DOI: 10.1113/EP088109

L-citrulline supplementation improves glucose and exercise tolerance in obese male mice

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Running Title: L-Citrulline and Metabolic Homeostasis

Word Count: 5,860

Figures: 7

This is an Accepted Article that has been peer-reviewed and approved for publication in the Experimental Physiology, but has yet to undergo copy-editing and proof correction. Please cite this article as an Accepted Article; doi: 10.1113/EP088109.

Tables: 5

Key Words: L-citrulline, obesity, glucose homeostasis, mitochondrial function, exercise capacity

Abstract:

L-citrulline is an organic α-amino acid that has been shown to produce a number of salutary actions on whole-body physiology, which includes reducing muscle wasting and augmenting exercise/muscle performance. The latter has been suggested to arise from elevations in mitochondrial function. Because enhancing mitochondrial function has been proposed as a novel strategy to mitigate insulin resistance, our goal was to determine whether supplementation with L-citrulline could also improve glycemia in an experimental mouse model of obesity. We hypothesized that L-citrulline treatment would improve glycemia in obese mice, and this would be associated with elevations in skeletal muscle mitochondrial function. 10-week old C57BL/6J mice were fed either a low-fat (10% kcal from lard) or high-fat (60% kcal from lard) diet, while receiving drinking water supplemented with either vehicle or L-citrulline (0.6 g/L) for 15-weeks. Glucose homeostasis was assessed via glucose/insulin tolerance testing, while *in vivo* metabolism was assessed via indirect calorimetry, and forced exercise treadmill testing was utilized to assess endurance. As expected, obese mice supplemented with L-citrulline exhibited an increase in exercise capacity, which was associated with an improvement in glucose tolerance. Consistent with augmented mitochondrial function, we observed an increase in whole body oxygen consumption rates in obese mice supplemented with L-citrulline. Surprisingly, L-citrulline supplementation worsened insulin tolerance and reduced insulin signaling in obese mice. Taken together, although L-citrulline supplementation improves both glucose tolerance and exercise capacity in obese mice, caution must be applied with its broad use as a nutraceutical due to a potential deterioration of insulin sensitivity.

New Findings

- **What is the central question of the study?**

  L-citrulline has been shown to augment performance in animals and athletes, possibly via increasing mitochondrial function. We were curious whether L-citrulline's potential mitochondrial actions would translate to obese animals, and whether this would improve glycemia.

- **What is the main finding and its importance?**

  Chronic supplementation with L-citrulline improves not only exercise capacity, but also glycemia in obese mice, which would be beneficial as obese individuals are at increased risk for type 2 diabetes. However, L-citrulline supplementation also caused a mild impairment in insulin signaling and insulin tolerance in obese mice.

1. INTRODUCTION

L-citrulline is an organic α-amino acid found naturally in high quantities in watermelons, onions, and garlic. Furthermore, L-citrulline is synthesized almost exclusively in the intestine and requires arginine and glutamine for its biosynthesis in the Urea Cycle (Bahri *et al.*, 2013). It is also a by-product of nitric oxide synthase (NOS), which converts L-arginine into nitric oxide (NO) and L-citrulline. It can also serve as a precursor for L-arginine biosynthesis, and thus has been used as a supplement to increase circulating arginine concentrations in situations of arginine deficiency (Bahri *et al.*, 2013), though it also increases circulating arginine concentrations in healthy young and aged adults (Churchward-Venne *et al.*, 2014; Bailey *et al.*, 2015).

Of interest, studies in humans have shown that citrulline/malate supplementation may enhance performance, as it reduced muscle fatigue via promoting aerobic energy production in exercising muscle (Bendahan *et al.*, 2002). In addition, citrulline/malate supplementation in humans has also been shown to increase performance during high-intensity anaerobic exercise (Perez-Guisado & Jakeman, 2010). Similarly, studies in rats suggest that citrulline/malate enhances gastrocnemius muscle performance, as citrulline/malate supplementation reduced both the phosphocreatine and oxidative cost of contraction following electrically induced transcutaneous stimulation (Giannesini *et al.*, 2011). While the above studies do not discern where citrulline or malate is responsible for the effects on performance, a recent study by Villareal *et al*. revealed that L-citrulline supplementation increases exercise performance in mice due to upregulation of peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α) expression in skeletal muscle (Villareal *et al.*, 2018).

It is possible that citrulline's potential beneficial actions on exercise performance involve increases in mitochondrial function. Indeed, L-citrulline mediated increases in circulating L-arginine levels increase NO production (Sureda *et al.*, 2009; Bahri *et al.*, 2013; Lee & Kang, 2018), and NO has been demonstrated in numerous studies to augment mitochondrial biogenesis and subsequent mitochondrial function, possibly via augmenting PGC-1α activity (Nisoli *et al.*, 2004; McConell *et al.*, 2010; Tengan *et al.*, 2012). Furthermore, a number of studies have demonstrated that increasing mitochondrial function through a variety of approaches (e.g. exercise, heat therapy, genetic-based strategies, etc.) protects against obesity-induced impairments in glucose homeostasis (Seth *et al.*, 2007; Dube *et al.*, 2008; Gupte *et al.*, 2009). It has also been demonstrated that mice with a whole-body deletion of endothelial NO synthase, exhibit impaired mitochondrial function, which results in glucose intolerance and insulin resistance (Duplain *et al.*, 2001; Le Gouill *et al.*, 2007).

Taken together, as increases in skeletal muscle mitochondrial function are frequently associated with improved glucose homeostasis, we anticipated that in addition to augmenting exercise performance, citrulline may have additional salutary actions that result in improved glycemia. We hypothesized that treatment with L-citrulline would not only improve exercise tolerance in mice subjected to experimental obesity, but would also improve glucose homeostasis, both of which would be due to increases in skeletal muscle mitochondrial function.

2. METHODS

2.1 Ethics approval

All experimental procedures performed in mice were approved by the University of Alberta Health Sciences Anima Welfare Committee under user protocol number AUP00001412, in

accordance with the guidelines of the Canadian Council on Animal Care. In addition, all possible steps were taken by the investigators to minimize pain and suffering of animals, and euthanasia was conducted in accordance with approved institutionalized protocols at the Health Sciences Laboratory Animal Services Facility of the University of Alberta. All animals were euthanized via intraperitoneal (IP) injection of sodium pentobarbital (Euthansol®; 0.3 mL/kg body weight), and once loss of consciousness and a lack of toe-pinch reflex was observed (within 5 min), all animals underwent cardiac puncture for collection of blood, following which their tissues were extracted for further analyses. Our studies fully comply with the ethical principles and animal ethics checklist of *Experimental Physiology*.

2.2 Animal care

Ten-week-old male C57BL/6J mice (Jackson Laboratory) were fed either a low-fat diet (LFD, 10% kcal from lard, Research Diets D12450J) or a high-fat diet (HFD, 60% kcal from lard, Research Diets D12492) and supplemented with either vehicle or L-citrulline (0.6 g/L) in their drinking water for 15-weeks. This dose of L-citrulline amounts to a mouse on average consuming ~100 – 150 mg/kg per day based on studies in our lab indicating that C57BL/6J mice consume ~8 mL of L-citrulline supplemented drinking water per day (8.39 mL ± 0.40 mL vehicle control drinking water versus 8.19 mL ± 0.78 mL L-citrulline supplemented drinking water). All experimental groups were subjected to several physiological assessment throughout the 15-weeks, and upon study completion all animals were euthanized via IP injection of sodium pentobarbital following a 6-hr fast and at 15 min post-administration of saline or insulin. Tissues (e.g. gastrocnemius muscle, liver) were subsequently extracted and immediately snap frozen in liquid N_2 using Wollenberger tongs precooled to the temperature of liquid N_2, and stored at -80^{o}C.

2.3 Cell culture

All reagents were purchased from Sigma. C2C12 (American Type Culture Collection) cells were cultured in six-well plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were incubated in a water-jacketed CO_2 incubator maintained at 37^{o}C with 95% O2 and 5% CO_2. Upon confluency C2C12 cells were differentiated into myotubes via growth in DMEM containing 2% horse serum (HS) and 1% penicillin/streptomycin as previously described (Ussher *et al.*, 2009). After 5 days differentiation, C2C12 myotubes were treated with either 0.1% FBS supplemented DMEM containing 30 μM, 300 μM, or 1 mM L-citrulline, 100 μM or 200 μM L-arginine, or saline for 24 hr. In a separate cohort of C2C12 myotubes, following 24 hr treatment with saline, L-citrulline, or L-arginine, all cells were subsequently treated with vehicle control (saline) or insulin (0.1 or 1 nM) for an additional 2 hr.

2.4 Glucose Homeostasis Assessment

Intraperitoneal glucose and insulin tolerance tests (GTTs/ITTs) were performed in overnight or 6-hr fasted mice, respectively, using a glucose dose of 2 g/kg body weight for the GTT, and an insulin dose of 0.7 U/kg body weight for the ITT. A 6-hr fast was chosen for the ITT, as lean C57BL/6J mice are highly insulin sensitive and may become hypoglycemic during an ITT following an overnight fast, due to their baseline glucose levels decreasing to ~4 mM. Blood glucose was measured at 0, 15, 30, 60, 90, and 120 min post-glucose or insulin administration from mouse tail whole-blood using the Contour Next blood glucose monitoring system (Bayer) as previously described (Al Batran *et al.*, 2018; Al Batran *et al.*, 2019). In addition, blood samples were collected in tubes containing the anticoagulant agent (EDTA) at 0 and 30 min post-glucose administration, in order to measure plasma insulin levels via

use of a commercially available kit (Alpco Diagnostics) as previously described (Al Batran *et al.*, 2018).

2.5 Exercise Capacity Assessment

During the assessment of exercise tolerance, mice were run on a calibrated, motor-driven treadmill (Columbus Instruments) at a starting speed of 3 m/min for 2 min, followed by increasing speeds of 4 m/min for 2 min, 5 m/min for 2 min, 6 m/min for 6 min, 8 m/min for 10 min, 10 m/min for 3 min, 12 m/min for 3 min,14 m/min for 3 min, 16 m/min for 3 min, 18 m/min for 3 min, 20 m/min until exhaustion. Animals were ran until they reached exhaustion, which was defined as when an individual mouse would no longer run for more than 5 sec at a time, and chose to remain on the apparatus shock grid even when assisted back onto the treadmill. The first 6 min is not included in our exercise capacity assessment, as this period is used for the mice to acclimatize and become familiar with the treadmill.

2.6 Indirect Calorimetry

An Oxymax comprehensive lab animal monitoring system (Columbus Instruments) was used to assess *in vivo* energy metabolism via indirect calorimetry. After a 24 hr period of acclimatization in the system, the subsequent 24 hr period was utilized for data collection on whole-body oxygen consumption rates, respiratory exchange ratios, heat production, and ambulatory activity as previously described (Al Batran *et al.*, 2018).

2.7 Magnetic Resonance Imaging (MRI) Body Composition Analysis

An EchoMRI 4in1/700 body composition analyzer was utilized to assess body composition for quantifying lean and fat mass in fully conscious mice via specialized nuclear magnetic resonance relaxometry-based technology.

2.8 Western Blotting

Frozen soleus or gastrocnemius tissue (20 mg), or C2C12 myotubes cells were homogenized in buffer containing 50 mM Tris HCl (pH 8 at 4^{o}C), 1 mM EDTA, 10% glycerol (w/v), 0.02% Brij-35 (w/v), 1 mM DTT, protease and phosphatase inhibitors (Sigma), following which extracted protein samples were denatured and subjected to western blotting protocols as previously described (Gopal *et al.*, 2017). Protein kinase B (Akt) and Phospho-Akt (9272S and 4060L (Cell Signaling)) antibodies were prepared in a 1/1000 dilution in 5% bovine serum albumin.

2.9 Real-Time PCR Analysis

First-strand cDNA was synthesized from TRIzol-extracted RNA using the SuperScript III synthesis system (Invitrogen, Carlsbad, CA). Real-time PCR was carried out with the CFX Connect real-time PCR machine (Bio-Rad Laboratories Inc.) using custom designed Sybr Green primers (Table 1). Relative mRNA transcript levels were quantified with the $2^{-\Delta\Delta Ct}$ method using peptidylprolyl Isomerase A (*Ppia*/*PPIA*) as an housekeeping internal control gene as previously described (Livak & Schmittgen, 2001).

2.10 Determination of Mitochondrial Respiration Levels

Mitochondrial oxygen consumption was assessed in fresh saponin-permeabilized gastrocnemius muscle using a Clark oxygen electrode connected to an Oxygraph Plus recorder (Hansatech Instruments Ltd., Norfolk, England) as previously described (Kuznetsov *et al.*, 2008). Permeabilized muscle was loaded onto a chamber containing 2 ml of respiration medium at 30°C. Saline, 30 μM L-citrulline, 1 mM L-citrulline, 100 μM L-arginine or 200 μM L-arginine was added to the chamber, following which basal respiration, complex I substrates glutamate (10mM) and malate (5mM) were added and oxygen consumption was recorded (State 4 respiration). Then, 1 mM ADP was added and the ADP-stimulated respiration rate was determined (State 3 respiration). Respiration rate was represented as nmol O_2 consumed per mg protein per minute. The respiratory control ratio (RCR) was calculated as the ratio between ADP-stimulated and basal respiration rates.

2.11 Statistical Analysis

All values are presented as means ± standard deviation (SD). Significant differences were determined by the use of an unpaired, two-tailed Student's *t*-test, or a two-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc analysis. Differences were considered significant when $P < 0.05$.

3. RESULTS

3.1 L-citrulline supplementation improves obesity-induced glucose intolerance but worsens insulin sensitivity

C57BL/6J mice were fed either a LFD or a HFD and supplemented with either vehicle or L-citrulline (0.6 g/L) in their drinking water for 15-weeks, which had no effect on food intake

(*data not shown*), body weight (Table 2), or lean body mass (Table 3). While L-citrulline supplementation had no impact on random fed ad libitum blood glucose levels in both lean and obese mice, it did result in a lowering of fasting blood glucose levels in obese mice (Table 4). Moreover, glucose tolerance was improved only in the obese mice following 11-weeks of L-citrulline supplementation (Figure 1a,b), which was associated with a trend to increased circulating insulin levels when comparing the change in insulin levels at the 30-min versus 0-min time points of the GTT (Figure 1c).

Conversely, L-citrulline supplementation for 9-weeks worsened insulin tolerance in obese mice (Figure 2a,b). This worsening was associated with alterations in insulin signaling, as baseline Akt phosphorylation appeared elevated in muscles from lean mice supplemented with L-citrulline, whereas in obese mice, insulin-stimulated Akt phosphorylation in gastrocnemius but not soleus muscles was nonexistent when compared to vehicle supplementation (Figure 2c,d). Of interest, these adverse actions of L-citrulline on skeletal muscle insulin tolerance/signaling may be indirectly mediated, since direct treatment of differentiated C2C12 myotubes with pharmacological concentrations of L-citrulline during serum starvation (0.1% FBS) did not worsen insulin-stimulated Akt phosphorylation (Figure 3).

3.2 L-citrulline supplementation increases exercise capacity in both lean and obese mice

To evaluate the effect of L-citrulline supplementation on exercise performance, both lean and obese mice supplemented with L-citrulline for 8-weeks were run on a forced exercise treadmill at gradually increasing speeds until exhaustion. As we anticipated, L-citrulline improved exercise performance, as we observed significant increases in the time and

distance that both L-citrulline supplemented lean and obese mice were able to run on the forced exercise treadmill (Figure 4).

3.3 L-citrulline supplementation increases whole-body consumption rates but does not impact substrate preference in obese mice

We next assessed *in vivo* energy metabolism in lean and obese mice following 10-weeks of L-citrulline supplementation via indirect calorimetry, which revealed significant increases in whole-body oxygen consumption rates in obese mice during the initial hours of the dark cycle (Figure 5a,b). This increase was associated with increased ambulatory activity (Figure 5c), but was not associated with changes in substrate preference, as respiratory exchange ratios were similar in both lean and obese mice supplemented with L-citrulline (Figure 6a,b). To further support our observations that L-citrulline supplementation does not modify substrate preference, and to assess whether L-citrulline has direct actions on muscle mitochondria that enhance respiration, we quantified respiratory control ratios in permeabilized fibres from gastrocnemius muscles of lean mice. Direct treatment of permeabilized fibres with pharmacological concentrations of L-citrulline had no impact on mitochondrial respiration rates or the respiratory control ratio (Table 5). Because L-citrulline can be recycled into L-arginine, which may enhance mitochondrial respiration via increasing nitric oxide formation, we also treated permeabilized gastrocnemius fibres with pharmacological concentrations of L-arginine, though once again saw no change in the respiratory control ratio (Table 5).

3.4 L-citrulline supplementation reverses obesity-induced impairments in the expression of key regulators of mitochondrial function/biogenesis

We next quantified mRNA expression for a number of factors associated with the regulation of mitochondrial function and/or biogenesis, as we posited these factors may contribute to how L-citrulline improves muscle performance and aerobic energy metabolism. Experimental obesity resulted in significant reductions or trends to reductions in the mRNA expression of peroxisome proliferator activated receptor gamma coactivator-1 α (*Ppargc1a*), nuclear respiratory factor 1 (*Nrf1*), and mitochondrial transcription factor B2 (*Tfbm2*) in gastrocnemius but not soleus muscles (Figure 7a,b). Of interest, L-citrulline supplementation prevented the obesity-induced reduction in gastrocnemius muscle *Ppargc1a*, *Nrf1*, or *Tfbm2* expression, while also increasing mitochondrial transcription factor A (*Tfam*) expression, but had no effect on this expression profile in soleus muscle (Figure 7a,b). To determine whether the actions of L-citrulline on gastrocnemius muscle mRNA expression profiles were due to direct actions on the muscle, we treated differentiated C2C12 myotubes during serum starvation (0.1% FBS) with increasing concentrations of L-citrulline. Similar to our *in vitro* observations assessing insulin signaling, direct treatment of C2C12 myotubes with L-citrulline had negligible influence on the mRNA expression of *Ppargc1a*, *Nrf1*, *Tfam*, or *Tfbm2* versus their saline treated counterparts (Figure 7c). Likewise, the *in vivo* changes in gastrocnemius *Ppargc1a*, *Nrf1*, *Tfam*, and *Tfbm2* mRNA expression are not due to L-citrulline mediated increases in L-arginine levels, as direct treatment of C2C12 myotubes with L-arginine failed to increase *Ppargc1a*, *Nrf1*, *Tfam*, or *Tfbm2* mRNA expression (*data not shown*).

4. DISCUSSION

Our current study supports previous findings from others demonstrating that L-citrulline supplementation may improve exercise performance, as both lean and obese mice supplemented with L-citrulline in the drinking water were able to run ~28% and ~47% longer on a forced exercise treadmill, respectively. In addition, we report here for the first-time novel

actions of L-citrulline supplementation on obesity-induced dysglycemia, where L-citrulline supplementation improves glucose homeostasis but surprisingly worsens insulin tolerance and insulin signaling in obese mice. Such observations should be taken into consideration for individuals choosing to consume L-citrulline as a nutraceutical supplement in attempts to improve their exercise performance.

The increase we observed in exercise performance in both lean and obese mice supplemented with L-citrulline was expected, as previous studies in humans have reported similar observations, as citrulline/malate co-supplementation reduced muscle fatigue in 18 men performing finger flexions at 1.5 second intervals lifting a 6-kg weight (Bendahan *et al.*, 2002). It was postulated that this benefit was due to augmented aerobic energy production in exercising muscle, as ^{31}P magnetic resonance spectroscopy (^{31}P MRS) studies revealed a 34% increase in oxidative ATP production and 20% increase in the rate of phosphocreatine recovery within the exercising muscle. Likewise, citrulline/malate co-supplementation also reduced muscle soreness and repetition number during flat barbell bench presses in 41 male volunteers (Perez-Guisado & Jakeman, 2010). In preclinical studies, citrulline/malate supplementation improves muscle efficiency in electrically stimulated gastrocnemius muscles from anesthetized Wistar male rats, as seen by decreases in both the phosphocreatine and oxidative costs of contraction during ^{31}P MRS studies (Giannesini *et al.*, 2011). Furthermore, studies in rats have demonstrated that citrulline supplementation enhances endurance capacity potentially via activating muscle protein synthesis and modulating substrate flux, though independent of a direct improvement of mitochondrial function (Goron *et al.*, 2017). Conversely, a single dose of L-citrulline failed to improve maximum number of repetitions over 5 sets, time to exhaustion, and maximal oxygen consumption during chest press exercise in 22 volunteer athletes (11 males/11 females) (Cutrufello *et al.*, 2015). Reasons for the discrepancy between these studies remains

unknown, though a possible explanation could stem from the fact that the latter study utilized L-citrulline supplementation alone versus citrulline/malate co-supplementation. Nonetheless, Takeda and colleagues have shown that L-citrulline supplementation improves time to exhaustion and reduces circulating lactate levels during swimming exercise in ICR mice (Takeda *et al.*, 2011).

The inclusion of malate as a co-supplement is thought to account for the potential improvement in aerobic energy metabolism, since malate is a key intermediate of the Krebs Cycle. Our results support the findings of Takeda and colleagues, however, since L-citrulline supplementation alone was sufficient to improve aerobic capacity on a forced exercise treadmill. Moreover, we observed increases in whole-body oxygen consumption rates only in mice subjected to experimental obesity, which impairs whole-body oxygen consumption (Ussher *et al.*, 2010), suggesting that mitochondrial function may potentially be improved via L-citrulline supplementation. Conversely, mitochondrial flux under submaximal conditions, as would primarily be the case for both our lean and obese mice, reflects the cellular demand for ATP generation (Holloszy, 2009), and hence the increase in oxygen consumption may not necessarily indicate an improvement in mitochondrial function. Nonetheless, we did observe reductions in mRNA expression of numerous factors linked to the regulation of various aspects of mitochondrial function in response to obesity, which were prevented via L-citrulline supplementation. Indeed, PGC-1α is a key regulator of mitochondrial function/energy metabolism in skeletal muscle (Handschin & Spiegelman, 2008), and L-citrulline supplementation was associated with enhanced *Ppargc1a* expression in obese mouse gastrocnemius muscle, though no differences were observed in lean mouse gastrocnemius muscle. Such observations contrast with studies in lean mice supplemented for 15-days with L-citrulline, as Villareal and colleagues demonstrated L-citrulline mediated increases in swimming exercise performance, which were associated with an upregulation of

PGC-1α expression in gastrocnemius muscles (Villareal *et al.*, 2018). The incompatibility of our observations with those of Villareal and colleagues could be due to the fact that our mice were not frequently exercising and we simply assessed performance during a single aerobic exercise challenge. In addition, our daily dose of L-citrulline supplementation was significantly lower, which could also explain why we did not observe increased gastrocnemius muscle *Ppargc1a* expression. Although L-citrulline did not have direct actions in lean mice resulting in increased *Ppargc1a* expression, L-citrulline supplementation reversed the impairment of experimental obesity on gastrocnemius but not soleus muscle *Ppargc1a*, *Nrf1*, *Tfam*, or *Tfbm2* mRNA expression. Because these genes all represent key regulators of mitochondrial function and subsequent energy metabolism (Handschin & Spiegelman, 2008; Dillon *et al.*, 2012), preventing downregulation of this mRNA expression profile in skeletal muscle may explain why whole-body oxygen consumption rates were not decreased in response to L-citrulline supplementation in obesity. Conversely, we observed mild increases in ambulatory activity in obese mice supplemented with L-citrulline, which could also explain the increase in whole-body oxygen consumption rates, thereby affecting the mRNA expression of these genes as a secondary response.

Reasons for why obesity does not decrease the expression of these genes in soleus muscle is not clear, but could be due to the fact that as a much more oxidative red muscle, the soleus has adaptive mechanisms in place to offset the detrimental actions of obesity on oxidative gene expression. It should be noted that the L-citrulline mediated increase in whole-body oxygen consumption rates was relatively mild despite being statistically significant, and was only seen during the initial hours upon the transition to the dark cycle of the mouse. As skeletal muscle accounts for ~20-30% of resting energy expenditure (Zurlo *et al.*, 1990), we posit that L-citrulline's actions on skeletal muscle, in particular white skeletal

muscle (e.g. gastrocnemius), may contribute to the increase in whole-body oxygen consumption rates we observed in obese mice.

We initially surmised that our *in vivo* observations in mice were due to direct actions on skeletal muscle, as studies have shown that L-citrulline acts directly on skeletal muscle myocytes *in vitro*, where it can enhance muscle protein synthesis and prevent wasting (Ham *et al.*, 2015; Le Plenier *et al.*, 2017). However, we observed no direct effects of L-citrulline treatment on mitochondrial respiration in permeabilized gastrocnemius muscle fibers. It is worth noting that all mice in these specific experiments were euthanized with sodium pentobarbital (Euthanyl), and it has been demonstrated that perfusion of isolated rat hearts with sodium pentobarbital can inhibit mitochondrial oxygen consumption (Bhayana *et al.*, 1980). Conversely, it has been demonstrated that high-dose pentobarbital for inducing euthanasia does not depress mitochondrial energetics in rat hearts (Takaki *et al.*, 1997). Nonetheless, direct comparisons between isolated hearts perfused with sodium pentobarbital, versus animals injected with high-dose sodium pentobarbital for the purpose of rapid euthanasia and tissue extraction is challenging. Moreover, all mice from all experimental groups received a similar dose of sodium pentobarbital, hence the potential depressive actions of sodium pentobarbital on mitochondrial respiration would be similar between groups.

In further support of L-citrulline lacking direct actions on skeletal muscle influencing mitochondrial function, differentiated C2C12 myotubes treated with L-citrulline demonstrated no changes in *Ppargc1a*, *Nrf1*, *Tfam*, or *Tfbm2* mRNA expression. This suggests that perhaps indirect actions account for the skeletal muscle phenotype we observed in obese mice supplemented with L-citrulline. L-citrulline supplementation has been proposed as a

novel approach to increase circulating arginine concentrations since arginine can be recycled from citrulline (Bahri *et al.*, 2013), while L-arginine is a precursor for NO synthesis, and NO has been shown in numerous studies to improve mitochondrial function (Le Gouill *et al.*, 2007; Tengan *et al.*, 2012). We therefore assessed whether increases in L-arginine could account for our in vivo observations following L-citrulline supplementation of obese mice, but treatment with pharmacological levels of L-arginine also had no effect on *Ppargc1a*, *Nrf1*, *Tfam*, or *Tfbm2* mRNA expression. In contrast, by increasing L-arginine levels and augmenting NO synthesis, L-citrulline could improve exercise tolerance via vasodilatory mechanisms that increase blood flow and nutrient delivery to the skeletal muscle (Clark *et al.*, 2003). Future studies are needed to discern the indirect mechanisms by which L-citrulline prevents the obesity-mediated decline in the expression of genes regulating mitochondrial function in white muscle, and whether this affects aerobic exercise performance.

Importantly, it has been suggested in numerous studies that interventions leading to increased mitochondrial function can protect against obesity-induced dysglycemia (Seth *et al.*, 2007; Dube *et al.*, 2008; Gupte *et al.*, 2009). Our results support this premise, as we observed that obese mice supplemented with L-citrulline had a significant decrease in blood glucose levels in response to a GTT. These findings are consistent with previous studies in Zucker Diabetic fatty rats, whereby 4-weeks of supplementation with 63% watermelon pomace juice lowered blood glucose levels, though changes in glucose and insulin tolerance were not assessed, nor was it confirmed whether these actions were due to increased L-citrulline levels (Wu *et al.*, 2007). Our observed improvements in glucose tolerance were associated with increased circulating insulin levels, consistent with previous findings demonstrating that both L-citrulline and L-arginine can act directly on β-cells to induce insulin secretion (Henquin & Meissner, 1981; Giugliano *et al.*, 1997; Nakata & Yada, 2003). While these actions of L-citrulline/L-arginine in response to L-citrulline supplementation may explain the improved glucose tolerance we observed in obese mice, other actions on

peripheral tissues (e.g. liver) contributing to glycemic control also need to be considered. For example, it has been demonstrated in healthy volunteers, that those individuals who experienced the largest increase in circulating citrulline levels in response to oral L-arginine administration, also had the greatest suppression of hepatic glucose production (Apostol & Tayek, 2003). Although we did not assess the hepatic actions of L-citrulline supplementation, we did assess whether L-citrulline may improve glucose homeostasis in obese mice via improving muscle insulin sensitivity, but to our surprise, L-citrulline supplementation worsened insulin tolerance in obese mice. This worsening was associated with impaired insulin stimulated Akt phosphorylation in gastrocnemius but not soleus muscle, which was also unexpected since gastrocnemius muscle was where we observed a prevention of the obesity-induced decline in *Ppargc1a*, *Nrf1*, *Tfam*, or *Tfbm2* mRNA expression. It is worth noting that despite numerous studies postulating that enhancing mitochondrial function can improve insulin sensitivity and glycemic control, others have suggested that increasing oxidative metabolism in the absence of elevated energy demand can actually overload mitochondria and impair insulin sensitivity and overall glucose homeostasis (Koves *et al.*, 2008; Muoio & Neufer, 2012). Although the mechanism by which L-citrulline supplementation impairs muscle insulin signaling/sensitivity remains unknown, it may involve mTORC1 signaling, as L-citrulline has been shown to activate mTORC1 and its downstream target S6K1 (Le Plenier *et al.*, 2017). Moreover, it has been reported that amino acid infusion increases activation of S6K1 and inhibits IRS-1 via phosphorylation on multiple serine residues, which prevents Akt activation and induces insulin resistance (Tremblay *et al.*, 2005). Similar to what we observed with our mRNA expression profiles, L-citrulline's action on muscle insulin signaling/sensitivity is likely indirectly mediated and not the result of increasing arginine concentrations, as we did not observe impaired insulin-stimulated Akt phosphorylation in differentiated C2C12 myotubes treated with either L-citrulline or L-arginine.

Taken together, our study supports findings from previous studies implicating the nutraceutical L-citrulline as an aerobic performance enhancer, with new findings demonstrating that these actions are preserved in obesity, and that L-citrulline also attenuates obesity-induced dysglycemia. Nevertheless, the deterioration in insulin sensitivity following L-citrulline supplementation in obese mice suggests that its broad use as a potential nutraceutical should be minimized, particularly in obese subjects.

COMPETING INTERESTS

The authors have no conflicts to disclose.

AUTHOR CONTRIBUTIONS

A.E. and J.R.U. contributed to conception and design of the work, the acquisition, analysis and interpretation of the data, and drafted and revised the work for important intellectual content. R.A., K.L.H., A.M.D., K.G., A.A.G., I.Z., H.A., F.E., E.E.M., J.E.C., and J.M.S. contributed to the acquisition, analysis and interpretation of the data, and revised the work for important intellectual content. J.R.U. is the guarantor of this work and had full access to all the data, and takes responsibility for the integrity of the data and accuracy of the data analysis.

FUNDING

This study was supported by a New Investigator Operating Grant from Diabetes Canada to E.E.M., a Project Grant from the Canadian Institutes of Health Research to J.M.S., a Career Development Award from the American Diabetes Association to J.E.C., and a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada to J.R.U. J.E.C. is a Borden Scholar and J.R.U. is Scholar of Diabetes Canada. A.E. is supported by a Scholarship from the Libyan Ministry of Higher Education.

ACKNOWLEDGEMENTS

The authors are grateful for the technical expertise and assistance of Mrs. Amy Barr, who operates the Oxymax comprehensive lab animal monitoring system in the Animal Physiology Core Facility of the Cardiovascular Research Centre at the University of Alberta.

COMPETING INTERESTS

The authors have no conflicts to disclose.

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Figure 1

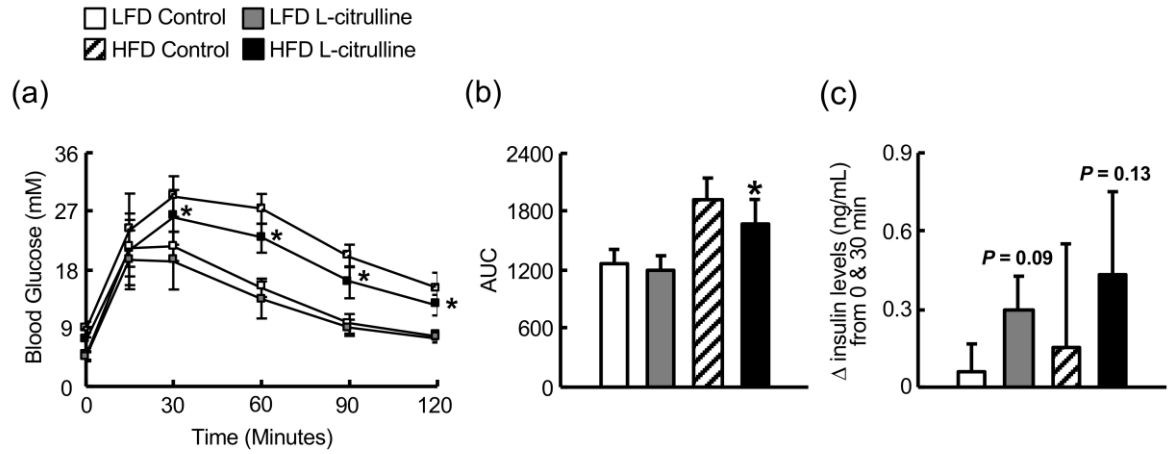

Figure Legends

FIGURE 1 L-citrulline supplementation improves glucose homeostasis in obese mice.

(a) Glucose tolerance in lean and obese mice treated with either vehicle or L-citrulline for 11-weeks (n = 12-15). (b) Area under the curve during the GTT (n = 12-15). (c) Plasma insulin levels during the glucose tolerance test at 0- and 30-min post-glucose administration (n = 5-11). Values represent means ± SD. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *P<0.05, indicates a significant difference versus the respective vehicle control treated counterpart.

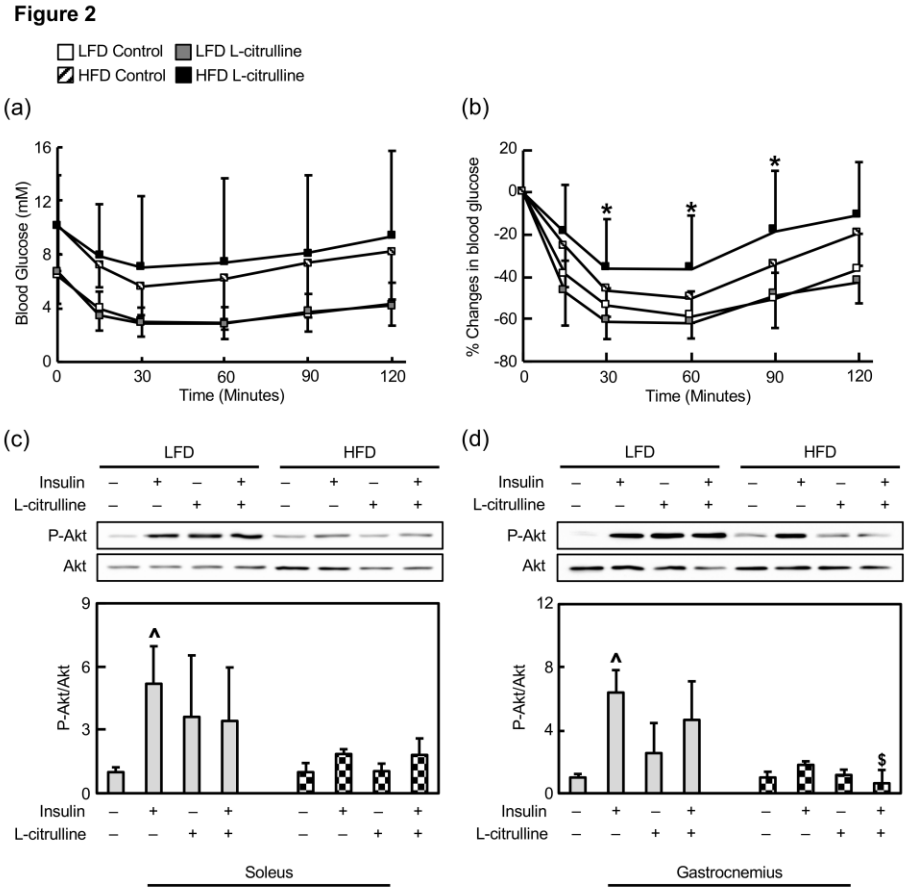

FIGURE 2 L-citrulline supplementation worsens insulin tolerance test in obese mice.

(a) Insulin tolerance in lean and obese mice treated with either vehicle or L-citrulline for 9-weeks (n = 7-17). (b) Changes in plasma glucose levels normalized to starting baseline glucose levels during the ITT (n = 7-17). (c) Insulin signaling (Akt phosphorylation) in soleus muscles from both lean and obese mice treated with either vehicle or L-citrulline during the ITT (n = 4). (d) Insulin signaling (Akt phosphorylation) in gastrocnemius muscles from both lean and obese mice treated with either vehicle or L-citrulline during the ITT (n = 4). Values represent means ± SD. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *P<0.05, indicates a significant difference versus the respective vehicle control treated counterpart. ^P<0.05, indicates a significant difference versus the LFD vehicle control no insulin group. $^{\$}P$<0.05, indicates a significant difference versus the HFD vehicle control + insulin group.

Figure 3

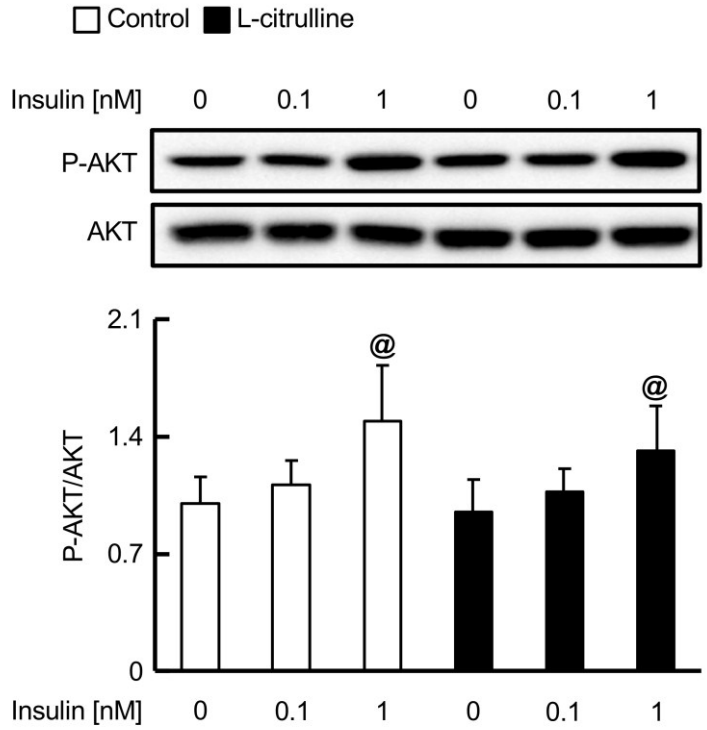

FIGURE 3 L-citrulline supplementation does not affect insulin signaling in C2C12 myotubes.

Insulin signaling (Akt phosphorylation) in C2C12 myotubes treated with either vehicle or L-citrulline for 24 hr and then stimulated with insulin for 30 min (n = 8). Values represent means ± SD. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. $^{@}P<0.05$, indicates a significant difference versus the respective no insulin counterpart.

Figure 4

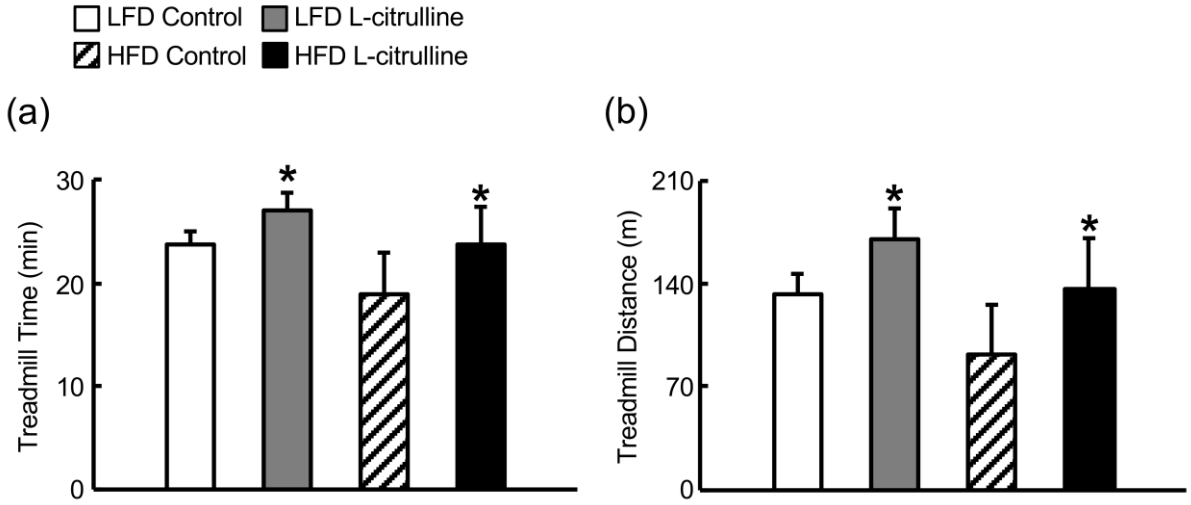

FIGURE 4 L-citrulline supplementation increases exercise capacity in both lean and obese mice.

(a) Treadmill time, and (b) Treadmill distance in lean and obese mice treated with either vehicle or L-citrulline for 8-weeks (n = 5-6). Values represent means ± SD. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *P<0.05, indicates a significant difference versus the respective vehicle control treated counterpart.

Figure 5

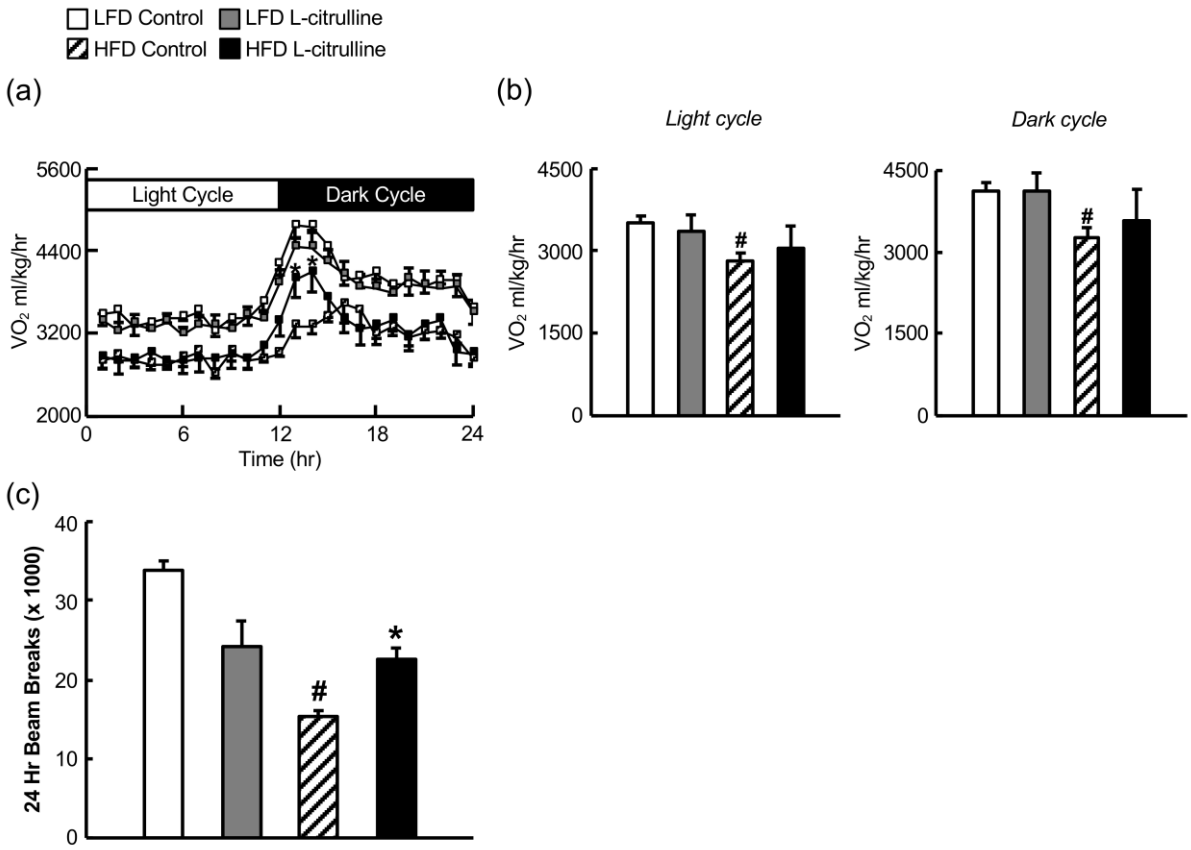

FIGURE 5 L-citrulline supplementation improves whole-body oxygen consumption rates in obese mice.

(a) 24-hr, (b) Light cycle and dark cycle whole-body oxygen consumption rates in lean and obese mice treated with either vehicle or L-citrulline for 10-weeks (n = 4-7). (c) Cumulative 24-hr ambulatory activity in lean and obese mice treated with either vehicle or L-citrulline for 10-weeks (n = 4-7). Values represent means ± SD. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *P<0.05, indicates a significant difference versus the respective vehicle control treated counterpart. $^{\#}P$<0.05, indicates a significant difference versus the respective LFD counterpart.

Figure 6

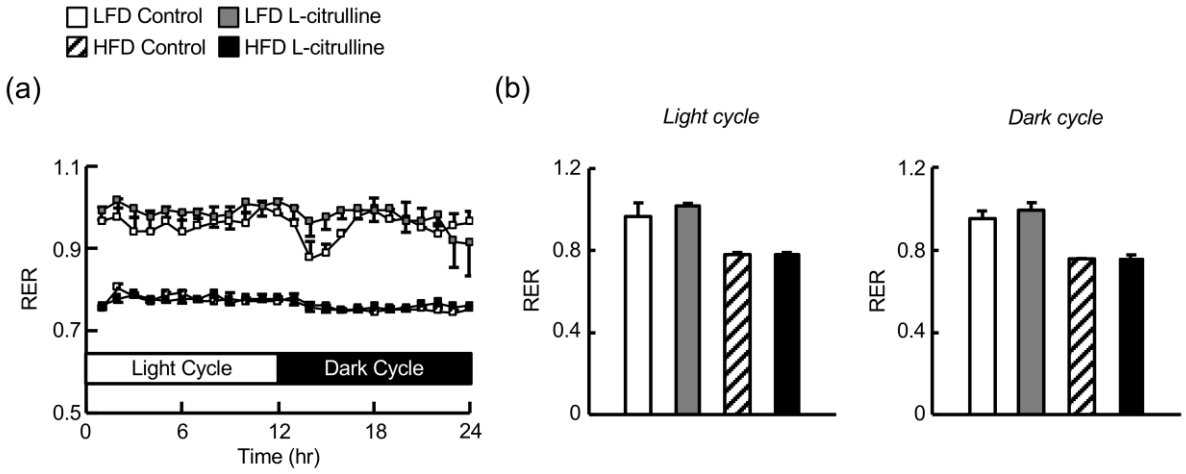

FIGURE 6 L-citrulline supplementation does not affect substrate preference.

(a) 24-hr, (b) Light cycle and dark cycle respiratory exchange ratios in lean and obese mice treated with either vehicle or L-citrulline for 10-weeks (n = 4-7). Values represent means ± SD.

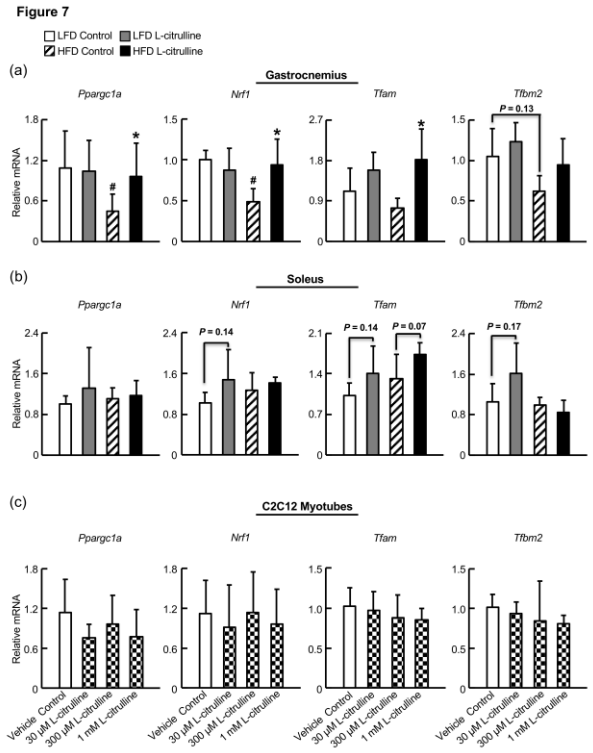

FIGURE 7 mRNA expression of factors that regulate mitochondrial function in mouse skeletal muscle and C2C12 myotubes.

(a) mRNA expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Ppargc1a*), nuclear respiratory factor 1 (*Nrf1*), mitochondrial transcription factor A (*Tfam*), and mitochondrial transcription factor b2 (*Tfbm2*) in gastrocnemius muscles of lean and obese mice treated with either vehicle or L-citrulline for 15-weeks (n = 5-6). (b) mRNA expression of *Ppargc1a*, *Nrf1*, *Tfam*, and *Tfbm2* in soleus muscles of lean and obese mice treated with either vehicle or L-citrulline for 15-weeks (n = 5-6). (c) mRNA expression of *Ppargc1a*, *Nrf1*, *Tfam*, and *Tfbm2* in C2C12 myotubes treated with either vehicle or L-citrulline for 24 hours (n = 4-6). Values represent means ± SD. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. **P*<0.05, indicates a significant difference versus the respective vehicle control treated counterpart. $^{\#}P$<0.05, indicates a significant difference versus the respective LFD counterpart.

Table 1: Gene expression real-time PCR primers

Gene Name	Forward	Reverse
Ppargc1a	TATGGAGTGACATAGAGTGTGCT	CCACTTCAATCCACCCAGAAAG
Nrf1	AGCACGGAGTGACCCAAAC	TGTACGTGGCTACATGGACCT
Tfam	GGAATGTGGAGCGTGCTAAAA	GCTGGAAAAACACTTCGGAATA
Tfbm2	CCCAGAAAGCGTTTACAGAT	GAGATGTATGTATATGGGTG
Ppia	GAGCTGTTTGCAGACAAAGTTC	CCCTGGCACATGAATCCTGG

Table 2: Effect of L-citrulline supplementation on body weight in lean and obese mice

	Body Weight (g)		
	Week 1	Week 7	Week 15
LFD control	24.7±0.5	28.2±0.6	29.2±1.5
LFD L-citrulline	23.9±0.8	27.3±1.1	28.4±2.8
HFD control	25.5±0.7	45.6±1.4$^{\#}$	50.6±1.4$^{\#}$
HFD L-citrulline	25.6±0.6	41.6±3.0$^{\#}$	49.0±4.5$^{\#}$

Body weight was assessed in mice supplemented with a LFD or HFD and either vehicle control or L-citrulline for 15-weeks (n = 5-8). Values represent mean ± SD. $^{\#}P<0.05$, indicates a significant difference versus the respective LFD counterpart. LFD = low fat diet, HFD = high fat diet.

Table 3: Effect of L-citrulline supplementation on lean body mass in lean and obese mice

	Total Lean Body Mass (g)
LFD control	22.6 ± 2.6
LFD L-citrulline	22.6 ± 2.4
HFD control	25.0 ± 1.3
HFD L-citrulline	25.4 ± 1.6

Lean body mass was assessed in mice supplemented with a LFD or HFD and either vehicle control or L-citrulline for 15-weeks (n = 4-8). Values represent mean ± SD. LFD = low fat diet, HFD = high fat diet.

Table 4: Effect of L-citrulline supplementation on blood glucose levels

	Fasting Blood Glucose (mM)	Ad Libitum Blood Glucose (mM)
LFD control	4.77 ± 0.66	7.48 ± 1.17
LFD L-citrulline	4.57 ± 0.70	7.05 ± 0.81
HFD control	8.77 ± 1.06$^{\#}$	11.50 ± 1.82$^{\#}$
HFD L-citrulline	7.12 ± 1.40$^{\#*}$	12.57 ± 4.10$^{\#}$

Blood glucose levels were assessed in mice supplemented with a LFD or HFD and either vehicle control or L-citrulline for 11-weeks, during an overnight fast or ad libitum (n = 8-10). Values represent mean ± SD. *P<0.05, indicates a significant difference versus the respective vehicle control treated counterpart. $^{\#}P$<0.05, indicates a significant difference versus the respective LFD counterpart. LFD = low fat diet, HFD = high fat diet.

Table 5: Mitochondrial respiration was assessed in permeabilized fresh isolated fibers with or without the addition of different concentrations of L-citrulline and L-arginine

	Basal Respiration (nmol O_2/min/mg)	ADP-stimulated (nmol O_2/min/mg)	Respiratory Control Ratio (RCR)
Vehicle Control	0.512 ± 0.251	1.667 ± 0.627	3.795 ± 1.861
30 μM L-citrulline	1.447 ± 1.702	5.259 ± 4.143	4.536 ± 2.361
1 mM L-citrulline	1.097 ± 0.652	5.044 ± 2.603	5.470 ± 4.251
100 μM L-arginine	2.485 ± 2.036˜	10.387 ± 7.898*	4.673 ± 1.682
200 μM L-arginine	1.587 ± 1.413	6.334 ± 4.781	4.395 ± 1.584

Oxygen consumption was assessed using a Clark electrode connected to an Oxygraph Plus recorder where malate and glutamate were used to stimulate basal respiration. Rates were presented as Respiratory Control Ratio (RCR), which is a ratio of ADP-stimulated to basal respiration. Values represent mean ± SD (n = 5-6). *P<0.05, indicates a significant difference versus the respective vehicle control treated counterpart. ˜P=0.19, versus the respective vehicle control treated counterpart.