Effects of loaded voluntary wheel exercise on performance and muscle hypertrophy in young and old male C57Bl/6J mice

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Accepted for publication 23 December 2014

This study compared the capacity of young and old male C57Bl/6J mice to exercise with increasing resistance over 10 weeks, and its impact on muscle mass. Young mice (aged 15–25 weeks) were subjected to low (LR) and high (HR) resistance exercise, whereas only LR was used for old mice (107–117 weeks). Weekly patterns of voluntary wheel activity, food consumption and body weights were measured. Running patterns changed over time and with age, with two peaks of activity detected for young, but only one for old mice: speed and distance run was also less for old mice. The mass for six limb muscles was measured at the end of the experiment. The most pronounced increase in mass in response to exercise was for the soleus in young and old mice, and also quadriceps and gastrocnemius in young mice. Soleus and quadriceps muscles were analyzed histologically for myofiber number and size. A striking feature was the many small myofibers in response to exercise in young (but not old) soleus, whereas these were not present after exercise in young or old quadriceps. Overall, there was a striking difference in response to exercise between muscles and this was influenced by age.

The age-related loss of skeletal muscle mass and function, called sarcopenia, adversely affects movement, mobility, posture, and metabolism (Cruz-Jentoft et al., 2010). The incidence of sarcopenia, reported as 14% by 65–69 years of age may reach greater than 50% by 80 years (Janssen, 2010) and the loss of muscle function may occur before loss of muscle mass (Clark & Manini, 2008; Chan & Head, 2010). Sarcopenia results in diminished independence and frailty that increases the risk of falls and fractures with escalating costs for the global health system (Cruz-Jentoft et al., 2010; Sayer et al., 2013). As the human population demographic is rapidly ageing, there is a compelling need to better understand the molecular mechanisms that cause sarcopenia, in order to design and implement the best early intervention strategies (Sayer et al., 2013).

Similar to humans, rodents (mice and rats) are affected by sarcopenia (Brooks & Faulkner, 1988; Daw et al., 1988; Holloszy et al., 1991; Blough & Linderman, 2000; Hamrick et al., 2006; Shavlakadze et al., 2010a; Sheard & Anderson, 2012; Ibebunjo et al., 2013). In C57Bl/6J mice, both males and females, sarcopenia is conspicuous at 24 months (m) of age (roughly equivalent to 70 years in humans (Flurkey et al., 2007), although the severity of muscle loss varies between muscles (Shavlakadze et al., 2010b; Sheard & Anderson, 2012) and sarcopenia is more pronounced in older (27–29 m) mice (Shavlakadze et al., 2010a).

Key features of sarcopenia in humans and animals, among others, are decreased myofiber size (atrophy) and myofiber death, remodeling of extracellular matrix with deposition of connective tissue and functional denervation of the ageing myofibers (Shavlakadze & Grounds, 2003; MacIntosh et al., 2006; Chai et al., 2011; Sayer et al., 2013). Recent comprehensive analyses of gene expression changes throughout the life of ageing rats (Ibebunjo et al., 2013) and mice (Barns et al., 2014) emphasize progressive alterations associated with myofiber denervation, metabolism, and extracellular matrix as well as the general dysregulation of transcription.

Whether muscle age can be reversed is debated; however, there is a consensus that sarcopenia can be reduced by exercise (reviewed in Hunter et al., 2004; Stewart et al., 2014). Exercise is widely recognized as a relatively simple and accessible intervention to reduce the decline of muscle mass and function and prolong independence and healthy living (Buford et al., 2010; Sayer et al., 2013). Human studies trialing progressive resistance training (PRT), whereby participants exercise against an increasing load, can maintain and increase muscle mass with benefits that vary between young and
old men and women. A standard 2–3 days/week PRT protocol can result in significant myofiber hypertrophy among elderly participants (≥ 60 years), with increases ranging between 13 to 52% among men (Frontera et al., 1988; Hikida et al., 2000; Trappe et al., 2000; Bamman et al., 2003; Leenders et al., 2012), and 5% to 38% among women (Charette et al., 1991; Häkkinen et al., 2001; Trappe et al., 2001; Bamman et al., 2003; Leenders et al., 2012) after 12 to 26 weeks of training. A recent review also demonstrated that elderly men and women (> 75 years) are capable of increasing muscle size (1.5–15.6%) and strength after a variety of resistance training interventions (reviewed in Stewart et al., 2014). Some studies suggest that elderly humans have a tendency to exhibit smaller relative muscle hypertrophy, or a blunted adaptive response to PRT than younger individuals (Kosek et al., 2006; Mero et al., 2012).

In rodent models, the use of voluntary running wheels paired with a progressive increase in resistance is appropriate for increasing muscle mass and myofiber size (Legerlotz et al., 2008). A number of different exercise protocols, employing both high and low levels of static resistance, have been used to assess the impact of an increasing load on skeletal muscle adaptation. Against a background of rapid postnatal growth, high impact static wheel loading (up to 173% of total body weight) increased muscle mass of young rats after only 6.5 weeks of training, with relative increases of 17%, 21%, 23%, and 29% observed in vastus lateralis, extensor carpi radialis longus and brevis, plantaris, and soleus muscle groups, respectively (Legerlotz et al., 2008). Similar increases in plantaris muscle mass (31% relative increase) have also been observed in young rats after 8 weeks of training, with a maximum load of 74% total body mass (no information on other muscles reported; Ishihara et al., 1998). Furthermore, administering a progressive wheel loading protocol in adult mice (up to either 17% or 41% of total body mass), while having no impact on plantaris, gastrocnemius or tibialis anterior, was sufficient to increase soleus muscle mass by ~20% after 7 weeks of training (Konhila et al., 2005).

Muscles of ageing rodents are also capable of exercise-induced muscle hypertrophy. Spontaneous activity, such as voluntary wheel running (no resistance; Brown et al., 1992; Gulve et al., 1993; McMahon et al., 2014), in addition to forced exercise techniques (weightlifting (Klitgaard et al., 1989) and treadmill running (Daw et al., 1988)) and athletic environmental enrichment (Brown et al., 2003) have shown that old rodents are capable of adaptive increases in mass when subjected to resistance exercise after continuous bouts of activity. As for humans, some studies suggest that the hypertrophic integrity of ageing rodent muscle in response to exercise is attenuated, with some exercise protocols unable to reduce the rate of atrophy associated with ageing (Farrar et al., 1981; Daw et al., 1988; Klitgaard et al., 1989). The capacity of old rodent muscle to hypertrophy in response to voluntary resistance wheel running has not been investigated.

Thus, we subjected young (aged 15 weeks) and old (aged 107 weeks) male C57Bl/6J mice to voluntary wheel running with increasing resistance for 10 weeks to characterize running patterns and determine the impact of resistance exercise on skeletal muscle mass and phenotype at both ages. Histological and morphometric analyses were performed on selected hind-limb muscles (quadriceps and soleus) of young and old mice.

Materials and methods
Mice and voluntary resistance wheel running protocols

Young (13 weeks, n = 32) and old (105 weeks, n = 24) male C57Bl/6J mice were obtained from the Animal Resources Centre, Western Australia and housed at the University of Western Australia under pathogen-free conditions. All experiments were conducted in accordance with the guidelines of the National Health and Medical Research Council, Australia and were approved by the Animal Ethics Committee of the University of Western Australia.

Mice were maintained on a 12-h light-dark cycle (lights turned on at 07:00 h), at 22 °C, with free access to meat-free rat and mouse diet (protein, 20%; total fat, 4.8%; total fiber, 28.8%; total carbohydrate, 59.4%) fortified with vitamins and minerals (Specialty Feeds, Perth, WA, Australia) and drinking water. Mice were acclimated for 2 weeks until they reached 15 weeks and 107 weeks of age and were assigned to the following groups: (1) young sedentary (SED; 15 weeks, n = 10); (2) young low resistance (LR; 15 weeks, n = 7); (3) young high resistance (HR; 15 weeks, n = 7); (4) old sedentary (SED; 107 weeks, n = 9); (5) old low resistance (LR; 107 weeks, n = 7). A group of n = 8 mice was also sampled at 105 weeks of age to obtain baseline data for old mice prior to starting the exercise regime. Old mice were not subjected to HR exercise because of limited numbers. An additional group (n = 4) young mice (aged 12 weeks) were exercised for 1 week (without resistance) and then sampled to test whether recent myogenesis (indicative of necrosis and regeneration) had occurred after such mild exercise. Where ages are also expressed as months, this represents calendar months, so that 15, 25, 105, 107, and 117 weeks corresponds to approximately 4, 6, 24, 25, and 27 months, respectively. The age of young mice at time of tissue collection was 25 weeks (~6 months) and for old mice 105 (~24 months), and 117 weeks (~27 months).

SED mice were housed individually in standard mouse cages with transparent walls (19.5 cm × 28 cm) for the duration of the experiment. Exercising mice were housed individually in Lafayette Mouse Activity Wheel Chambers (23.5 cm × 35.3 cm; Model 80820; Lafayette Instrument, IN, USA) equipped with a 12.7 cm diameter exercise wheel with a 5.72 cm wide running surface (Model 80820RW, Lafayette) and an adjustable servo-brake (Model 86070-B1) to control resistance application and wheel function. Each chamber was equipped with an activity wheel counter (Model 86070A) to monitor wheel revolution, distance travelled (set at 0.40 m/revolution) and speed (m/min). The Activity Wheel Monitoring (AWM) Software (Model 86065) was used to record all data sets. Wheel loading was determined by hanging known weights on each individual wheel and adjusting the brake to hold each selected weight (per manufacturer’s instructions). These wheels are considered to be low resistance, or free spinning wheels, given that wheel inertia is very low (< 1 g).

Voluntary resistance exercise began at 15 weeks for young mice and at 107 weeks for old mice and lasted for 10 weeks, until mice reached 25 weeks and 117 weeks, respectively. Exercise protocols for LR and HR groups are detailed in Fig. 1. LR exercise groups
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Fig. 1. Experimental protocols for low (LR) and high (HR) resistance voluntary wheel running over 10 weeks. Young mice were exercised with LR and HR protocols from 15 weeks of age until sacrifice at 25 weeks. Old mice (107 weeks) were exercised with the same LR protocol and sacrificed at 117 weeks (see text for details).

ran without resistance for the first 2 weeks, with a 1 g increase in resistance commencing at the start of every 2 weeks (up to a maximum of 4 g; Fig. 1). The HR group ran without resistance for the first week, and was then subjected to a 1 g increase in resistance at the start of every week for the first 4 weeks (up to 3 g), and a further increase of 1 g every 2 weeks for the remaining 6 weeks (up to 6 g) (Fig. 1). Values for distance run and speed were recorded every hour, for each mouse, throughout the duration of the study by AWMSoftware and the data shown as an average value over 5 days. Body weights and food consumption were recorded for each mouse three times a week for 10 weeks. Food consumption was calculated by weight of food remaining in the feeding tray.

Haematoxylin and eosin (H&E) and nicotinamide adenine dinucleotide nitro-blue tetrazolium (NADH-TR) staining

Transverse frozen muscle sections (8 μm) of soleus and quadriceps muscles were stained with H&E to assess general tissue architecture. NADH-TR histochemical staining was used to identify fast and slow myofiber types in quadriceps transverse sections (Shavlakadze et al., 2005).

Laminin immunostaining

Polyclonal rabbit anti-PAN laminin antibody (L9393, Sigma, Australia; dilution 1:300) was used to label myofiber basement membrane (Shavlakadze et al., 2005). The primary antibody was detected by goat anti-rabbit ALEXA594 (A-11012, Invitrogen, Molecular Probes, Oregon, USA; dilution 1:500).

Image acquisition and morphometric analyses

Tiled images of transverse muscle sections stained with H&E, NADH-TR and laminin were captured at ×10 magnification using a Nikon Eclipse Ti inverter microscope equipped with Nikon DS-Fi2 camera (Nikon Corporation, Tokyo, Japan) for bright field imaging (H&E and NADH-TR) and CoolSNAP EZ camera (Roper Scientific Photometrics, Ottobrunn, Germany) for fluorescence imaging (laminin). Images were captured using NIS-Elements BR 4.1 software. Nontiled images of transverse muscle sections were captured at 40× magnification using a Nikon 90i microscope equipped with Nikon DS-Fi2 camera. Images were captured using NIS-Elements AR 3.0 software (Laboratory Imaging Ltd., Czechoslovakia, http://www.lim.cz/en/). Colour enhancements on H&E and laminin images were performed using Adobe Photoshop (Adobe Systems Incorporated) Version 7.

All morphometric analyses were carried out with ImagePro Plus 4.5 (Media Cybernetics, MD, USA) software. The soleus was selected for detailed analysis because it showed the most pronounced hypertrophic response to LR exercise at both ages. Tiled images of entire soleus muscles stained with laminin were portioned into four equal quadrants and the cross-sectional area (CSA) of 60 myofibers were measured in each quadrant, totaling 240 myofibers for each muscle section. Individual myofiber numbers were counted on entire soleus cross sections stained with laminin. The number of myofibers with displaced myonuclei were counted on entire soleus cross sections stained with H&E.

The number and size of different myofiber types were quantified on tiled transverse sections of the quadriceps stained with NADH-TR, that differentiates fast, intermediate, and slow-type myofibers (Shavlakadze et al., 2005). Quadriceps was selected for this analysis since it showed a loss of muscle mass with ageing and a hypertrophic response to LR wheel exercise in young mice. The quadriceps is predominantly a mixed fibre muscle and the percentage distribution of myofiber types differ between the four muscles that comprise it (rectus femoris and vastus medius, lateralis and intermedius). Tiled images of entire quadriceps muscle stained with NADH-TR were portioned into two equal parts, deep (close to the bone) and superficial (peripheral to the bone). The deep region was used to measure slow-type myofibers and the superficial region to measure fast-type myofibers. Intermediate type myofibers were excluded from the analyses. Two hundred myofibers were measured each in deep and superficial layers. Individual myofiber numbers were counted on entire quadriceps cross sections stained with NADH-TR.

Statistical analyses

Longitudinal analyses for body weights, average daily food consumption, running distance, and speed were performed with a repeated measure analysis of variance (ANOVA) using Genstat v15 (VSN International Ltd., Hemel, Hempstead, UK), with all experimental groups included in the treatment structure statement. Post-hoc multiple comparisons were performed using Tukey’s method (GenStat, 2003). The effect of age on SED mouse phenotype or muscle mass (15, 105, and 117 weeks) was analyzed using a one-way ANOVA (Genstat v15). Post-hoc tests of least significant difference were used for direct mean comparisons. The same treatment structure was used to analyze the effect of different exercise protocols on young mice (SED, LR, and HR). Data comparisons between SED and LR exercising young and old mice were analyzed with a two-way ANOVA (Genstat v15) using age (young and old) and activity (SED or LR) as sources of variance. Where no interaction between factors (age and activity) was detected, differences were determined by independent samples t-test (two-tailed). Any additional analyses by...
**Results**

**Body weights**

At the start of the experiment (week 1; W1), young (15 weeks) mice were lighter than old (107 weeks) mice, weighing on average 28.3 g and 31.6 g, respectively ($P < 0.001$; Fig. 2(a)). For all young mice, body weights increased progressively from 15 to 25 weeks of age (23% for SED, 13% for the LR and 11% for the HR; $P < 0.001$). Old SED mice did not change body weight over the 10 weeks of study, and old LR mice did not alter body weight significantly from SED controls. At the end of the experiment (W10), young SED mice were heavier than all other groups ($P < 0.001$; Fig. 2(a)).

**Food consumption**

Overall, the food consumed by all groups decreased (17%) over 10 weeks of the study ($P < 0.001$). However, young mice consumed more food (18%) compared with old mice ($P < 0.001$) and there was a tendency ($P < 0.1$) for the old LR group to consume more food (9%) compared with the old SED group (Fig. 2(b)).

**Running distance**

Distances run by young mice increased from W1 to peak at W2 (at least $P < 0.05$) before declining over the remainder of the study ($P < 0.001$). The distance run by old mice declined between W1 and W10 ($P < 0.01$) and was significantly lower than that of young mice during the first 3 weeks (Table 1).

**Running speed**

Average nightly speeds achieved by young mice either increased (HR, $P < 0.05$), or tended to increase (LR, $P < 0.1$) from W1 to peak at W2, before declining over the remainder of the study ($P < 0.001$). Nightly speeds among old mice also declined from W2 to W10 ($P < 0.05$) and were lower than that of the young mice during the first three weeks of the study (Table 2).

**Running patterns**

Average hourly distances run by young and old (LR) mice over 24 h throughout each fortnight are shown in Fig. 3. Overall, young mice subjected to LR or HR exercise ran further than old mice given LR exercise ($P < 0.01$). Mice are nocturnal and exercise mainly at night. Accordingly, in both young and old mice, most of the wheel activity coincided with the dark phase (19:00–07:00 h). Young mice had two major intervals of activity. The first occurred during the dark phase, where running distance peaked 2 h after lights out, before progressively declining throughout the remainder of the phase (Fig. 3(a–e)). The second was initiated 2 h before the
light phase and peaked within 1 h after lights on. In contrast, running activity in old mice peaked 2 h into the dark phase, but there was no second phase of activity before lights were switched on (Fig. 3(a–e)). Young mice given HR exercise had a similar pattern of activity to that of young LR mice (Supporting Information Fig. S1). When running activities at equal resistance were compared (Supporting Information Fig. S1(a–e)), HR mice outperformed LR mice at both a 1 and 2 g load (W2 and W3, respectively; Supporting Information Fig. S1(b,c)), but were similar for the 3 and 4 g load (Supporting Information Fig. S1(d,e)).

**Phenotypic characterization of ageing SED mice**

Body weights of old mice aged 105 and 117 weeks were 11% lower than for young adults aged 25 weeks (P < 0.05) and this was due in part to a marked decrease in epididymal fat pad weight (62% and 58%, respectively, between 25 weeks and 105/117 weeks, P < 0.05; Table 3). Because tibial length was smaller (2.2% and 3.3%) in old (105 and 117 weeks) compared with young (25 weeks) mice, indicating that the older cohort had smaller body size, all muscle weights were standardized to tibia length to account for the difference in body size (Table 3).

The relative muscle mass of quadriceps and gastrocnemius was reduced from 25 to 105 weeks of age by 15.5% (P < 0.05) and 8% (P < 0.05), respectively (Table 3). There was no further significant mass reduction for these muscles between 105 and 117 weeks of age. In addition, the relative mass of TA was reduced by 10.3% between 25 and 117 weeks (P < 0.05). Muscle mass for EDL, soleus, and triceps did not change with ageing (from 25 to 117 weeks; Table 3).

**Impact of LR exercise on muscle and fat mass in young and old mice**

Voluntary LR exercise for 10 weeks increased the mass of selected limb muscles in both young and old mice, compared with age-matched SED controls. For quadriceps, gastrocnemius, and soleus muscles, a two-way ANOVA showed an effect of age (P < 0.001), activity state (P < 0.005), and an interaction between these factors (P < 0.001; Fig. 4(a–c)).

The standardized weights of quadriceps, gastrocnemius, and soleus muscles from young LR (25 weeks) mice were heavier by 16.5%, 23.5%, and 52%, respectively, compared with SED young mice (P < 0.001, independent sample t-test; Fig. 4(a–c)). Exercise did not affect the mass of young TA, EDL, or triceps muscles (Fig. 4(d–f)). Unlike young mice, only soleus increased in mass (18%) in old mice subjected to LR exercise compared with SED age-matched controls (P < 0.04, independent sample t-test; Fig. 4c). Such LR exercise in old mice did not affect the muscle mass of quadriceps, gastrocnemius, TA, EDL nor triceps (Fig. 4(a,b,d–f)).

Epididymal fat pad mass was influenced by age (P < 0.001) and activity state (P < 0.001) with an interaction between these factors (P = 0.05; Fig. 4(g)). Young mice subjected to LR exercise had ~50% less epididymal fat compared with age-matched SED mice (P < 0.002; independent samples t-test). In contrast, the weights of epididymal fat pads were similar for old LR exercised and SED mice, but were less compared with young SED mice.

**Impact of HR exercise on young mice**

HR exercise increased the mass of quadriceps and soleus muscles, to the same extent as did LR exercise (Fig. 4(a,c)). Quadriceps and soleus muscles from young HR exercised mice were heavier by 14% and 37.5% compared with muscles from young SED mice (P = 0.004 and P < 0.001, respectively; independent samples t-test; Fig. 4(a,c)). Unlike LR exercise, HR wheel running did not increase the gastrocnemius mass (Fig. 4(b)). HR exercise decreased epididymal fat pad weight to the same extent as LR exercise, and contributed to a 42% decrease relative to SED controls (P = 0.005; independent samples t-test; Fig. 4(g)). Given the similar impact of HR and LR exercise on muscle mass, only LR exercised muscles were analyzed in depth.

**Soleus muscles: myofiber number and size in SED and LR exercised young and old mice**

The number and size of individual myofiber profiles were quantified on transverse sections of soleus muscles immunostained with laminin antibody, which defines individual myofiber contours. The soleus was selected for detailed myofiber analyses because of the significant increase in size after LR exercise for both young and old mice.
The number of myofiber profiles in soleus was increased by 28% in young LR mice compared with SED controls ($P = 0.05$; independent samples $t$-test; Fig. 5(a)). This was associated with a striking (21%) increase in myofibers with displaced myonuclei ($P = 0.01$; independent samples $t$-test; Fig. 5(b)); where myonuclei were not in the normal peripheral juxtasarclemmal position (Fig. 6(a)), but were instead located centrally or well within the sarcoplasm (Fig. 6(b); arrows). Myofiber numbers were similar in the soleus muscles of young and old SED mice (Fig. 5(a)); however, the number of myofibers that contained displaced myonuclei increased by $-3\%$ with age alone ($P = 0.04$; independent sample $t$-test; Fig. 5(b); Fig. 6(c); arrows). LR exercise did not further increase the number of myofibers with displaced nuclei in old soleus muscles (Fig. 5(b); Fig. 6(d); arrows). Overall, no change in average myofiber CSA was detected by two-way ANOVA in the soleus (Fig. 5(c)). Analysis by independent samples $t$-test showed a trend for decreased myofiber CSA between young and old SED mice ($P = 0.065$; Fig. 5(c)).

Further analysis of myofiber CSA distribution and of histology of soleus muscles showed that young SED mice have a normally distributed myofiber profile (clustered at 2000 $\mu$m$^2$; Fig. 5(d)) and uniform muscle architecture (Fig. 6(a)). LR exercise in young mice shifted myofiber CSA toward smaller myofibers, with 13.5\% of all myofibers measured approximating $\leq 500\mu$m$^2$ (Fig. 5(d)). This is likely due to the branching of myofibers, and contributes to loss of structural uniformity and clustering of smaller myofibers throughout sections of the young exercised soleus (compare Fig. 6(a, b)). Age also influenced the distribution of myofiber CSA, with both SED and LR cohorts of old mice displaying an overall shift in myofiber CSA toward smaller myofibers (Fig. 5(d)), compared with young mice. As for young mice, old LR mice had a modest shift toward larger myofibers relative to old SED controls.
Central myonuclei and branched myofibers in soleus of young LR mice

To further investigate the presence of small myofibers and central myonuclei in young LR mice, serial sections from the soleus stained with H&E or pan-laminin and Hoechst were analyzed (Fig. 7). Figure 7(a) shows two myofibers with central myonuclei and subsequent serial sections stained with laminin show that the sarcolemma starts to invaginate into these myofibers (Fig. 7(b–c)) and progresses to where two distinct myofiber profiles are present (Fig. 7(d–f)); each still retaining a central myonucleus.

Additional young mice aged 12 weeks were exercised for 1 week on a voluntary running wheel (0 g resistance) and soleus muscles from these exercised and age-matched sedentary controls were sectioned and stained with H&E (Fig. 8(a,b)). Control sedentary soleus had normal architecture (Fig. 8(a)), whereas the exercised soleus showed many small and large myotubes/young myofibers with central myonuclei, confirming that myonecrosis had occurred rapidly in response to the unaccustomed exercise with resultant regeneration over the 7 days (Fig. 8(b)).

Quadriceps muscles: myofiber number and size in young and old SED and young LR mice

The quadriceps was selected for detailed analyses because of the decline in relative muscle weight with age, and increased muscle mass in young LR mice. As there was no increase in mass of old LR quadriceps, myofiber number and CSA were not further investigated.

No difference in total myofiber number was detected between quadriceps of young and old SED mice (Supporting Information Fig. S2(a)). Thus, the decline in quadriceps weight with age can be attributed in part to a significant reduction in the CSA of both fast- (19%) and slow- (20%) type myofibers (P = 0.009 and P = 0.031 respectively; independent samples t-test; Supporting Information Fig. S2(b)). Percentage distribution of fast-type myofibers demonstrated a striking shift in the CSA of all myofibers toward smaller sizes with age (Supporting Information Fig. S2(c)), while for slow-type myofibers, there was a selective loss of larger myofibers (Supporting Information Fig. S2(d)).

LR exercise in young mice contributed to an 18.5% increase in the number of myofibers in quadriceps compared with young SED controls (P = 0.02; independent samples t-test; Supporting Information Fig. S3(a)). Only a trend increase in fast-type myofiber CSA was observed between young LR and SED quadriceps (P = 0.085; independent samples t-test; Supporting Information Fig. S3(b)). The young LR quadriceps showed a striking shift in the CSA of all fast-type myofibers, and a modest shift in slow-type myofibers toward larger sizes relative to young SED controls (Supporting Information Fig. S3(c,d)).

There was no evidence of myofiber splitting in the quadriceps of young SED or old SED/LR mice (Supporting Information Fig. S4(a,c,d)), although this was seen for young LR mice (Supporting Information Fig. S4(b)). Central/displaced myonuclei were conspicuous on H&E sections of the quadriceps (in rectus femoris and vastus lateralis, medialis, and intermedialis) of young LR mice (but not young SED; compare Supporting Information Fig. S4(a,b)). While myofibers contained displaced myonuclei in old (SED and LR) mice (Supporting Information Fig. S4(c,d)), there appeared to be little myofiber necrosis, since myofibers with fragmented sarcoplasm

### Table 3. Phenotypic characterization of muscle weights standardized to tibia length in S57Bl/6J mice aged 25, 105, and 117 weeks

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Absolute mass</th>
<th>Relative mass, mg/tibia length, cm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 weeks (n = 10)</td>
<td>105 weeks (n = 8)</td>
</tr>
<tr>
<td>Body weight (g)**</td>
<td>35.9 ± 0.7</td>
<td>32.0 ± 1.2 #</td>
</tr>
<tr>
<td>Epididymal fat (g)***</td>
<td>1.2 ± 0.1</td>
<td>0.46 ± 0.1 #</td>
</tr>
<tr>
<td>Tibia length (mm)*</td>
<td>17.9 ± 0.14</td>
<td>17.5 ± 0.15#</td>
</tr>
</tbody>
</table>

Values are means ± SE; asterisk (*) indicates an effect of age. **P < 0.05; ***P < 0.001. Hash (#) indicates significantly different from 25 weeks, P < 0.05. No significant differences were observed between mice aged 105 and 117 weeks.

n, number of mice; n/a, not applicable; SED, sedentary.
and inflammatory cells were not seen (and areas with myoblasts and small myotubes resulting from early regeneration as a result of myonecrosis were not evident) in old muscles.

**Discussion**

When young (15–25 weeks) and old (107–117 weeks) mice were subjected to increasing resistance exercise using voluntary running wheels for 10 weeks, the old mice ran less distance, were slower, and there was a striking age-related difference in their diurnal running patterns. While some skeletal muscles responded to the resistance exercise by muscle hypertrophy, others did not, and the increase in muscle mass was more pronounced in young, compared with old, mice.

Because all exercise was voluntarily and old mice ran less, the intensity and duration of exercise were not...
equivalent between young and old groups. Clearly, this aspect can influence the extent of hypertrophy in response to resistance exercise and we are aware that this complicates comparisons between young and old mice in our study.

Exercise performance and running patterns in young and old mice

Young mice were subjected to two resistance protocols: either low (LR) or high (HR) resistance. Only LR
Exercise was tested in old mice because of limited numbers of aged mice. Exercise performance and running patterns were clearly affected by ageing with decreased distance and speed for old mice. In addition, while two distinct peaks of activity were seen in young mice over 24 h (one 2 h after lights out (19:00 h) and the other at 2 h prior to lights on (07:00 h)), old mice ran almost exclusively during the dark phase (night) with activity peaking around 19:00 h. An age-related decline in wheel running activity is well documented in rodents (Ingram, 2000; Cheng et al., 2013; McMahon et al., 2014). In our study, shorter distances run by old mice were attributed to a decrease in speed and, although not measured in this study, a decline in the amount of time spent running is also common for ageing mice (McMahon et al., 2014). The different diurnal patterns of running between young and old mice are similar to those reported by others in C57Bl/6J males (Houtkooper et al., 2011). The precise reason why old mice did not display the second peak of activity (around 07:00 h) seen in young mice is not clear, although contributing factors may be age-related diminished endurance (decreased oxygen consumption, $V_{O_2\text{max}}$ and maximal exercise capacity; Schefer & Talan, 1996) and altered circadian rhythms (Valentinuzzi et al., 1997), since there are strong circadian patterns of gene regulation that control metabolism (Shavlakadze et al., 2013). Testosterone is also a strong driver for exercise in mice, as removal of endogenous testosterone by orchietomy results in almost complete cessation of voluntary wheel running (Ibeunjo et al., 2011). Whether testosterone concentrations decline in old male mice is unclear. One study showed no difference in serum testosterone of 6- and 29-month-old C57Bl/6J mice (Hamrick et al., 2006), whereas another reported that serum testosterone concentrations are halved in 24-month-old C57Bl/6J mice compared with 4 months (Kovacheva et al., 2010). It is possible that testosterone is high in young 4-month-old mice and declines by 6 months, with no further decline with ageing. While administration of testosterone to 28-month-old male C57Bl/6J mice in combination with low-intensity exercise (treadmill three times/week) showed many benefits and it was stated that testosterone concentrations declined at this late age, no measurements of testosterone levels were provided (Guo et al., 2012).

While C57Bl/6J mice are less avid runners than some other mouse strains (Irintchev & Wernig, 1987; Lightfoot et al., 2004; Nehrenberg et al., 2009), male C57Bl/6J mice aged 8–26 weeks run approximately 7.5 km per night on wheels without resistance (Allen et al., 2001; Lerman et al., 2002). This distance is similar to our study where young male mice ran approximately 6–7 km per night on voluntary running wheels (without resistance), with decreased distance when a 2–4 g load was applied. The inverse relationship between applied resistance and exercise performance (distance run and speed) has been demonstrated previously (Konhilas et al., 2005); although young adult (∼3 months) C57Bl/6J male mice tolerated a 7 g increase in wheel load (∼25% of total body weight) before the distance run decreased significantly (Konhilas et al., 2005). The difference between these two studies (using similar mice) regarding the tolerance to increasing resistance

![Fig. 6. Transverse sections of soleus muscles stained with H&E from young SED (a), young LR (b), old SED (c), and old LR (d) mice. Small myofibers with displaced (e.g., central) myonuclei (arrows) are visible in young LR mice (b). Myofibers with displaced myonuclei were also seen in old soleus muscles from SED (c) and LR (d) mice (arrows). Scale is 50 μm.](image)
may be due to different exercise protocols (the timing of resistance application) and the wheel setups used.

The initial distance run by old male mice on wheels without resistance in our study (~3 km over 24 h) was about half that for young mice, although old mice maintained stable levels of daily running distance over many weeks (W1 to W8). Decreased voluntary exercise performance with age has been previously reported by our group for male FVB mice (McMahon et al., 2014) and by others in female (Cheng et al., 2013) and male (Valentinuzzi et al., 1997) C57Bl/6J mice. In the present study, while old mice ran less than young mice, they had the capacity to run voluntarily even when a load of ~13% of total body weight (4 g) was applied to the wheels. Notably, the most enthusiastic old male mouse maintained initial levels of running activity (W1) well into W6 when 2 g of wheel resistance was applied, and remained the highest achiever until W8 (3 g).

Impact of resistance exercise on adult and old mice

Food consumption in adult and old sedentary and exercised mice

The apparent food consumption of all mice was measured (by removal of food from the feeder box) throughout the 10 weeks study. It should be noted that because our mice were housed on pine shavings, instead of wire bottom cages, we were unable to quantify the amount of dropped food: yet, old mice are messy eaters and drop more food and this confounds many measurements of food consumption in ageing rodents (reviewed in Starr & Saito, 2012). A detailed comparison of food intake in sedentary C57Bl/6J male mice aged about 2, 6, 12, 20 and 29 months showed that the amount of food removed from the feeder box was constant throughout life, apart from a significant (transient) increase at 20 months (Starr & Saito, 2012). This age corresponded to the time when food dropping...
dramatically increased (doubled) for all older mice and thus there is a tendency to overestimate the amount of food consumed by mice aged more than 20 months. This study concluded that actual food consumption only decreased in the very old mice aged 29 months, although the amount of food intake per body weight was constant for all mice aged from 6 to 29 months. Despite this caveat, during our 10-week study, young SED mice (aged 4–6 months) consumed more food and their body weight increased significantly (probably because of increased adiposity), compared with less apparent food intake by old SED mice (aged 25–27 months).

The voluntary resistance exercise protocols (LR and HR) in our study did not increase food intake in young mice, but did slow the rate of weight gain and reduce epididymal fat. In contrast, for old LR mice there was a trend for increased food intake, although no impact on body weight or epididymal fat weight was detected. Long-term access to a voluntary running wheel (greater than 4 weeks) increased food intake and decreased adiposity among adult male and female mice (Swallow et al., 2001) and rats (Tokuyama et al., 1982). However, studies in adult male C57BL/6J and DBA/J mice (strains considered to have lower running activity) found no difference in food consumption after 3 to 13 weeks of voluntary wheel exposure (Harri et al., 1999; Jung et al., 2010).

In young mice, LR exercise increased the mass of quadriceps, gastrocnemius, and soleus muscles, whereas HR increased the mass of only quadriceps and soleus muscles (with similar efficacy to that of LR exercise). The lack of impact of HR exercise on gastrocnemius muscle mass may be explained by the decrease in distance run with HR compared with LR. Similar to our study, no additional benefit of HR (capped at 12 g) compared with LR exercise (capped at 5 g) was reported for increasing soleus muscle mass in adult male C57BL/6J mice (Konhilas et al., 2005; Table 4).

In our study, the soleus muscle showed the most hypertrophic response to resistance exercise in all mice and this accords with studies in rats and mice subjected to resistance wheel running, where the soleus hypertrophied more than other limb muscles (Konhilas et al., 2005; Legerlotz et al., 2008; Table 4). In contrast, voluntary wheel running protocols (no resistance) ranging between 1 and 8 weeks were insufficient to increase the mass of soleus, gastrocnemius or TA in adult C57BL/6J mice (Allen et al., 2001; Konhilas et al., 2005; Pellegrino et al., 2005; Table 4). The lack of a hypertrophic response by many hindlimb muscles to resistance wheel running in our study (TA, EDL) also agrees with other reports (Konhilas et al., 2005; Landisch et al., 2008; Legerlotz et al., 2008). Although not analyzed in this study, the plantaris is widely studied in rats and shows a strong hypertrophic response to exercise (Table 4).

While the impact of resistance wheel running has been studied in juvenile and adult mice and rats (Table 4), we could not find any reports of studies using resistance wheels for old rodents. Whether old muscle has a diminished capacity to undergo a compensatory increase in mass and strength in response to increased loading compared with young muscle is not clear. In humans, progressive resistance training matches exercise intensity between young and old participants by measuring each individual’s one-repetition maximum, with every increase in resistance/intensity then calculated as a percentage of that maximum (as in Kosek et al., 2006; Mero et al., 2012). Thus, young and old participants perform the same number of repetitions and sets, at the same relative intensity, allowing for a fair comparison between both ages. The difficulty in matching exercise intensities between young and old mice under voluntary conditions complicates the comparison of the outcome of exercise in animals of different ages.

The benefits of exercise on ageing muscles have been described for many animal models and human subjects. Long-term exercise, even without resistance, initiated in young adult rats (Brown et al., 1992) and mice (McMahon et al., 2014) benefits selected skeletal muscles with ageing. Short-term voluntary wheel running of old mice for 1 month (from 22 to 23 months)
prevented the loss of neuromuscular junctions (Valdez et al., 2010), as did long-term exercise for 4 or 10 months (from 21 to 25 months or 18 to 28 months, respectively; Cheng et al., 2013), plus life-long exercise (4 to 28 months) prevented sarcopenia in quadriceps, with variable benefits on other muscles (McMahon et al., 2014). An important question that we attempted to address in the present study was whether exercise introduced later in life will reduce the age-related loss of muscle mass (sarcopenia). Even though exercise duration and intensity was not matched between young and old mice, and the old mice ran shorter distances, hypertrophy of soleus muscles did occur in both ages, although to a lesser extent in the old mice.

We carried out detailed histological analyses on soleus and quadriceps muscles to more fully understand the cellular impact of exercise on the young and old mice. The age-related loss of muscle mass in old mice is accounted for by myofiber atrophy and reduced numbers of myofibers that varies in extent with gender and between different muscles (Sheard & Anderson, 2012). Our detailed histological analyses showed similar numbers of individual myofiber profiles in transverse sections of young and old soleus and quadriceps muscles, with reduced myofiber size (CSA) only evident in quadriceps. It should be noted that in transverse sections, numbers of myofiber profiles do not necessarily reflect the total number of myofibers because myofiber branching or splitting can be a confounding factor (Ontell, 1986; Shavlakadze et al., 2010b), and such split myofibers can appear with age (Pichavant & Pavlath, 2014).

Myofibers with displaced (nonperipheral and often central) myonuclei were also increased in old SED and LR soleus muscles. The precise reasons for this are hard to determine since such central myonuclei can result from myonecrosis and subsequent regeneration of adult myofibers (McGeachie et al., 1993) as well as from...
Exercise and muscle hypertrophy in old mice

Resistance exercise is widely proposed as a strategy to combat age-related loss of muscle mass and function (sarcopenia) in humans. This is the first study to assess the impact of increasing resistance exercise using voluntary wheel running on muscles of old mice. The low resistance exercise regime (with weight increasing from 1–4 g) over 12 weeks showed that old male C57Bl/6J mice ran less than young mice, and had only a single peak of nocturnal running activity. In response to exercise, the most striking increase in mass (hypertrophy) was seen for the soleus of young mice and was associated with histological evidence of exercise-induced myonecrosis and muscle regeneration. Exercise-induced hypertrophy also occurred for the quadriceps and gastrocnemius muscles in young, but not old, mice: other muscles did not hypertrophy at either age. Hypertrophy was less pronounced and there was no evidence of myofiber damage in old mice: this might reflect reduced exercise capacity with age. While resistance exercise resulted in some hypertrophy of old muscles, exercise may also have benefits on innervation which would help maintain other aspects of muscle function and coordination, although this was not evaluated in the present study. It is emphasized that gender needs to be considered in ageing rodents and that benefits cannot be generalized across all muscles.

**Key words:** Exercise, ageing, hypertrophy, old skeletal muscle, myofiber splitting.
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Acknowledgements

This study was supported by funding from the Australian Research Council (Grant LP120100520 to M. G.) and postgraduate research scholarships from the University of Western Australia and the Centre for Cell Therapy and Regenerative Medicine Top-Up Scholarship, School of Medicine and Pharmacology, University of Western Australia and Harry Perkins Institute of Medical Research, Perth, Western Australia (Z. S.).

Author contributions


Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Figure S1. Average distance run by young HR mice per hour over 24 h. Each hourly value represents an average over 1–2 weeks. Values are calculated for week (W) 1 with resistance set at 0 g (A); W2 (1 g) (B); W3 (2 g) (C); W4 (3 g) (D); W5 and W6 (4 g) (E); W7 and W8 (5 g) (F); W9 and W10 (6 g). Where young LR mice ran at the same resistance (G), distances run per hour over 24 h were added for comparison (A–E). Asterisk (*) indicates significant differences between young LR and HR mice at $P < 0.05$; ($*$) $P < 0.01$; (***) $P < 0.001$.

Figure S2. Morphometric characterization of quadriceps muscles from young and old, SED mice. Entire transverse sections of soleus muscles stained with NADH-TR were used to quantify number of individual myofiber profiles (A), average myofiber size as cross-sectional area (CSA) (B) and myofiber size distribution of both fast (C) and slow (D) type myofibers. Asterisks (*) indicates significant differences between young and old SED mice at $P < 0.05$. Data are mean ± SEM. $n = 4–7$ mice per group.

Figure S3. Morphometric characterization of quadriceps muscles from young SED and LR mice. Entire transverse sections of soleus muscles stained with NADH-TR were used to quantify number of individual myofiber profiles (A), average myofiber size as cross-sectional area (CSA) (B) and myofiber size distribution of both fast (C) and slow (D) type myofibers. Asterisk (*) indicates significant differences between young SED and LR mice at $P < 0.05$. Data are mean ± SEM. $n = 4–7$ mice per group.

Figure S4. Morphometric characterization of quadriceps muscles from young and old, SED and LR mice. Transverse sections of quadriceps muscles (rectus femoris) stained with H&E from young SED (A), young LR (B), old SED (C) and old LR (D) mice (40x magnification). Myofibers with displaced (e.g., central) myonuclei (arrows) are visible in young LR mice (B). Myofibers with displaced myonuclei were also seen in old quadriceps muscles from SED (C) and LR (D) mice (arrows). Scale bar is 50 μm.

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