Increased physical activity does not improve obesity-induced decreases in muscle quality in zebrafish (Danio rerio)

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Recommended levels of aerobic exercise do not restore obesity-induced decreases in muscle quality

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Abstract

Obesity has a negative effect on muscle contractile function, which may cause physical disability and impair mobility. These effects of obesity on muscle are not reversed by weight loss. It is therefore important to determine how muscle function can be restored, and exercise is the most promising approach. The Centre for Disease Control and Prevention, USA recommends a minimum level of 30 min of moderate physical activity per day to mediate health benefits from exercise. We tested the hypothesis (in zebrafish, *Danio rerio*) that exercise at the recommended levels will alleviate the negative effects of obesity of muscle function and help reverse these effects during weight loss. We show that obesity leads to decreased muscle force production per unit area (stress), and slowed muscle contraction and relaxation rates. These effects were not reversible by weight loss or exercise, or their interaction. However, swimming performance and myosin heavy chain concentrations were not affected by obesity. The latter data are at variance with our earlier work. Re-analysing our earlier data together with the current data set led us to the conclusion that there is a proportional effect of obesity and, unlike contractile function which is more sensitive, locomotor performance declines gradually as body mass index increases. Our data suggest that more intense aerobic exercise and a combination between modes of exercise, such as endurance and strength exercise, may be necessary to improve muscle quality during obesity and following weight loss.

Introduction

Skeletal muscle is the largest organ in the vertebrate body and has a profound influence on metabolic phenotypes, posture and mobility [1]. Obesity (defined as body mass index [BMI] > 30; [2]) can have a negative effect on muscle contractile function principally by disruption of calcium cycling and activity of the AMP-activated protein kinase (AMPK) [3]. As a result, there is a shift from slow to fast muscle fibres, and a decrease in force production and power output of skeletal muscle [4,5]. We have shown previously that obesity led to a decrease in muscle power output, force production per cross-sectional area, and muscle relaxation rate in zebrafish (*Danio rerio*). These changes were accompanied by a decrease in locomotor performance. Interestingly, these decreases in muscle contractile function were not reversed by weight loss [6]. Hence, weight loss alone is not always a sufficient intervention to treat the adverse effect of obesity.

Regular aerobic exercise has the opposite effects of obesity, and it leads to a shift from fast to slow muscle fibres, greater oxidative capacities, and a leaner phenotype [7,8]. Exercise is an effective treatment for a broad range of medical conditions, ranging from heart disease to cancer and psychological conditions [9]. Increased exercise also increases energy expenditure and therefore facilitates weight loss. Knee extension strength of active obese adult humans was greater than in sedentary obese or lean groups [10], indicating that physical activity can improve muscle contractile function. There is often an increase in strength of weight-bearing muscles in obese individuals as a result of the greater load muscles experience [11], although overall muscle quality decreases with obesity [3]. The obesity-induced decrease in muscle quality is likely to decrease mobility and energy expenditure [12], and thereby facilitate further weight gain. It is important, therefore, to determine whether

exercise intervention alleviates the negative effects of obesity on muscle function. The recommended levels of exercise to achieve health benefits (by the Center for Disease Control and Prevention, USA) are 30 min of moderate aerobic exercise on five days per week plus additional strength training [13,14]. These recommendations are based on comparisons between sedentary and regularly exercising individuals. Obesity exacerbates the effects of sedentary behaviour, and it has additional, specific impacts on muscle contractile function [3] which may modify the effects of exercise. The aim of this study therefore was to determine whether weight loss combined with aerobic exercise restores muscle contractile function in zebrafish. We tested the hypotheses that a) weight loss accompanied by recommended levels of moderate aerobic exercise will shift muscle fibre types from fast to slow and improve muscle contractile function (stress, and activation and relaxation rates), and b) that even obese individuals that perform regular exercise will show less decline in muscle contractile function compared to sedentary obese individuals.

Materials and Methods

Study animals and treatments

All procedures were performed with the approval of the University of Sydney Animal Ethics Committee (approval #723). Adult zebrafish (*Danio rerio*) were obtained from a commercial supplier (Livefish, Bundaberg, Australia) and maintained in plastic tanks (600 x 450 x 250 mm; 1-2 fish l^{-1}) with dechlorinated water at 22^{o}C, and a 12h dark:12 h light photoperiod for two weeks before experimentation, and fed with commercial fish flakes (Wardley's, The Hartz Mountain Company, Secaucus, USA; 46% protein, 6% fat). After two weeks fish were randomly allocated to one of the experimental groups. The experiment comprised two independent factors, obesity with three levels (obese, lean, obese - lean), and exercise with two levels (exercise and control). Fish were kept in circular containers

(dimensions) with a central column that created an annulus. All tanks contained a submersible pump (model), which were switched on in the exercise treatments but not in the control treatments (see below). There were 10-15 fish in each tank, and there were five tanks for each obesity x exercise treatment combination.

The obesity treatments were: 1) lean fish, which were fed to satiety once a day for six days per week for 9-10 weeks; 2) obese fish fed to satiety three times per day for six days per week, and once per day on the seventh day for 9-10 weeks; 3) obese-lean fish, which were fed as obese fish for 4-5 weeks, and then switched to the lean diet for 4-5 weeks. For subsamples of 10-15 fish per treatment, we took photos (with an Exilim camera, Casio, Japan) to determine standard length (in ImageJ software, NIH, USA), and we weighed fish at the beginning of treatments, and again at the time when obese-lean fish were switched to the lean diet. We weighed and measured all experimental fish at the end of the treatments immediately before measurements were taken.

In the exercise treatment, we aimed to implement at least the levels of exercise that confer health benefits as recommended by the Center for Disease Control and Prevention, USA [13]. The recommendations stipulate a minimum of 150 min of moderate aerobic exercise per week, where moderate is defined as exercise inducing energy expenditure that is 3-6 times that of an inactive animal. We exercised fish for 30 min daily, somewhat more than the minimum recommended. The pumps created a flow of around 0.08 m s^{-1} (2-3 body lengths s^{-1}) when switched on [15]. Our previous data on cost of transport in zebrafish show that this flow rate increases metabolic rate to 3-4 times that of resting rates [16]. We verified that fish in the flow treatment exercised significantly more than fish in still water by filming groups of fish at the same density as that used for experiments (10-15 fish in each of n = 10 tanks) with pumps switched on and off. From the videos, we counted tail beat frequencies (in Tracker Video Analysis software, https://physlets.org/tracker/) of five fish per tank with the pumps on

and off. With the pumps off, tailbeat frequencies were 55.5 (± 0.6 [s.e.]) beats per minute, which increased to 170.7 (± 1.1 [s.e.]) beats per minute with the pumps on.

Swimming performance

Sustained swimming performance was measured (in n = 15-16 fish per treatment combination) as critical sustained swimming speed (U_{crit}) in a Blazka-type swimming flume according to published protocols [17]. The U_{crit} protocol uses an incremental increase in speed (U_i) for predetermined time intervals (T_i) until fish are fatigued as a measure of maximum locomotor capacity.

Muscle biomechanics

Fish (n = 10 per treatment group) were euthanized cervical dislocation. The skin was removed and a section of rostral (anterior dorsal) muscle fibres of 5 to 7 myotomes in length was dissected from one side of the fish in cooled (<5°C) aerated fish Ringer's solution (composition in mmol l^{-1}: NaCl 115.7; sodium pyruvate 8.4; KCl 2.7; $MgCl_2$ 1.2; $NaHCO_3$ 5.6; NaH_2PO_4 0.64; HEPES sodium salt 3.2; HEPES 0.97; $CaCl_2$ 2.1; pH 7.4 at 20°C) [18]. The spine was removed from most of the muscle preparation leaving one myotome attached to the residual amount of spine at either end.

We conducted isometric studies to determine the twitch and tetanus kinetics of the isolated muscle according to published protocols [6]. In our previous work, we found that isometric tetanus force gave similar results to work-loop measurements [6] so that we determined tetani only here. We calculated rates of force production as peak tetanic stress (force per cross-sectional area) divided by twice the time to half peak tetanus, and muscle relaxation as peak tetanic stress divided by twice the time from last stimulus to half relaxation as measures of the contractile performance of muscle. After 5 min rest following tetanus

measurements, fatigue resistance was determined by subjecting the muscle preparation to a series of tetani, each of 150 ms stimulation duration, at a rate of one tetanus per second for 25 s. For each muscle, fatigue resistance was calculated as the maximal force produced in the 25^{th} tetanus as a percentage of the maximal force produced in the 1^{st} tetanus for the same muscle. Ten minutes after the fatigue run each preparation was stimulated to produce a further tetanus to determine recovery from the fatigue run. The mean recovery of all 30 muscle preparations was 81.1% indicating that reversible fatigue had been induced.

At the end of the muscle mechanics experiments, bone and connective tissue were removed and each muscle preparation was blotted on absorbent paper to remove excess Ringer's solution. Wet muscle mass was determined to the nearest 0.1 mg using an electronic balance (LA120S, Sartorius, Australia). Mean muscle cross-sectional area was calculated from muscle length and mass assuming a density of 1060 kg m^{-3}. The overall mean cross-sectional area ± s.e. of all muscle preparations was 2.65 ± 0.17 mm^2. Maximum isometric muscle stress (kN m^{-2}) was then calculated for each tetanic response as the maximum tetanic force within a trial divided by mean cross-sectional area.

Myosin heavy chain concentrations

We froze muscle samples (from n = 8 fish per treatment) collected from the dissections for the muscle mechanics measurements immediately in liquid nitrogen and stored them at -80°C. For the assays, muscle samples were homogenised (in a TissueLyser LT; Qiagen, Venlo, Netherlands) in 9 volumes RIPA buffer (20mM TrisCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP40, 1% sodium deoxycholate) and protease inhibitor cocktail (cOmplete, EDTA-free; Roche Life Sciences, Germany) solution. The identification and quantification of slow and fast myosin heavy chain (MHC) isoforms was performed by capillary electrophoresis in a "Wes" Simple Western System (ProteinSimple,

CA, USA) following the manufacturer's instructions. The antibodies (all from Developmental Studies Hybridoma Bank, University of Iowa, USA) we used were: EB165 to determine fast MHC concentrations; BA-F8 to determine slow MHC concentrations; 12G10 (α-tubulin) as internal control. We expressed normalised MHC concentrations by dividing MHC peaks by α-tubulin peaks measured for the same sample on the same plate. The concentrations of protein extracts was determined using a Bradford assay kit (Sigma-Aldrich, Castle Hill, Australia) following the manufacturer's instructions.

Statistical analysis

We analysed all data with permutational analyses in the R package lmPerm [19]. Permutational analyses do not make assumptions about underlying data distributions, but use the data per se to infer significant differences. This approach is preferable to parametric tests, especially for sample sizes that are small relative to the total population of all possible samples[20]. We analysed all dependent variables (body mass index, U_{crit}, muscle mechanics, and MHC concentrations) in a fully factorial design with exercise (exercise and control) and obesity (lean [L], obese [O], obese then lean [OL]) as factors. We used 'tank' and 'sex' as a random variables to control for tank effects and sex differences. In the event, neither was significant (all $p > 0.9$). In analyses of U_{crit} (in m s^{-1}) we used body length as covariate, but we show data in units of body lengths per second to facilitate comparisons. In case of significant results, we used pair-wise permutational tests for post hoc comparisons using marginal means (p-values given in text). We used $p < 0.05$ to indicate significant differences between treatment groups. Sample sizes were based on the power we achieved using similar techniques on zebrafish in past experiments [6,21].

The part of the experiment testing the effect of obesity treatment on muscle performance, MHC concentrations, and locomotor performance repeated a portion of our

earlier published experiments [6]. We used this opportunity to assess the extent to which experimental results were repeatable, and to explore possible causes for discrepancies in case they were not. Hence, we used permutational regression analysis (in lmPerm) to analyse changes in swimming performance with BMI using both datasets, and we re-analysed MHC concentrations (with permutational analyses of variance as described above) on the combined data set from both studies.

Results

Body mass index

Body mass index (BMI) was significantly higher in the obese group than in the lean or obese-lean groups, but there was no effect of exercise or by the interaction between obesity and exercise (Table 1; Fig. 1). There was no difference between the lean and obese-lean groups ($p = 0.10$), but the BMI of both groups were lower than that of the obese group (both $p < 0.0001$).

Overall, the BMI of fish in this experiment was lower than in our previous experiment, where BMI was: lean = 0.055 ± 0.0029 [s.e.], obese = 0.075 ± 0.0044 [s.e.], and obese-lean = 0.052 ± 0.0019 [s.e.] [6].

Exercise did not improve obesity-induced declines in muscle contractile properties

Exercise did not have a significant effect on tetanic stress (Table 1). However, obesity led to significantly decreased stress (Table 1). Post-hoc tests on marginal means showed that obese fish had significantly lower stress than lean fish ($p = 0.007$), but obese-lean fish did not differed from either lean ($p = 0.11$) or obese ($p = 0.14$) fish (Fig. 2A).

Obesity caused a significant decrease of muscle activation rates, but exercise or their interaction did not (Table 1; Fig. 3B). Lean fish were not significantly different from obese-

lean fish ($p = 0.27$), and obese-lean fish also did not differ from obese fish ($p = 0.073$). Activation rates of lean fish were significantly greater than in obese fish ($p = 0.0032$).

Similar to activation rates, obesity significantly reduced relaxation rates, but neither exercise nor the interaction between obesity and exercise influenced relaxation rates (Table 1; Fig. 3D). Relaxation rates of lean fish did not differ significantly from obese-lean fish ($p = 0.064$), but were significantly greater than those of obese fish ($p = 0.0038$). Obese-lean fish did not differ from obese fish ($p = 0.15$).

Neither obesity nor exercise or their interaction had a significant effect on fatigue resistance (Table 1; Fig. 3D).

Swimming performance was not affected by obesity or exercise

Neither obesity nor exercise nor their interaction had a significant effect on U_{crit} (Table 1; Fig. 2A). These results contradict our earlier results [6], and we explored whether the lower BMI in the present experiment may have contributed to this discrepancy by regressing swimming performance against BMI in the lean and obese groups from both experiments (Fig. 2B). There was considerable variation between individuals within each the lean and obese groups. Over the range of values within the lean group, U_{crit} did not change with BMI, while there was a significant decrease in U_{crit} with increasing BMI within the obese group ($p < 0.05$). The lower range of BMI values of fish of fish in the present experiment (indicated below the x-axis in Fig. 2B) combined with among-individual variation can explain why we did not detect that decline in the present experiment alone.

Myosin heavy chain composition did not change with obesity or exercise

Similar to swimming performance, obesity and exercise, or their interaction did not influence MHC concentrations or their ratio significantly (Table 1; Fig. 4). This result

contradicts our earlier finding [6] showing that obesity had a significant effect on MHC concentrations. However, using an increased sample size by combining data from both studies showed that at a one-tailed significance there was an effect of obesity on slow myosin heavy chain concentrations ($p = 0.070$), where lean fish had greater concentrations. However, obesity did not affect fast MHC concentrations, or the ratio between slow and fast MHC in the combined data set (both $p > 0.10$).

Discussion

We have shown that obesity decreases muscle contractile function, and that weight loss does not reverse this effect. Aerobic exercise at minimum levels recommended to confer health benefits did not alleviate the effects of obesity and did not improve muscle contractile function after weight loss. However, the effects of muscle contractile function were not mirrored by changes in locomotor performance, which did not decrease with obesity. The latter result is at variance with our previous study [6], and analysing the combined data sets revealed an interesting pattern: locomotor performance decreases gradually with increasing BMI. Similarly, MHC concentrations did not change with obesity and exercise in this experiment, but the combined data from both studies indicates that obese and obese-lean fish have lower slow MHC, as expected from the literature. Together, these data indicate that muscle contractile function is most sensitive to weight gain, but changes are independent from MHC concentrations and do not affect locomotor performance at least at "low" obese BMI. There is also a cautionary tale in these data: if we did not repeat part of the earlier experiment and thereby increased sample sizes and the range of BMI values, we would not have detected underlying patterns.

Exercise has a wide range of health benefits [9,22] and endurance exercise, in particular, is essential to trigger signalling programs that lead to healthy phenotypes in humans [23]. The

recommended minimum level of exercise for adult humans were recommended both with the ensuing health benefits [13] and energy expenditure and weight loss [14] in mind. However, our data indicate that 30 min per day of moderate aerobic exercise that raises metabolic rates to 3-4 times that of resting levels is not sufficient to improve muscle contractile function or alleviate the negative effects of obesity. We interpret these data as indicating that the intensity or mode of exercise, or both, were not effective in stimulating muscle performance. Of course, the exercise regimen we implemented may have been beneficial for other physiological responses, such as cardiovascular performance, which we did not measure. The mode of exercise may be important. High intensity exercise, with several short sessions per week on a stationary bicycle for 6-8 weeks, improved several metabolic and cardiovascular risk factors, such as decreased blood glucose levels, improved insulin response, and reduced blood pressure, in obese humans compared to non-exercised control [24,25]. Weight loss intervention combined with a multicomponent exercise program comprising endurance and resistance exercise as well as flexibility improved muscle quality (knee torque) in overweight and obese older women [26]. Similarly, the combination of aerobic and strength exercise following bariatric surgery facilitated weight loss and improved muscle strength [27,28]. Physical activity can also increase muscle strength of obese individuals without weight loss [10]. Compared to resistance training, endurance exercise is more effective for weight loss [29], and in promoting fatigue resistance [30] by shifting muscle fibre types from fast to slow [31]. Endurance exercise may thereby counteract the obesity-induced shift to fast muscle fibre types. Together, these data indicate that the recommended level of exercise we implemented for our zebrafish was not sufficient to affect muscle quality in the control or obese groups or following weight loss. More intense aerobic exercise and a combination between modes of exercise, such as endurance and strength exercise, seem to be a more effective intervention to improve muscle quality during obesity and following weight loss.

We were surprised not to observe an effect of obesity on locomotor performance given our previous data [6], and the fact that obese fish in our study showed decreased contractile function in isolated muscle. Zebrafish are an established model for obesity, and diet-induced obesity causes pathophysiological responses in zebrafish as in mammals [32-34]. A BMI of 0.06 in zebrafish, similar to the one we achieved in the present study, was sufficient to elicit responses typical of obesity in mice and humans, and lead to significantly increased plasma triglyceride levels [32]. Hence, our dietary treatment achieved an obese phenotype. Nonetheless, locomotor performance and myosin heavy chain composition were not affected negatively by that level of obesity. Our data, including the re-analysis of our earlier study, combined with the literature indicate that there are different levels of sensitivity of different physiological responses to obesity: metabolic responses [lipid metabolism, [32]], and muscle contractile function are more sensitive than locomotor performance and muscle composition. Contractile function of isolated muscle can be affected by the activity of calcium handling proteins such as dihydropyridine and ryanodine receptors and sarco-endoplasmic reticulum ATPase (SERCA), in addition to myosin heavy chain composition. We suggest therefore that the effects of obesity on calcium handling in skeletal muscle may explain the decrease in contractile function in a similar way as in cardiac muscle [35]. This suggestions is reasonable, because obesity in our zebrafish slowed contraction and relaxation rates, which are largely mediated by calcium release from the sarcoplasmic reticulum via ryanodine receptors, and calcium re-sequestration into the sarcoplasmic reticulum by SERCA, respectively [36,37].

Obesity changes whole-body and joint kinematics, which can lead to physical disability and decreased mobility. These effects are mediated by the increased body mass, as well as by the decrease in muscle quality [3,5,11]. It is important therefore to reverse decreases in muscle contractile function to maintain quality of life and prevent complicating pathophysiological conditions in obese subjects, particularly with increasing age [3]. Exercise

is one of the most promising approaches both to mediate weight loss and maintain muscle function [26]. The recommended levels of exercise can be effective in mediating weight loss [14], but our data show that they are not sufficient to preserve muscle quality. An important future direction will be to determine the combination of exercise regimens that will improve muscle function, and more intensive exercise such as interval training appears to be promising.

Acknowledgments

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Table 1 Summary of statistical results. We analysed dependent variables (left column) with permutational analyses, and permutational probabilities are shown for each factor (Obesity, Exercise) and their interaction (Ex*O, d.f. = 1). Significant results are shown in bold, and d.f. for each factor and response variable are shown as subscripts.

	Obesity$_1$	**Exercise$_2$**	**Ex*O$_1$**
Body mass index$_{87}$	**<0.0001**	0.18	0.13
U$_{crit87}$	0.77	0.29	0.65
Stress$_{54}$	**0.013**	0.26	0.58
Activation rate$_{54}$	**0.018**	0.76	0.69
Relaxation rate$_{54}$	**0.0042**	0.88	0.74
Fatigue resistance$_{54}$	0.55	0.66	0.78
Slow MHC$_{43}$	0.60	0.98	0.67

Fast MHC_{43}	0.56	0.96	0.98
Slow:fast MHC_{43}	0.39	0.90	0.73

Figure captions

Fig. 1 Body mass index (BMI) of the different treatment groups. BMI was significantly greater in obese (O) fish compared to lean (L) or obese-lean (OL) fish. The exercise (black bars) treatment did not differ from the control (grey bars). Significant differences between levels of the obesity treatment are indicated by horizontal bars with different letters.

Fig. 2 Muscle mechanics in the different treatment groups. Muscle stress (force/muscle cross-sectional area) was significantly greater in lean (L) compared to obese (O) fish. Obese-lean (OL) fish did not differ from any of the other groups (A). There was an interaction between the exercise (exercise = black bars, control = grey bars) and obesity treatments that

determined muscle activation rate (B). Obesity treatment had a significant effect on muscle relaxation rate (C), but fatigue resistance was not affected significantly by either obesity or exercise treatments (D). Significant differences between exercise and control are indicated by an asterisk, and significant differences between levels of the obesity treatment are indicated by horizontal bars with different letters (the hash superscript indicates a one-tailed probability).

Fig. 3 The effect of obesity on swimming performance. Swimming performance (U_{crit}) in the present study was not affected by exercise (A; exercise treatment = black bars, control = grey bars) or obesity (L = lean, O = obese, OL = obese-lean). Combining data from obese and lean fish from the present study with our earlier published data [6] (B) shows that U_{crit} did not change with increasing BMI over the range of values within the lean group (green circles and regression line). However, U_{crit} decreased significantly with increasing BMI in the obese fish (red circles and regression line). The lower values on BMI of fish in the present study combined with considerable among-individual variation may explain why we did not detect an effect of obesity on swimming performance in the present study; mean BMI (vertical lines) and BMI ranges (horizontal lines) for the two studies are indicated below the x-axis.

Fig. 4 Myosin heavy chain concentrations. There was no effect of exercise (light grey columns A-C), or obesity treatment (L = lean, O = obese, OL = obese-lean) on slow (A), or fast (B) myosin heavy chain concentrations (MHC, normalised to α-tubulin), or their ratio (slow:fast, C). Combining data from this experiment with our previously published data for obesity treatments (D-F) indicates that there is a decrease in slow MHC in the O and OL treatments (D) compared to control at a one-tailed significance level [indicated by ($*$)].

Means $\pm$ s.e. are shown and n = 8 for A-C, and n = 14 for D-F.

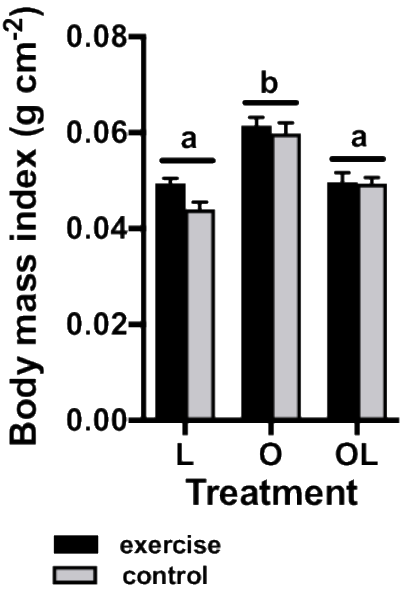
Body mass index (g cm-2)
0.08
0.06
0.04
0.02
0.00
a
b
a
L
O
OL
Treatment
exercise
control

Fig. 1

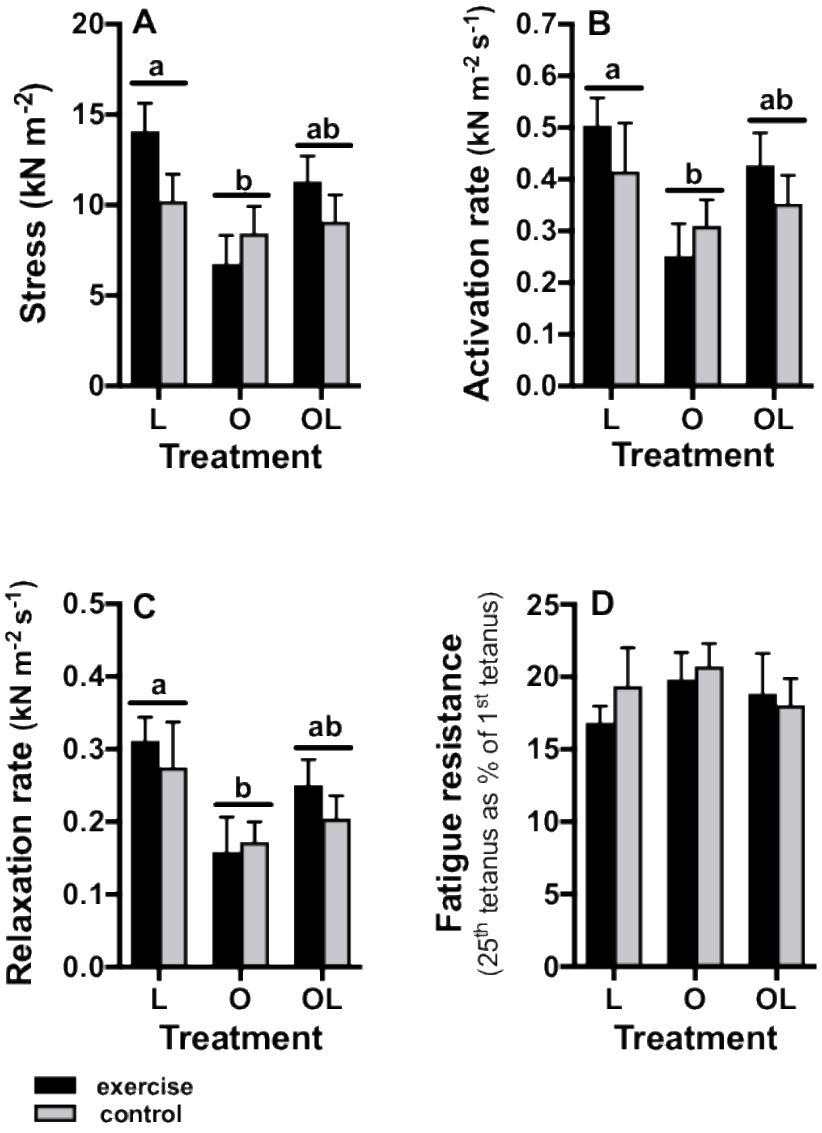
A
Stress (kN m-2)
20
15
10
5
0
a
b
ab
L
O
OL
Treatment
B
Activation rate (kN m-2 s-1)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
b
ab
L
O
OL
Treatment
C
Relaxation rate (kN m-2 s-1)
0.5
0.4
0.3
0.2
0.1
0.0
a
b
ab
L
O
OL
Treatment
D
Fatigue resistance
(25th tetanus as % of 1st tetanus)
25
20
15
10
5
0
L
O
OL
Treatment
exercise
control

Fig. 2

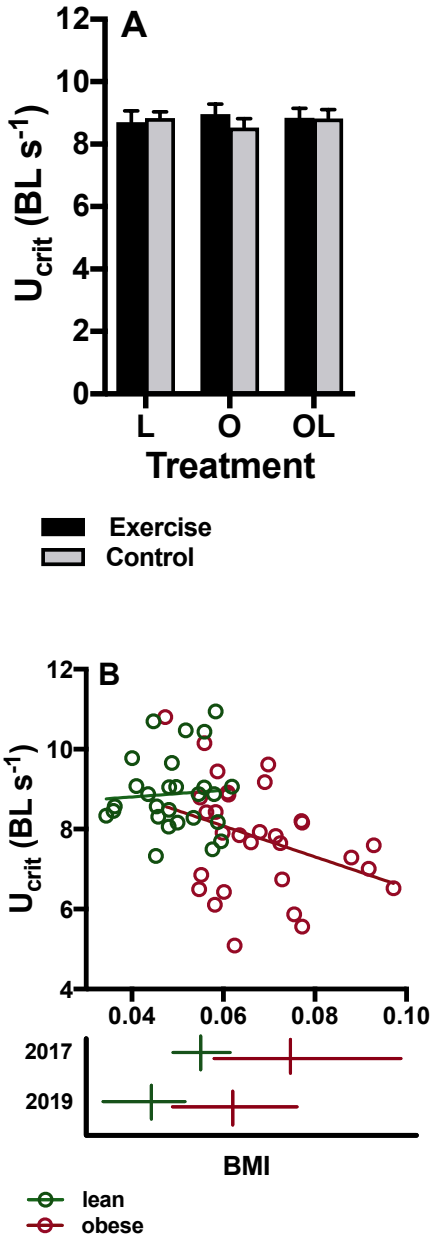
A
12
10
8
6
4
2
0
U_{crit} (BL s^{-1})
L
O
OL
Treatment
Exercise
Control
B
12
10
8
6
4
U_{crit} (BL s^{-1})
0.04
0.06
0.08
0.10
2017
2019
BMI
lean
obese

Fig. 3

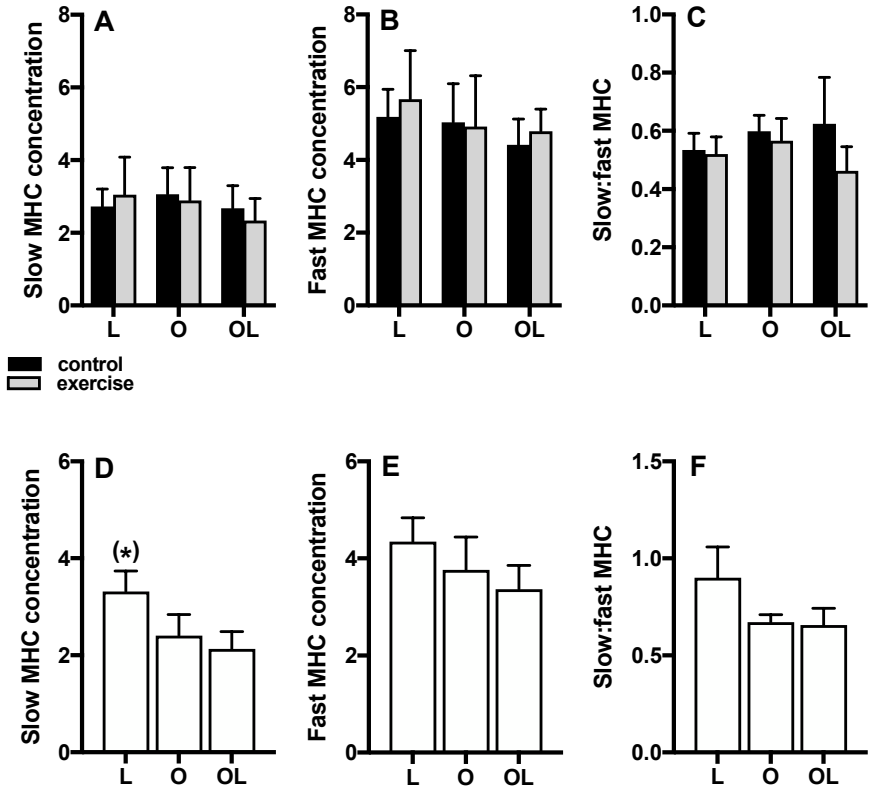
A
B
C
D
E
F
Slow MHC concentration
Fast MHC concentration
Slow:fast MHC
L
O
OL
control
exercise
(*)

Fig. 4