- and late- recruited) among different age groups and sexes. The early-recruited group (left panel) consisted of the motor units recruited up to 20% MVC (group 1 and 2) while the late-recruited group (right panel) consisted of the motor units recruited in the range 20-40% MVC (group 3 and 4). Statistical significances are reported in black for comparison between sex and with the respective color for comparison among age groups.



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Figure 3 Experimental Setup. a) Instrumented leg extension machine for isometric contractions at a knee angle of 120° (where 180° is the full extension). A monitor displayed the time course of the recorded torque and the target torque level for visual feedback. b) Position of the 64-electrode HDEMG grid over the VL muscle at 50% of the femur length measured as the distance between the greater trochanter and the distal boundary of the lateral femoral condyle.

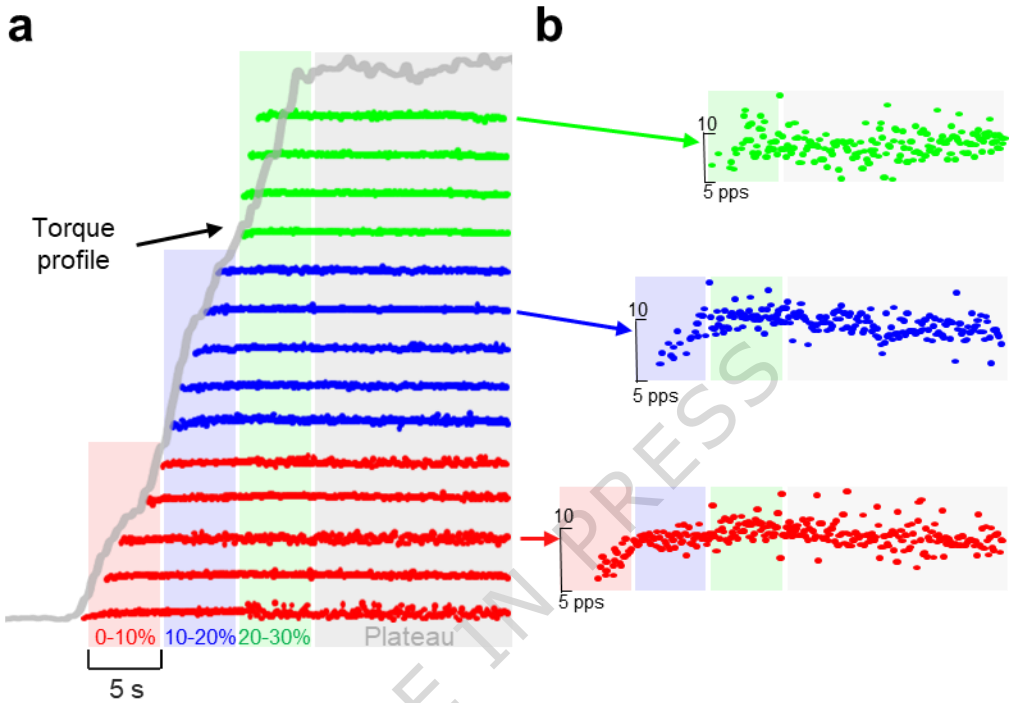


Figure 4 Example of decomposition results and analysis for a representative 30% MVC contraction. a) The ramp was segmented into intervals corresponding to 10% of the MVC to identify b) groups of motor units recruited at different contraction levels (red 0-10% MVC, blue 10-20% MVC, green 20-30% MVC).

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Table 1 Results of the measurements of torque, muscle morphology, physical activity.

	Young (YG)		*Middle-Aged (MA)*		*OLD*	
	Male (n = 8)	*Female (n = 8)*	*Male (n = 17)*	*Female (n = 29)*	*Male (n = 24)*	*Female (n = 11)*
Torque						
MVC (Nm) #	284.7 ± 15.1 *ab	205.3 ± 15.5 *ab	212.6 ± 8.9 *ac	133.3 ± 8 *ac	147.3 ± 10.2 *bc	67.9 ± 10.4 *bc
Muscle morphology						
Muscle thickness (mm) #	25.5 ± 1.1 *ab	22.8 ± 1.2 *	22.7 ± 0.7 *a	20.0 ± 0.5 *	21.7 ± 0.7 *b	18.9 ± 0.7 *
CSA (cm^2) #	N.A.	N.A.	18.8 ± 0.7 *c	13.3 ± 0.6 *	16.4 ± 0.6 *c	11.0 ± 0.7 *
Subcut. thickness (mm) #	4.2 ± 1.1 *	11.5 ± 1.1 *a	5.6 ± 0.6 *	12.9 ± 0.5 *ac	3.5 ± 0.6 *	10.8 ± 0.7 *c
Physical activity						
TAC x1000 (Counts/day)	2952.5 ± 380.6	3057.0 ± 855.7 b	2529.6 ± 531.9 *c	3316.9 ± 659.9 *bc	2052.6 ± 449.1 c	2599.1 ± 1058.0 c
Active time (min/day)	504.5 ± 72.1	503 ± 86.9 a	470.3 ± 101.5 *	600.2 ± 69.5 *ac	420.1 ± 85.9	496.8 ± 144.4 c

Data are presented as means ± SD. Significance p<0.05: * Males vs Females; [a] YG vs MA; [b] YG vs OLD; [c] MA vs OLD. # adjusted with BMI. MVC: maximum voluntary contraction; CSA: cross-sectional area, TAC: total activity counts.

Table 2 Motor unit decomposition yield and quality.

	Young (YG)		*Middle-Aged (MA)*		*OLD*	
	Male (n = 8)	*Female (n = 8)*	*Male (n = 17)*	*Female (n = 29)*	*Male (n = 24)*	*Female (n = 11)*
Valid HDEMG trials						
at 30%MVC, n	16	16	32	58	47	22
at 50%MVC, n	16	16	32	54	44	21
Analyzed motor unit						
Total, n	391	227	589	661	861	306
at 30%MVC	13.8 ± 2.2	7.8 ± 1.9	10.0 ± 4.0	6.4 ± 2.6	10.3 ± 3.5	7.0 ± 3.6
at 50%MVC	10.7 ± 2.2	6.4 ± 2.9	8.4 ± 3.2	5.4 ± 2.2	8.5 ± 3.5	7.3 ± 3.3
per participant	48.9 ± 6.3	28.4 ± 8.1	34.6 ± 11.3	22.8 ± 8.5	35.9 ± 11.0	27.8 ± 13.6
Decomposition quality						
SIL	0.859 ± 0.035	0.856 ± 0.028	0.863 ± 0.036	0.859 ± 0.030	0.880 ± 0.044	0.862 ± 0.039

Data are presented as means ± SD, unless otherwise specified. Valid HDEMG trials refer to the number of subject × contraction recordings contributing at least one retained motor unit at the specified intensity. Analyzed motor units are reported either as pooled counts ("Total") or as mean ± SD per the contraction and participant. "At 30% MVC" and "at 50% MVC" refer to the number of retained motor units averaged across valid contractions performed at the corresponding target intensity, whereas "per participant" refers to the total number of retained motor units averaged across participants. SIL is the silhouette-based decomposition quality metric calculated for retained motor units after quality control and editing.

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Table 3 Anthropometric measurements of the 97 participants divided by sex and age.

	Young (YG)		*Middle-Aged (MA)*		*OLD*	
	Male (n = 8)	Female (n = 8)	Male (n = 17)	Female (n = 29)	Male (n = 24)	Female (n = 11)
Anthropometry						
Age (years)	27.4 ± 3.7	25.9 ± 3.3	58.7 ± 3.3	57.8 ± 3.2	77.3 ± 4.7	77.1 ± 4.1
Height (cm)	178.8 ± 7.5	167.1 ± 9.4	178.2 ± 5.4	163.7 ± 5.4	171.4 ± 8.1	155.6 ± 6.2
Body mass (kg)	71.9 ± 9.8	56.4 ± 10.7	86.4 ± 12.3	64.4 ± 10.6	80.3 ± 12.1	62.9 ± 9.4
BMI (kg/m^2)	22.4 ± 2.0	20.1 ± 2.5	27.2 ± 3.8	24.0 ± 3.7	27.2 ± 2.6	26.1 ± 4.7

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