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Muscle characteristics and substrate energetics in lifelong endurance athletes

Running title: Muscle in younger and older athletes

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ABSTRACT

Purpose: The goal of this study was to explore the effect of lifelong aerobic exercise (i.e. chronic training) on skeletal muscle substrate stores (intramyocellular triglyceride [IMTG] and glycogen), skeletal muscle phenotypes, and oxidative capacity (ox), in older endurance-trained master athletes (OA) compared to non-competitive recreational younger (YA) athletes matched by frequency and mode of training.

Methods: Thirteen OA (64.8±4.9 yo) exercising ≥ 5 times/week were compared to 14 YA (27.8±4.9 yo) males and females. IMTG, glycogen, fiber types, succinate dehydrogenase (SDH) and capillarization were measured by immunohistochemistry in *vastus lateralis* biopsies. Fat-ox and carbohydrate (CHO)-ox were measured by indirect calorimetry before and after an insulin clamp and during a cycle ergometer graded maximal test.

Results: $\dot{V}O_2$peak was lower in OA than YA. OA had greater IMTG in all fiber types and lower glycogen stores than YA. This was reflected in greater proportion of type I and less type II fibers in OA. Type I fibers were similar in size, while type II fibers were smaller in OA compared to YA. Both groups had similar SDH content. Numbers of capillaries per fiber were reduced in OA but with a higher number of capillaries per area. Metabolic flexibility and insulin sensitivity were similar in both groups. Exercise metabolic efficiency was higher in OA, but no differences in substrate use were observed during submaximal exercise. At peak exercise, CHO-ox was lower in OA but with similar Fat-ox.

Conclusion: Lifelong exercise is associated with higher IMTG content in all muscle fibers and higher metabolic efficiency during exercise that are not explained by differences in muscle fibers types and other muscle characteristics when comparing older to younger athletes matched by exercise mode and frequency.

KEYWORDS

Aging, chronic exercise, IMTG, muscle fibers, proteins, capillary density, resting energy expenditure, carbohydrate oxidation, fat oxidation, insulin sensitivity

INTRODUCTION

Aging is associated with a decline in physical capacity and modifications of muscle phenotype (34) leading to increased overall morbidity and risk for development of cardiometabolic diseases. Aerobic training interventions suggest that aged skeletal muscle remains malleable to sustain the functional and metabolic demands of exercise (6) demonstrated by a shift towards higher content of type I fibers and relative decrease in type IIx fibers (29), increased fiber cross sectional area (22), enhanced oxidative capacity (39), capillary angiogenesis (35) and elevated glycogen stores (33). Further, we have previously demonstrated that chronic aerobic training in older adults increases intramyocellular triglyceride (IMTG) stores (9) and reliance on fat metabolism (2) during exercise.

Despite the growing body of literature demonstrating alterations in skeletal muscle substrate content and capacity for oxidation in previously sedentary subjects, few studies have compared chronic aerobic training adaptations in young and old athletes. Current evidence supports the notion that being physically active throughout a person's life (lifelong) protects oxidative fiber number and size, as well as mitochondrial function when compared to younger trained (39) and older sedentary (1, 45) subjects. These retained muscle adaptations to exercise seem to provide functional benefits such as improved balance, gait speed and ability to get up from a chair (45), which in turn are likely to improve quality of life and reduce risk of falling. Yet, the impact of lifelong aerobic training on skeletal muscle metabolism within the context of whole-body substrate oxidation and insulin sensitivity is still largely unknown.

The primary goal of this study was to determine skeletal muscle substrate storage and capacity for oxidation, as well as exercise metabolic efficiency in older masters athletes and younger subjects matched by frequency and mode of training. A secondary goal was to determine if differences in skeletal muscle substrate storage was associated with differences in substrate oxidation under different physiological conditions. We hypothesized that despite

lower peak aerobic capacity in older master athletes, lifelong aerobic training in this group would result in similar skeletal muscle substrate storage compared to the younger athletes matched by exercise mode and frequency, as well as similar oxidative capacity, metabolic efficiency and substrate oxidation under same physiological conditions.

METHODS

Subjects

Fourteen younger (age 18-39) and 13 older (age 60-75) endurance-trained athletes were recruited for this cross-sectional comparison. To be included, older women and men were training 5 or more structured aerobic exercise sessions per week either in running, cycling, swimming, or aerobic dancing (fitness classes). Younger athletes were non-competitive recreational athletes matched by frequency and mode of training with at least 3 years of uninterrupted (>3 months) training. Habitual physical activity was self-reported and discussed during the screening visit medical interview, including exercise mode, frequency and training years. All subjects were in general good health, non-smokers, weight stable and training stable for the last 6 months. The University of Pittsburgh Institutional review board approved the protocol and all volunteers gave written consent.

Body composition

Total body fat-free (FFM), fat mass (FM) and percent body fat were measured by dual-emission X-ray absorptiometry (Lunar Prodigy; GE Healthcare, Milwaukee, MI).

Physical fitness

$\dot{V}O_2$peak was assessed by a graded exercise test on an electronically braked cycle ergometer (Excalibur, Lode B.V., Groningen, The Netherlands) in conjunction with indirect calorimetry (Moxus, AEI Technologies, Pittsburgh, PA). The initial workload was set depending on the sex and age of the individual (50 W for younger and older women, 75 W for older men, 100

W for younger men) for the first 2 minutes and then increased by 50 W (men) or 25 W (women) every 2 minutes thereafter until volitional exhaustion or one of the established criteria for $\dot{V}O_2$peak had been reached (38). Heart rate, blood pressure, and ECG were recorded before, during and immediately after this test.

Skeletal muscle biopsies

Percutaneous muscle biopsies were obtained from the *vastus lateralis* as described previously (1). Subjects were asked to refrain from exercise in the last 48 hours before the biopsy. Subjects were admitted to the Clinical and Translational Research Center (CTRC) in the evening and received a standard dinner (7.5 kcal·kg^{-1} of body weight, 50% carbohydrate, 30% fat and 20% protein). The biopsy was performed the following morning at 7 AM after an overnight fast. Samples were trimmed of all visible adipose tissue with a dissecting microscope (Leica EZ4, Leica Microsystems, Wetzlar, Germany) and blotted dry. The muscle specimen was mounted on a small piece of cork with mounting medium, placed in liquid nitrogen cooled isopentane and then placed into liquid nitrogen. All samples were stored at -80 degrees Celsius until analysis.

Immunohistochemistry

Histochemichal analyses were performed on 10 μm serial sections using methods previously described (9). IMTG content was determined by Oil Red O (ORO) and fiber type costain (1) allowing fiber specific IMTG measurements and cross sectional area. Succinate dehydrogenase (SDH, complex II of the electron transport chain) staining was used as a marker of oxidative capacity (40). Glycogen content was measured using a standard Shiffs reagent protocol (23). Capillary density was determined as previously described (9). Capillary density was computed as total number of capillaries per cross sectional area of tissue (capillaries/area). The number of fibers in the cross sectional area of tissue is reported as the ratio fiber/area and the number of capillaries per fiber as the ratio capillaries/fiber.

133 *Whole body substrate oxidation and exercise efficiency*

134 Indirect calorimetry was used to measure $\dot{V}O_2$ and $\dot{V}CO_2$ under three physiological
135 conditions: 1) in the fasted state between 6 and 7 AM (prior to the biopsy described above), 2)
136 in the post-prandial state at the end of an hyperinsulinemic euglycemic clamp, and 3) during
137 the graded exercise test described above. Systemic rates of fat oxidation (Fat-ox) and
138 carbohydrate (CHO-ox) were calculated using the adapted stoichiometric equations of Frayn
139 (13):

140 $$\text{Fat-ox (mg/min)} = 1.67\ \dot{V}O_{2(ml/min)} - 1.67\ \dot{V}CO_{2(ml/min)}$$

141 $$\text{CHO-ox (mg/min)} = 4.55\ \dot{V}CO_{2(ml/min)} - 3.21\ \dot{V}O_{2(ml/min)}$$

142 To compute the proportion of energy expended from carbohydrates or fat, Fat-ox and
143 CHO-ox were transformed in kilocalories per minute and expressed as a proportion of resting
144 energy derived from fat or carbohydrates as used previously (2). Protein oxidation rates were
145 not included based on our laboratory's prior work demonstrating that rates of urinary nitrogen
146 excretion were similar in different body phenotypes during resting conditions (19) and on the
147 assumption that the amount of protein oxidized, as well as other metabolic processes, such as
148 gluconeogenesis from protein, ketone body formation, and lipogenesis during exercise, are
149 quantitatively negligible compared with glucose and fatty acid oxidation (37).

150 To account for possible aging and sex biases, all physiological data were normalized
151 to FFM. Glucose uptake (glucose oxidase, [YSI, Yellow Springs, Colorado]) and plasma
152 insulin (ELIZA, [Millipore, Billerica, MA]) were used to calculate insulin sensitivity
153 ($mg \cdot kgFFM^{-1} \cdot min^{-1} \cdot unit\ insulin^{-1}$) during the steady state of the clamp.

154 During the graded exercise test, metabolic efficiency was measured as delta efficiency
155 in percent for each consecutive stages as the difference in watts divided by the difference in
156 $\dot{V}O_2$ (14). This was performed for each submaximal stage using the average $\dot{V}O_2$ for the last
157 30 seconds of each stage. Further, to obtain overall delta efficiency ($\Delta\eta$), linear regressions

158 were drawn for each subject using all the submaximal stages. The average slopes and
159 intercepts for each group were used to define the relationship $\dot{V}O_2 = b\ \dot{W} + a$, where b is the
160 slope and a the intercept. The inverse of the slope $1/b = \Delta\dot{W}/\Delta\dot{V}O_2$ is $\Delta\eta$ (12).
161 *Statistical Procedures*
162 Subject characteristics are presented as means ± SD, all other data are presented as
163 means ± SEM. After checking normality and equality of variance, two tailed independent *t*-
164 tests were performed to examine group differences. If the equality of variance assumption was
165 not met, comparisons between groups were performed with the Welch corrected *t*-test. If the
166 normality assumption was not met, comparisons between groups were performed with the
167 non-parametric Median test. For substrate oxidation comparisons in fasted and fed conditions,
168 2x2 mixed MANOVA were performed. For substrate use during the graded exercise test,
169 repeated mixed MANOVA were used with group X time. When needed pair-wise post hoc
170 analyses were used to identify the significant difference.
171
172 **RESULTS**
173 ***Subject characteristics***
174 Subject characteristics are presented in Table 1. Training years were between ~ 35-40 years
175 for the older masters athletes and 5-13 years for younger subjects. FFM, FM and percent body
176 fat were not different between age groups. Younger athletes had a higher $\dot{V}O_2$peak than older
177 athletes with a magnitude of ~25% when expressed relative to FFM. Self reported activities
178 were on average 6 sessions/week with running as the most common physical activity (62%),
179 followed by biking (23%), brisk walking and aerobic fitness classes (both 8%). In addition of
180 their main exercise mode, cross-training and seasonal activities included skiing, golfing and
181 swimming.
182

Skeletal muscle lipid storage is greater in older compared to younger endurance-trained athletes

Chronic aerobic training increases skeletal muscle substrate storage in young and old previously sedentary subjects. Yet the effects of lifelong aerobic training on skeletal muscle adaptations are largely unknown. Older athletes had higher content of IMTG in each fiber type measured (Figure 1, Panel A), as well as overall greater content of IMTG. Glycogen content (Figure 1, Panel B) was higher in young athletes compared to old, while no differences in SDH (Figure 1, Panel C) were noted.

Oxidative fibers are higher in older compared to younger endurance-trained athletes

Older athletes had higher proportion of type I fibers and lower type IIa fibers than younger athletes (Figure 2, Panel A). The proportion of type IIx fibers was not different between groups. Mean area of type I fibers was similar in both groups, while younger athletes had larger IIa and IIx fiber area (Figure 2, Panel B). These data suggest that lifelong physical activity may not prevent the proposed age related decline in type II fiber area (31).

Capillary density is lower in older compared to younger endurance-trained athletes

As skeletal muscle capillary density is affected by aging and type 2 diabetes (21) and is associated with oxidative capacity (9), we next determined if capillary density was associated with the observed differences in oxidative fibers. While the number of capillaries per fiber was higher in the younger (Figure 3, Panel A) athletes, capillary density relative to muscle area was higher in the older athletes (Figure 3, Panel B). These data suggest that the decline in capillary density associated with sedentary aging (21) is attenuated with lifelong aerobic exercise.

Metabolic flexibility and insulin sensitivity are similar in older compared to younger endurance-trained athletes

Given the observed differences in skeletal muscle substrate composition and capacity for oxidation, we next examined whether or not these differences translated into changes in whole-body substrate oxidation and insulin sensitivity. Older athletes had higher resting energy expenditure in fasting condition, while younger athletes had higher energy expenditure in postprandial condition (Figure 4, Panel A, significant interaction P=0.01). The proportion of substrate use during both states was comparable in both groups (Figure 4, panel B). Metabolic flexibility, originally defined by the overall change in RQ from fasting to postprandial (28) was similar in both groups (Figure 4, Panel C, insulin effect P<0.0001). Insulin stimulated glucose uptake was similar in younger and older athletes (Figure 4, Panel D), with no differences in non-oxidative and oxidative disposal. Together these data suggest that lifelong endurance training protects older adults from declines in metabolic flexibility and insulin sensitivity. Moreover, relative fat- and carbohydrate-oxidation rates for basal and insulin-stimulated substrate use under non-exercising conditions are maintained throughout the lifespan with aerobic exercise.

Exercise metabolic efficiency is enhanced in older compared to younger endurance-trained athletes

We previously demonstrated that exercise training resulted in improved skeletal muscle oxidative capacity (9) and exercise efficiency (2) in previously sedentary older adults. Based on the differences in peak aerobic capacity and substrate storage in older athletes, we next calculated exercise metabolic efficiency during a graded exercise test. Older athletes had higher exercise metabolic efficiency compared to younger athletes ($\Delta\eta$ of 9.03±0.32 and 8.03±0.26%, P=0.02). Regression curves for each group, including slope and intercept are

presented in Figure 5, Panel A (P=0.02 [older] and P=0.13 [younger]). Stage by stage delta efficiency is presented in Figure 5, Panel B (2x5 MANOVA not significant, point by point independent T tests are presented in the figure).

Peak exercise carbohydrate oxidation rates are lower in older compared to younger endurance-trained athletes

At higher relative intensities, younger athletes had greater rates of carbohydrate oxidation compared to older (Figure 6 Panel A). No differences in fat oxidation where observed (Figure 6 Panel B). To account for the possible changes in the size of the bicarbonate pool during maximal exercise, CHO and fat oxidation rates were also computed with the modified equations proposed by Jeukendrup et al. (26) adapted for intensity of the exercise (different equations for RER < or > 1). These confirmed exact same significant differences between Y and O at peak exercise and during the stage by stage analyses (data not shown). Together these data suggest that the observed increase in IMTG and oxidative fibers may contribute to the enhanced exercise metabolic efficiency. Further, these data support the notion that younger endurance trained athletes are better suited for higher intensity exercise as evidenced by the higher rates of peak carbohydrate oxidation.

DISCUSSION

The overall goal of this study was to investigate chronic aerobic exercise training on skeletal muscle substrate adaptations, as well as systemic oxidation in young and older endurance trained subjects. To achieve this goal we examined skeletal muscle phenotypes, as well as whole-body substrate utilization using indirect calorimetry in two cohorts of subjects with similar endurance training regimens. We found that, despite lower peak aerobic capacity, lifelong master athletes have higher intramyocellular triglyceride (IMTG) and proportion of

oxidative fibers compared to younger athletes. These differences were reflected in enhanced exercise metabolic efficiency with lower reliance on carbohydrate oxidation during exercise in the older subjects (at higher intensities). Together the data suggest that lifelong aerobic exercise, not only attenuates the age associated decreases in muscle oxidative potential, but also provides older endurance-trained subjects with an enhanced capacity for fatty acid oxidation.

Age-induced increases in intramyocellular lipids have been observed in previous human studies. Under sedentary conditions, this phenomenon is associated with a decline in muscle mass and strength (8, 16), as well as decreased insulin action (36). While decreases in muscle mass, fiber cross sectional area, and shifts in fiber type composition may explain, in part, intramyocellular lipid deposition in sedentary conditions (8, 18), this is not the case for the chronically trained older individuals in the current study. We have previously exposed that the "athlete's paradox" observed in younger endurance trained athletes (17) was also present in older endurance trained athletes compared to sedentary controls (1). A key novel finding in the present study is that older endurance trained athletes have greater lipid, yet lower carbohydrate stores, compared to younger athletes with similar training regimens. While aging *per se* has been associated with increased lipid uptake (44), chronic exercise training increases factors associated with IMTG turnover (i.e. storage and lipolysis) (1). We hypothesize that the combination of these age- and exercise-related alterations in IMTG turnover likely mediates, in part, the increased IMTG in this cohort. Proteins involved in IMTG storage are elevated in exercise-trained muscle (1, 4, 10) (amati, diabetes, 2011; dube, diabetologia 2011, Bergman, JAP, 2010). Additional studies are needed to investigate whether these, or other mechanisms for the increased IMTG storage, are altered in older endurance athletes.

In contrast to higher IMTG levels, older subjects demonstrated lower muscle glycogen stores compared to younger subjects. Although controversial, there is a suggestion that glycolytic activity (5), as well as type II fiber proportion and size(discussed below) may be reduced with aging. However, aerobic exercise training in previously sedentary older adults has been demonstrated to increase muscle glycogen content (9). Possible explanations to the lower glycogen content in older trained subjects is that younger endurance athletes may engage in relatively more frequent high-intensities and/or that younger athletes may have altered post-exercise carbohydrate consumption relative to older athletes, thus providing the necessary stimulus for enhanced glycogen storage (25). Nevertheless, lower glycogen content in our older athletes did not contribute to alterations in basal or insulin-stimulated rates of substrate oxidation. Rather, the functional relevance was only observed at maximal intensity exercise. These data support the notion that lifelong endurance training may better position older athletes for moderate intensity activities with relative higher fat oxidation, while young athletes may be positioned for high intensity exercise (i.e. higher glycogen). Thus the capacity for moderate high fat oxidation activity may be enhanced with lifelong endurance training.

Based on our novel demonstration of increased lipid stores with lifelong exercise training, we next examined the potential mechanisms associated with this phenomenon. While several studies have suggested that aging results in the atrophy of type II fibers (20, 39), with a relative increase of the area occupied by type I fibers (30), this is not without controversy. Our data suggest that lifelong exercise training is accompanied by a shift toward greater slow oxidative fibers with no change in the overall size of these fibers (45). Interestingly, not only was the relative percentage of glycolytic fibers decreased in older trained subjects, the mean area was also decreased. These data suggest that if an aging decrease in glycolytic fibers occurs, perhaps exercise training promotes a compensatory increase in oxidative fibers. This new harmony between type I and type II fibers observed in the aging and trained muscle may

307 explain, at least in part, the distinction in substrate stores between older and younger muscle
308 of endurance trained athletes witnessed in this study.

309 Previous studies have demonstrated that, while master athletes have significantly
310 higher peak fitness levels compared to sedentary age-matched controls (41), the age-related
311 decline in fitness persists despite continuous training. Thus, as expected, $\dot{V}O_2$peak, both
312 absolute and adjusted to fat free mass, was higher in younger than older athletes. Peak fitness
313 may be limited by two key peripheral factors, capillarization (3, 24) and mitochondrial
314 capacity (3). While capillary density, relative to the number of fibers, was lower in older
315 trained subjects, adjusting the data to the lower number and cross sectional area of glycolytic
316 fibers suggests that capillary density is not different between the cohorts (7). This
317 interpretation is in accord with previous studies that found similar adaptations in
318 capillarization between older and younger adults undergoing an exercise intervention (15, 35).
319 With respect to mitochondria, it has been reported that mitochondrial respiration (21),
320 mitochondrial biogenesis (32), and perhaps oxidative capacity and energy production decline
321 with aging. However, it's generally accepted that aerobic exercise training, in both older (9)
322 and younger (11) previously sedentary subjects, results in enhanced mitochondrial oxidative
323 capacity. In agreement with data from Proctor et al. (39), we did not observe any differences
324 in mitochondrial capacity between the cohorts in this study. Thus, the difference in $\dot{V}O_2$peak
325 observed in our younger and older athletes seems to be explained mostly by the central
326 component. This is in agreement with previous studies suggesting that peripheral factors play
327 an important role in the elderly in the response to endurance exercise training (33). Together
328 our data suggest that while lifelong exercise training may not prevent the age-associated loss
329 of skeletal muscle capillarization, the overall capacity for substrate oxidation, as well as
330 overall fitness is enhanced relative to sedentary subjects regardless of age (1).

Based on our demonstration of enhanced lipid stores and similar capacity for oxidation, we next examined whole-body substrate utilization under different physiological conditions. Previous studies have reported age-related declines in the capacity of skeletal muscle to oxidize fat in the fasting state and during exercise (42, 44). In this study, higher energy expenditure at rest was not associated with differences in substrate selection in the older athletes. These data are in stark contrast to previous reports from sedentary subjects (27) demonstrating a significant reduction in resting energy expenditure in older subjects adjusted for fat free mass. We speculate that the increased basal energy expenditure may be due to the modest but not significant BMI and gender difference between the groups (see bellow). Nevertheless, our data clearly indicate the lifelong training preserves basal energy expenditure, as well as rates of both fat and carbohydrate oxidation in the basal and insulin-stimulated conditions. Thus, lifelong exercise training preserves metabolic flexibility and substrate selection with aging.

During exercise, both groups used similar sources of nutrients for energy for submaximal stages, but not for maximal intensity where the younger burned significantly more carbohydrates. These data are in agreement with our demonstration of greater muscle glycogen content in younger subjects. Intervention studies have concluded that previously sedentary older subjects undergoing endurance exercise interventions of 16 weeks were able to improve their reliance of fat during a one hour submaximal exercise (2, 43), thus our data may be explained by the maintenance of substrate oxidation in older athletes as well as by the shift towards type I fibers. Interestingly in our cohort, the higher muscle efficiency observed in the older athletes during the graded exercise test cannot be explained by different substrate use during exercise, but may be influenced by the greater number of capillaries per fibers and the higher proportion of type I fibers (2). Together these data suggest that lifelong aerobic

exercise preserves, or perhaps enhances, resting exercise expenditure, as well as metabolic flexibility and substrate oxidation under physiological conditions.

This study is not without limitations. First, training regimens (frequency, mode) were self-reported. However, our data are in accord with previous reports of overall fitness and body composition in older and younger athletes (7, 39). Although we attempted to include equal numbers of males and females, males represent 50% in the younger group and 69% in the older group. While the chi-square test for sampling distribution was not significant, this discrepancy may influence some of the results. We believe that if so, this would have been in disfavor of the older group as women have relative lower exercise capacity and higher insulin sensitivity than men and thus, if the gender balance was important, we would have probably seen unequal insulin sensitivity and markers of oxidative capacity between our two groups.

In summary, the results of the present study demonstrate that lifelong endurance training results in increased skeletal muscle lipid stores and shift toward greater numbers of oxidative fibers. Despite lower glycogen and glycolytic fiber content in older endurance trained subjects, exercise metabolic efficiency was enhanced and substrate selection was comparable to younger trained subjects. We conclude that these physiological adaptations to chronic aerobic training in older subjects may place them in an optimal position for moderate high-fat oxidation activity. Moreover, these data provide further evidence against triglyceride-mediated impairments in metabolic function. Conversely, the demonstration of higher muscle glycogen content in younger subjects supports the notion of a higher capacity for high-intensity training, supported by enhanced carbohydrate oxidation observed in this study. Our studies raise further questions on lifelong adaptations to exercise in terms of increased efficiency without modifying the balance between sources of substrate oxidation. Additionally, these data further emphasize the importance of chronic exercise throughout life to attenuate the deleterious effects of aging and sedentary lifestyle.

AUTHORS CONTRIBUTIONS

J.J.D. researched data, contributed to the study concept, design and wrote the manuscript. N.T.B and A.D. researched data. F.G.S.T and M.S.R. performed biopsies. B.H.G. contributed to the study concept, interpretation of the data and edited the manuscript. F.A. researched data, contributed to the study concept, design, analysis, and interpretation of the data; and wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. The results in the present study do not constitute endorsement by ACSM.

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FIGURE LEGENDS

Figure 1: Skeletal muscle fiber type proportion (panel A) and cross sectional area (panel B) in younger and older athletes. MHC= myosin heavy chain. *P<0.05 **P<0.001 two tailed independent *t*-test.

Figure 2: Intramyocellular triglycerides (panel A), glycogen (panel B) and SDH content (panel C) in younger and older athletes. MHC= myosin heavy chain, A.U.= arbitrary units. *P<0.05 **P<0.001 two tailed independent *t*-test.

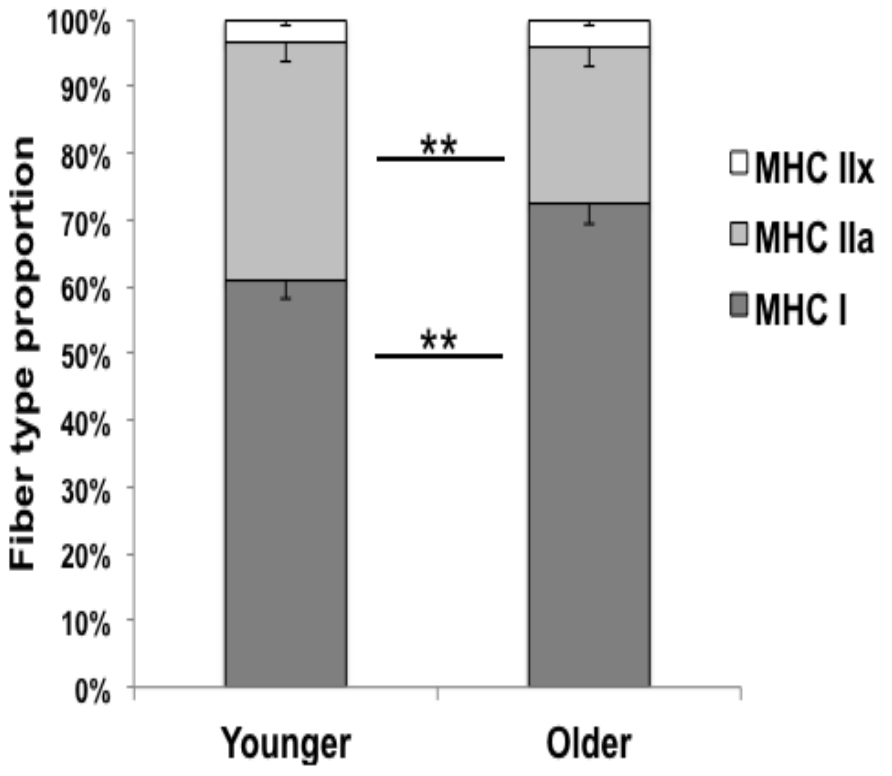
Figure 3: Skeletal muscle capillary density: Number of capillaries per fiber (panel A), number of fibers per area and capillaries per area (panel B). **P<0.001 two tailed independent *t*-test, §<0.05 non parametric Median test.

Figure 4: Energy expenditure (panel A) and substrate use at rest in the fasted and post-prandial phase (panel B), metabolic flexibility (panel C) and insulin-stimulated glucose uptake (panel D). FFM=fat free mass, RQ=respiratory quotient, CHO=carbohydrate. *Significant interaction effect, **Significant effect of time in 2x2 mixed MANOVA.

Figure 5: Delta efficiency during graded exercise test in older and younger endurance trained athletes. Panel A represents the regression lines defining oxygen uptake as a function of power output. The insert is the magnification of the origin of the axis (box). *Significant difference on the slope but not on the intercept. Panel B is delta efficiency between consecutive stages. Panel C represents substrate use at peak. Panel D is substrate use stage by stage. CHO=carbohydrate**.** *P<0.05, #=0.09 two tailed independent *t*-test.

553 **Figure 5: Substrate use during graded exercise test in older and younger endurance**
554 **trained athletes.** Panel A represents carbohydrate and fat oxidation as a function of relative
555 intensity of peak oxygen consumption. Panel B is the magnification of the fat oxidation data.
556 *P<0.05 two tailed independent *t*-test, #=0.08 in Panel A and 0.06 in Panel B.
557

Figure 1, Panel A



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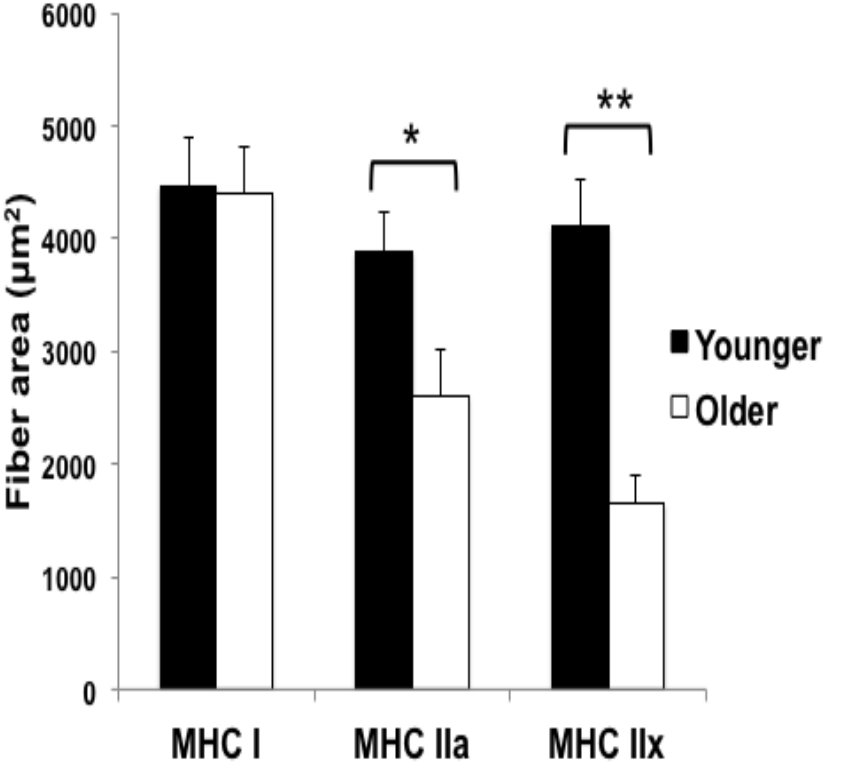

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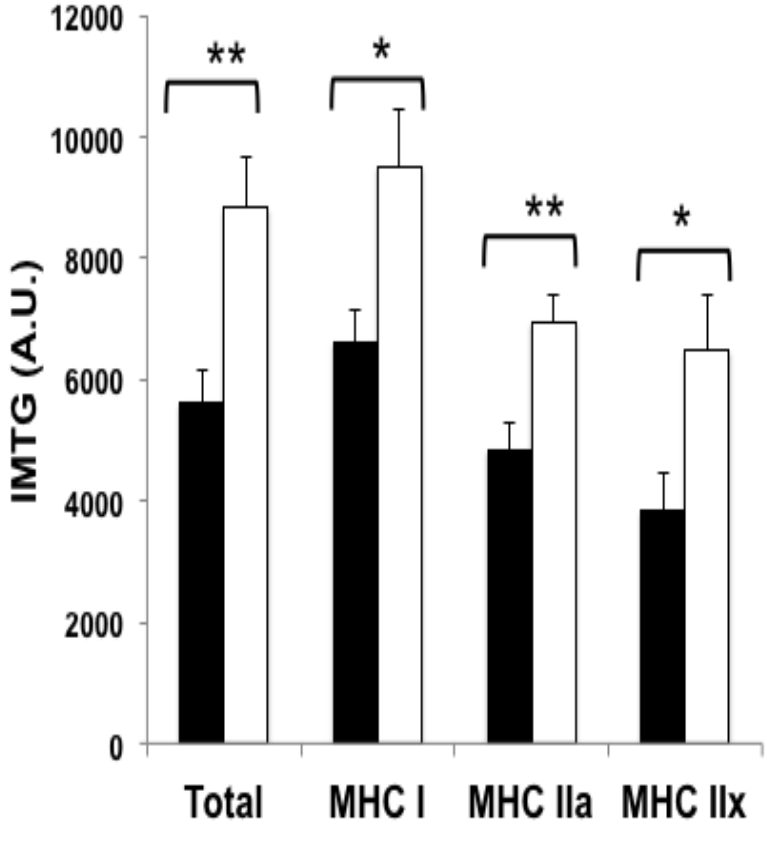

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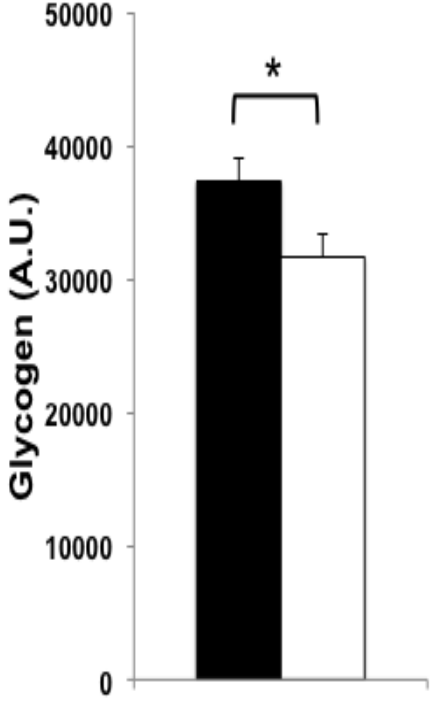

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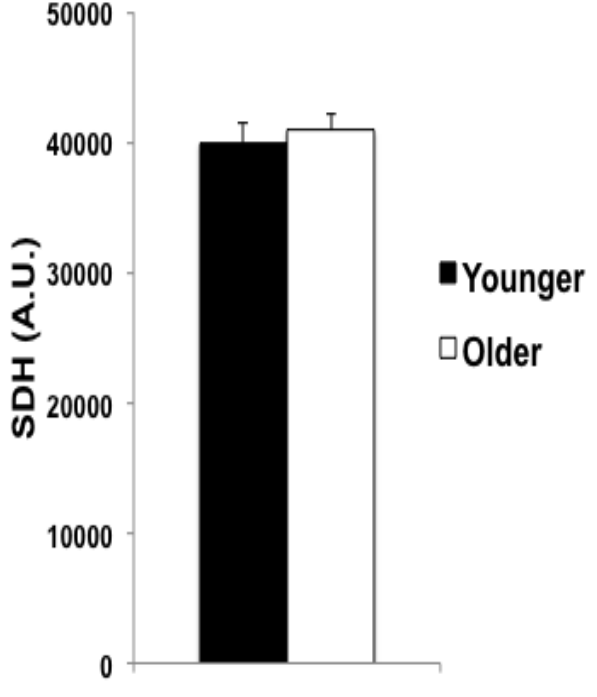

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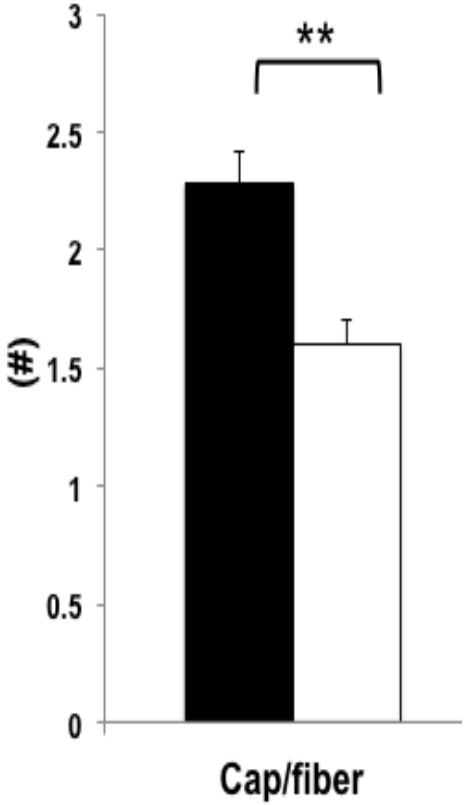

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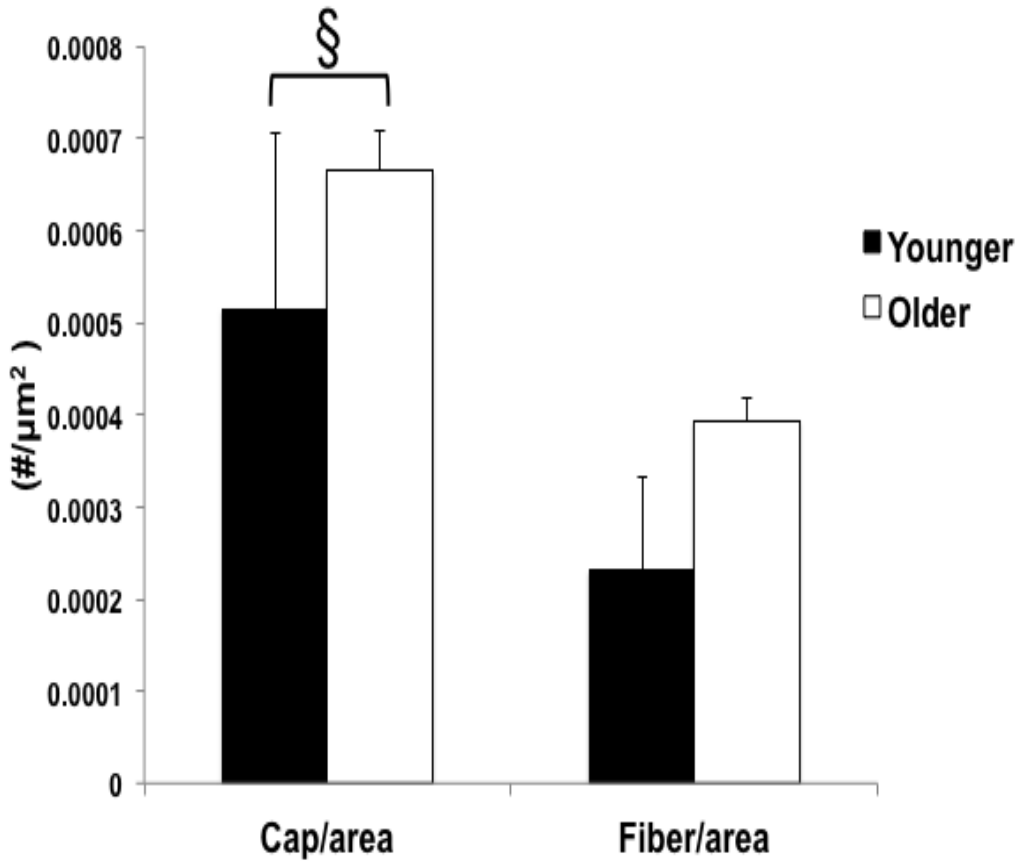
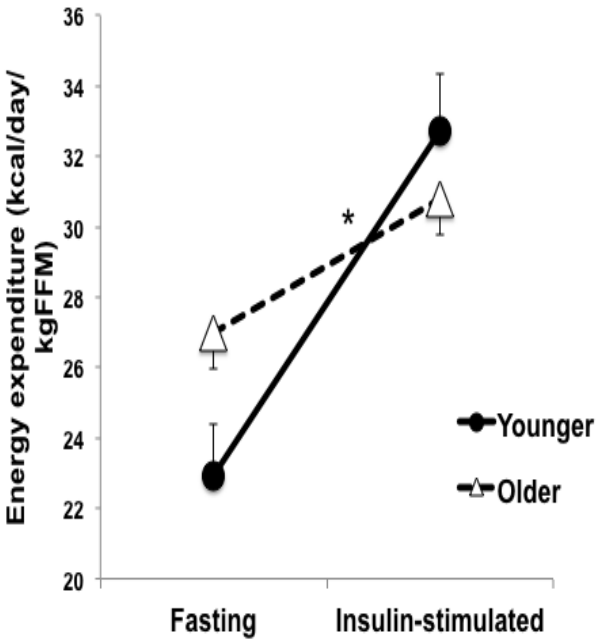


Figure 4, Panel A



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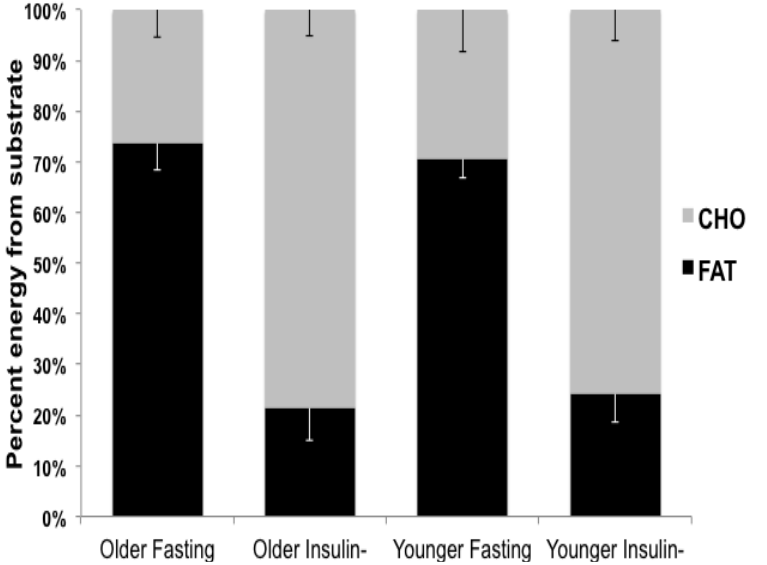

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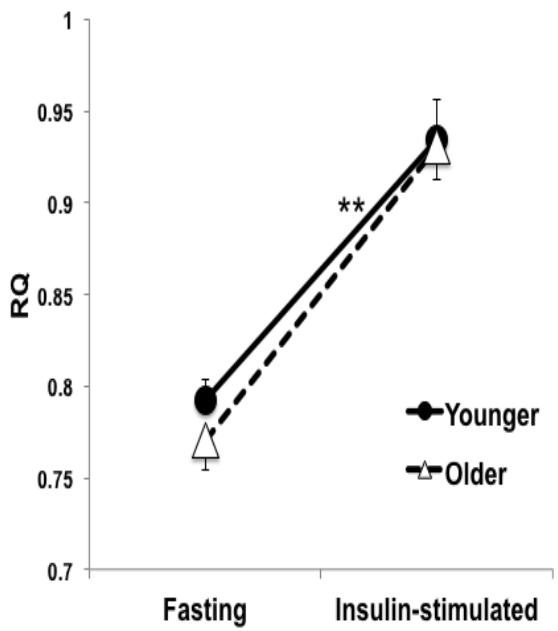

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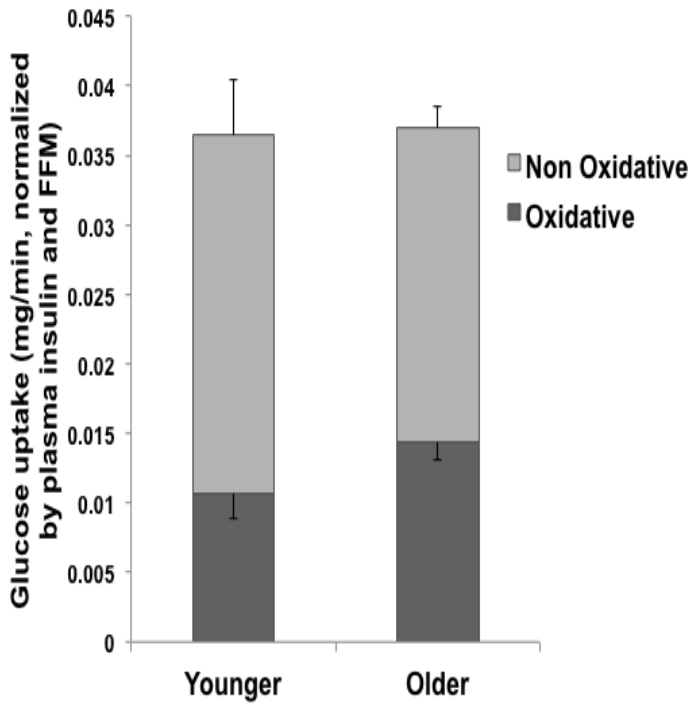

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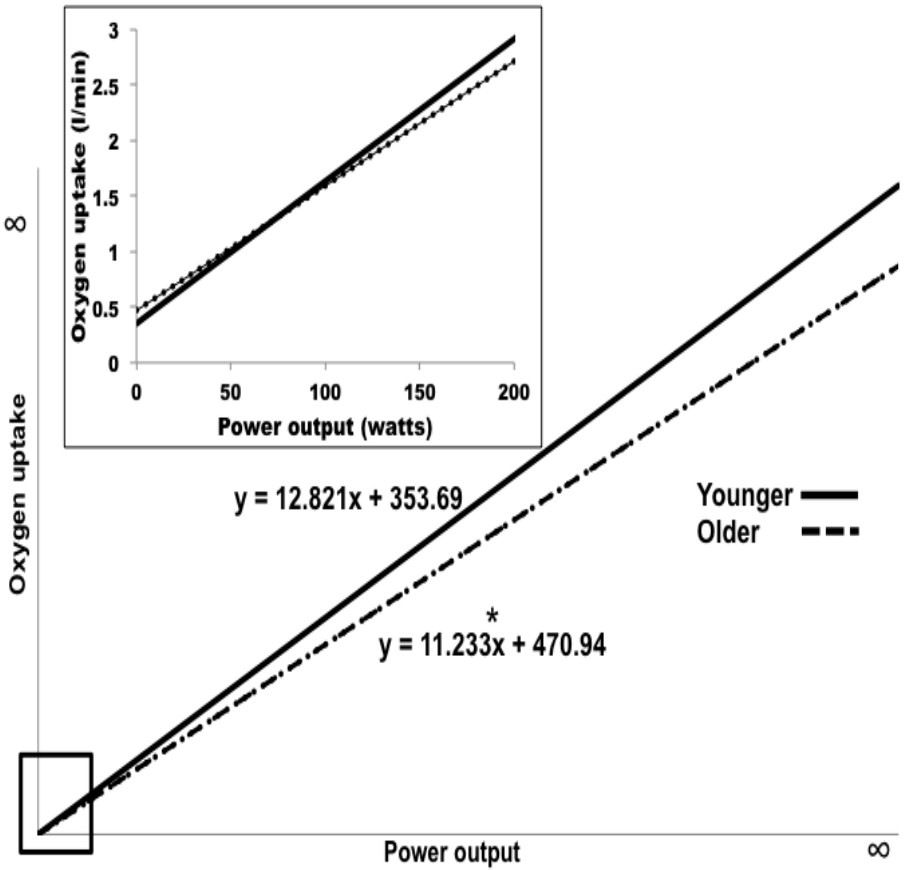

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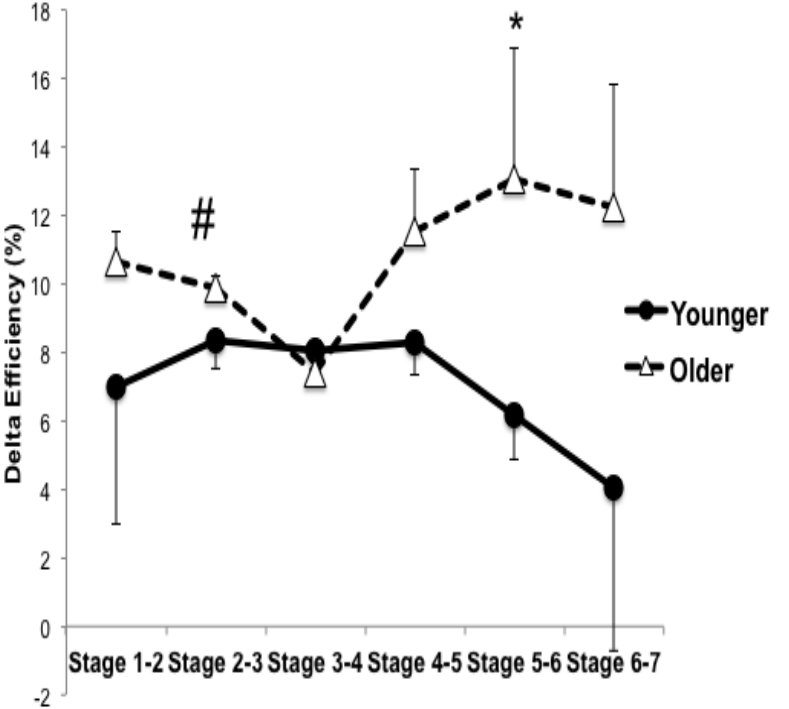

N Younger	13	13	13	13	13	9
N Older	14	14	13	12	7	4

Figure 6, Panel A

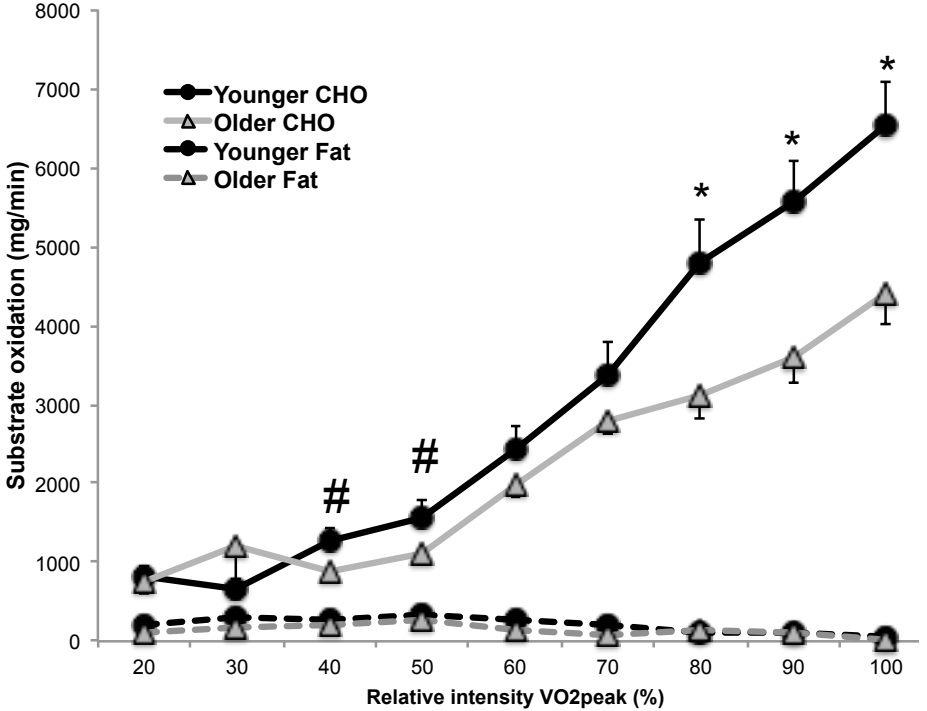

Figure 6, Panel B