Reduced non-bicarbonate skeletal muscle buffering capacity in mice with the mini-muscle phenotype

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ABSTRACT

Muscle pH decreases during exercise, which may impair function. Endurance training typically reduces muscle buffering capacity due to changes in fiber type composition, but existing comparisons of species that vary in activity level are ambiguous. We hypothesized that High Runner (HR) lines of mice from an experiment that breeds for voluntary wheel running would have altered muscle buffering capacity as compared with their non-selected control counterparts. We also expected that six days of wheel access, as used in the selection protocol, would reduce buffering capacity, especially for HR mice. Finally, we expected a subset of HR mice with the "mini-muscle" phenotype to have relatively low buffering capacity due to fewer type IIb fibers. We tested non-bicarbonate buffering capacity of thigh muscles. Only HR mice expressing the mini-muscle phenotype had significantly reduced buffering capacity, females had lower buffering capacity than males, and wheel access had no significant effect.

KEY WORDS: Artificial selection, Buffering Capacity, Endurance, Voluntary Exercise, Skeletal muscle, Wheel running

INTRODUCTION

During exercise, metabolic acidosis may occur from by-products of muscle contraction and ATP production. This acidosis may impair muscle function in various ways, including decreased rates of glycolysis and glycogenolysis due to inhibition of glycogen phosphorylase and phosphofructokinase activities, and through decreased maximum force (see Cairns, 2006 for review; Chase and Kushmerick, 1988; Chasiotis et al., 1983; Donaldson and Hermansen, 1978; Fitts, 1994; Trivedi and Danforth, 1966).

Organisms ameliorate decreases in muscle pH in at least two ways. One is to remove protons generated in the cytosolic compartment by transporting them to the mitochondria or out of the cell entirely. Transport of protons into the mitochondria using monocarboxylate transporter 1 (MCT1) also transports lactate, which can be used by the mitochondria and converted to pyruvate (intracellular lactate shuttle; Brooks, 1998; Brooks et al., 1999). Proton transport out of the cell is accomplished by numerous mechanisms, but MCT proteins are of particular interest because lactate may then be taken up by other muscle cells via MCT1 (usually by type I or type IIa fibers; Donovan and Pagliassotti, 2000) and converted into pyruvate via lactate dehydrogenase.

The other way to mitigate decreased pH is to buffer it, which is especially important in the blood and muscle of organisms that must undergo an oxygen deficit, such as during some types of exercise or diving. Organisms accomplish this in numerous ways, including the use of HCO_3^- and imidazole-containing (histidine-containing) compounds (carnosine, anserine, etc.), production of lactate, the hydrolysis of phosphocreatine (and subsequent production of P_i), and behavioral changes (e.g., increased ventilation). As might be expected, marine mammals and pelagic fish have higher buffering capacity than terrestrial mammals or deep-sea/shallow water

fish, respectively (see Abe, 2000 for review; Castellini and Somero, 1981; Okuma and Abe, 1992). Greyhounds and thoroughbred horses, which regularly perform extended bouts of exercise, have increased buffering capacity as compared with humans of unspecified training status (Harris et al., 1990). In humans, buffering capacity changes in response to training, and differentially depending on the type of training (e.g., high-intensity interval training increases buffering capacity; Parkhouse et al., 1985; Sahlin and Henriksson, 1984; Weston et al., 1997). Possible mechanisms underlying the training responses in humans include increases in the amount of buffering compounds in the muscle (potentially by changing the number of fast glycolytic fibers), increases in the number of proton transporters (Juel et al., 2004a; Juel et al., 2004b; Juel et al., 2003; Pilegaard et al., 1999; Pilegaard et al., 1994), and/or increases in muscle capillarity (Jensen et al., 2004).

Given its association with athletic abilities, buffering capacity would be expected to evolve in concert (coadapt) when artificial selection targets forced or voluntary exercise (review in Swallow et al., 2009). The purpose of the present study was to examine muscle buffering capacity in replicate high-runner (HR) lines of mice that have experienced long-term breeding for high voluntary wheel running (Swallow et al., 1998). Several correlated responses in the HR lines seem to enhance capacity for endurance exercise, including more intermittent locomotion on wheels, altered stride characteristics during treadmill locomotion, increased maximal oxygen consumption (VO_2max), increased heart ventricle mass, and larger femoral heads (Claghorn et al., 2017; Garland and Freeman, 2005; Girard et al., 2001; Kelly et al., 2017; Kelly et al., 2006; Rezende et al., 2006).

A subset of the HR mice, known as mini-muscle individuals, have a 50% reduction in hindlimb muscle mass when homozygous for a SNP mutation in the *Myh4* gene (Burniston et al.,

2013; Kelly et al., 2014). This mutation leads to a severe reduction in IIb muscle fibers throughout the body (Talmadge et al., 2014), causes increased cost of transport, reduced maximal sprint speed, and differences in contractile properties of some muscles in the triceps surae complex (e.g., slower twitches; Dlugosz et al., 2009; Guderley et al., 2006; Syme et al., 2005). Mice with the mini-muscle phenotype also show increased capillarity in the medial gastrocnemius, increased VO_2max during hypoxia, and decreased plasma [lactate] during peak wheel running, but not during exhaustive exercise (Meek et al., 2009; Rezende et al., 2006; Wong et al., 2009). Thus, mini-muscle individuals may have altered buffering capacity.

MATERIALS AND METHODS

Experimental animals

All procedures in this study were approved by and are in accordance with guidelines set forth by the Institutional Animal Care and Use Committee at the University of California, Riverside. Mice used in the present study are the same as in Thompson et al. (2017), which came from generation 74 of an ongoing artificial selection experiment in which mice are bred for high voluntary wheel-running behavior (Careau et al., 2013; Swallow et al., 1998). The founding population was 224 outbred Hsd:ICR mice (*Mus domesticus*). After two generations of random mating, individuals were randomly assigned to one of eight closed lines; four lines designated as non-selected control (C) and 4 designated as high runner (HR) lines. In the selection protocol, all mice were given access to Wahman-type activity wheels (1.12 m circumference, 10 cm wide, 35.7 cm diameter) attached to home-cages with *ad libitum* food and water. The HR mice were bred based on their mean amount of wheel running on days 5 and 6 of a 6-day trial, while C mice were bred without regard to their wheel running. In all cases, sibling mating was not allowed.

This study used 50 male and 50 female mice (evenly split between C and HR) that were not allowed access to wheels, along with another 50 male and 50 female mice that were given access to running wheels for six days (also evenly split between C and HR) immediately prior to dissection. A period of six days was chosen in order to gain insight regarding their running abilities in a way that matched the criterion used for selective breeding. As in Thompson et al. (2017), experimental animals were on a reverse photoperiod with lights on from 19:00 to 07:00. Mini-muscle status was determined by dissection of the triceps surae muscle complex (Garland et al., 2002). Because the mini-muscle phenotype includes altered fiber type of the hindlimb muscles (Bilodeau et al., 2009; Guderley et al., 2006; Guderley et al., 2008; Talmadge et al., 2014), it was used as an additional main effect in all statistical analyses (see below).

Wheel running

For the half of the mice having access to wheels, revolutions were recorded for 23 hours/day by a computer that records revolutions in one-minute intervals (Careau et al., 2013; Swallow et al., 1998). Mice were removed from wheels during peak wheel running on day 6 and immediately sacrificed. Wheel freeness (measured by accelerating the wheel to a known velocity and recording the number of free-spinning revolutions) was used as a covariate in all analyses of wheel running (e.g., see Acosta et al., 2017; Copes et al., 2015; Kolb et al., 2010).

Home-cage activity

Home-cage activity (HCA, an indicator of spontaneous physical activity; Garland et al., 2011) was measured using passive infrared motion sensors placed inside each cage. As previously described (Acosta et al., 2017; Copes et al., 2015; Thompson et al., 2017), HCA sensors were

interfaced with a computer and record activity 3 times/second as a binary output (0 = no movement, 1 = movement) and then readings are averaged across every one-minute interval. As for wheel running, HCA was measured for 23h/day. Sensor sensitivity was used as an additional covariate in these analyses.

Buffering capacity

Right thigh muscles from all mice were studied for buffering capacity. All mice were sacrificed via cardiac puncture (under anesthesia) at the time when peak wheel running would occur on the sixth day, dissected, and thigh muscles were frozen and stored at -80ºC. (Age at sampling was 71-91 days.) Muscle buffering capacity (βm or Slyke; defined as the number of μmoles of base needed to change the pH of muscle homogenate by one pH unit per gram of wet weight of muscle) was determined using methods modified from Castellini and Somero (1981). Muscles were homogenized in 0.9% NaCl solution on ice (1g of muscle in 19 ml of NaCl solution), and 2 ml were then transferred to a scintillation vial to be tested for buffering capacity at a constant temperature of 37±0.5ºC. Initial pH of homogenized muscle was taken, and 0.5N HCl was used to reduce the pH to 6, then, 0.0125N NaOH was used in 10 μl increments to bring the pH up to 7. Preliminary studies showed no effect of total protein concentration on buffering capacity and therefore it was not measured in this experiment.

Statistics

Dependent variables were transformed as needed to improve normality and homoscedasticity of residuals. Following previous studies of mice from this selection experiment (e.g., Belter et al., 2004; Meek et al., 2009; Swallow et al., 1998; Thompson et al., 2017), buffering capacity was

analyzed by nested analysis of covariance (ANCOVA), with replicate line nested within linetype (HR vs C) using SAS Procedure Mixed. Analyses of wheel running and home-cage activity across 5 days were done by SAS Procedure Mixed repeated-measures ANCOVA. The main factors for all analyses were linetype (HR vs C), sex, and the mini-muscle phenotype (Thompson et al., 2017), and wheel access was also used when applicable. As noted above, a measure of wheel freeness and a measure of home-cage sensor sensitivity were used as covariates. For all analyses, age at sacrifice was used as a covariate. In preliminary analyses, body mass was never a significant predictor of home-cage activity, so it was removed for final analyses (following Thompson et al., 2017).

For analyses of buffering capacity (always log-transformed to improve normality of residuals), time at sacrifice was used as an additional covariate. Because previous studies have shown fiber type alteration within muscle due to as little as 1 week of training (Allen et al., 2001), we tested whether the amount of physical activity was a predictor of buffering capacity and if the level of activity would change the magnitude of the main effects or the interactions. Therefore, in some analyses of buffering capacity we also used the total amount of wheel running (assigning mice without wheels values of zero) and/or the total amount of home-cage activity as covariates (following Thompson et al., 2017). Note that the mini-muscle phenotype is absent from all C lines, fixed (100% of individuals have it) in one of the HR lines, and polymorphic in another HR line, so mini-muscle status is confounded with linetype. Therefore, we performed additional analyses removing line and linetype from the model and performing an ANCOVA of buffering capacity for the four sex X linetype groups of mice. Statistical significance was judged at $p < 0.05$.

RESULTS AND DISCUSSION

Wheel running

As shown previously (e.g., see Belter et al., 2004), HR mice ran significantly more revolutions across days 1-5 than their C counterparts (repeated-measures ANCOVA, $p = 0.0068$; see Fig. 1A and Table S1), with a strong effect of day ($p < 0.0001$) and a linetype*day interaction ($p < 0.0001$). Figure 1A shows that HR mice had a dramatic increase from ~8,000 and ~8,500 revolutions/day for males and females, respectively, on day 1 to ~11,000 and ~14,000 revolutions/day on day 5. In contrast, both sexes of C mice had a smaller absolute and proportional increase from ~4,000 revolutions on day 1 to ~5,000 on day 5. Figure 1A suggests that females tended to run more than males in HR lines, but not in C lines, but the linetype*sex interaction was non-significant ($p = 0.2431$). The pattern for total revolutions per day was largely mirrored by the pattern for average running speed (Fig. 1B), with a strong effect of linetype ($p = 0.0029$), day ($p < 0.0001$), and their interaction ($p < 0.0001$). The amount of time spent running was higher for HR than for C ($p = 0.0157$; Fig. 1C) and higher for females than males ($p = 0.0391$), with a strong effect of day ($p < 0.0001$), where most groups ran the most on day 1, likely an effect of novelty (Table S1). ANCOVA indicated a significant positive effect of mini-muscle phenotype on revolutions per day ($p = 0.0119$; Fig. 1D) and average running speed ($p = 0.0240$; Fig. 1E), but not running duration (Fig. 1F).

Home-cage activity

Similar to results from previous studies of animals housed without wheels (Acosta et al., 2017; Copes et al., 2015; Thompson et al., 2017), HR mice had higher total activity, minutes per day of activity, average activity per minute, and maximal activity for any one-minute interval (Fig 2,

left panels and Table S2: linetype $p = 0.0015$, $p = 0.0235$, $p = 0.0007$, and $p = 0.0018$, respectively). In addition, females without wheels had more total activity, more time spent active, more activity per minute, and higher maximal activity for any one-minute interval than male mice ($p = 0.0133$, $p = 0.0134$, $p = 0.0410$, and $p = 0.0207$, respectively; Fig. 2A-D and Table S2). Mini-muscle individuals without wheels had significantly lower total activity ($p = 0.0061$; Fig. 2E), activity/minute ($p = 0.0002$; Fig. 2G). Male mini-muscle mice without wheels had lower maximum activity in any 1-min interval than other mice (sex*mini interaction $p = 0.0114$; Fig. 2H). No other interaction terms were statistically significant (Table S2). All measures of activity tended to decline across days for all groups (all day $p < 0.0001$).

With access to wheels, the only linetype effect was that HR mice were active in cages for significantly more time than C mice ($p = 0.0402$; Fig 2B), while mini-muscle individuals were active for less time per day as compared with other mice ($p = 0.0044$; Fig. 2F). Again, all measures of activity tended to decline across days (all day $p < 0.0001$), at the same time that wheel running was increasing (Fig. 1), a phenomenon that has been reported previously for these mice (Acosta et al. 2017). No interaction terms were statistically significant for mice with access to wheels (Table S2).

Buffering capacity

Our hypothesis that HR mice in general would have reduced buffering capacity in skeletal muscle was not supported by any statistical model (Fig. 3A and Table S3). However, the subset of HR mice with the mini-muscle phenotype had significantly lower buffering capacity than normal-muscled mice (Fig. 3 and Table S3). The mini-muscle phenotype has been favored (unintentionally) by the selective breeding protocol, and hence can be viewed as an adaptive

response to selection, albeit one that occurs in only two of the four replicate HR lines because it was initially rare and the underlying Mendelian recessive allele was lost by random genetic drift in all other lines (Garland et al., 2002) . Although it has been favored by selection, the mini-muscle phenotype includes several differences as compared with normal-muscled individuals (e.g., increased fatigue resistance in medial gastrocnemius: see Introduction), and it is not yet clear which one(s) of these provides the primary benefit(s) for endurance running. Indeed, reduced buffering capacity might even be maladaptive for endurance running. For example, a study of endurance capacity during forced treadmill exercise found that the HR line fixed for the mini-muscle phenotype has relatively low endurance compared with the other three HR lines (Meek et al., 2009). However, in the context of the prevailing selective regime, this "cost" appears to be outweighed by other benefits inherent to mini-muscle individuals.

Regardless of its adaptive significance, what might underlie the reduced buffering capacity of mini-muscles? Many previously mentioned comparative studies have shown that fiber type of the muscle and a few histidine-containing compounds are very important in the non-bicarbonate buffering capacity of muscles (carnosine, anserine, and balenine). These compounds occur in most animals (Crush, 1970; Davey, 1960) but vary in their concentrations (among species; see Bate-Smith, 1938) and in different muscle fiber types (Dunnett and Harris, 1995). Much has been done on anserine and carnosine, which have pKa values of 6.83 and 7.04 (Bate-Smith, 1938; Davey, 1960), respectively, which would make them good buffering compounds. Dunnett and Harris (1995) showed that carnosine concentrations in the middle gluteal muscle of horses are increased in type IIa fibers, but highest in type IIb fibers. Mini-muscle mice have reduced numbers of type IIb fibers (Bilodeau et al., 2009; Guderley et al., 2008; Guderley et al., 2006; Talmadge et al., 2014), meaning that they should show reduced carnosine-mediated

241 buffering capacity. Taurine (an organic acid) was shown to have marked effects on increasing
242 buffering capacity as well, but its distribution is opposite of carnosine, being higher in type I
243 fibers, but still present in lower concentrations in type IIa fibers and type IIb fibers (Dunnett and
244 Harris, 1995). This evidence suggests that the differences in buffering capacity between mini-
245 muscle and normal-muscled individuals are unlikely to be caused by differences in taurine
246 concentrations.

247 Mini-muscle mice further differentiate themselves from normal-muscled mice by having
248 lower blood lactate during peak wheel running (Meek et al., 2009), which could be caused by
249 either reduced lactate production or increased usage within the mitochondria. If mini-muscle
250 mice are generating less lactate, then they should have reduced ability to resist changes in pH,
251 given that lactate acts as a buffering compound. Mini-muscle mice also have increased
252 mitochondrial densities (Guderley et al., 2006), but lower lactate dehydrogenase activity per
253 gram of muscle tissue (Houle-Leroy et al., 2003), which could prevent them from using the
254 intracellular lactate shuttle to convert lactate to pyruvate in the mitochondria of type I and type
255 IIa fibers. The increased capillarity of mini-muscle mice (Wong et al., 2009) may be more
256 effective at transporting H^+ away from the muscles (via MCT proteins), as suggested by Juel
257 (2008), which could mean they would not require increased buffering capacity.

258 Female mice had a lower buffering capacity than males ($p = 0.0051$; see Fig. 3A and
259 Table S3). The differences in buffering between the sexes in these mice may reflect lower
260 carnosine and/or anserine levels in muscles of females, as has been reported in some species of
261 mammals (e.g., humans and mice; Mannion et al., 1992; Peñafiel et al., 2004), but not in others
262 (i.e. rats; Peñafiel et al., 2004). The sex differences seem to be mediated by testosterone in mice,
263 given that treatment of females with testosterone propionate increases their carnosine to levels

similar to that seen in males, and hence may also equalize muscle buffering capacity (Peñafiel et al., 2004). Female mice also have lower levels of type IIb fibers in some hindlimb muscles (e.g., the tibialis anterior; see Figure 1 in Haizlip et al., 2015) which could also contribute to lowered buffering in those muscles.

Our results regarding the effects of sex and of mini-muscle phenotype were generally unaltered when we included measures of wheel running and/or of home-cage activity as covariates, and the activity metrics did not significantly predict buffering capacity in these statistical models (Table S3). However, in an analysis using only mice with wheel access, average running speed across 5 days negatively predicted buffering capacity ($p = 0.0407$). Similarly, the sum of the wheel revolutions during the 8 minutes prior to sacrifice negatively predicted buffering capacity ($p = 0.0440$) (preliminary analyses indicated that wheel running over this time interval had a higher predictive ability than over other intervals in the range of 1 through 120 min prior to sacrifice). Both of these results suggest that wheel-running activity may have some influence on muscle buffering capacity, i.e., cause training effects.

Training effects on muscle buffering capacity have been reported previously (Parkhouse et al., 1985; Sahlin and Henriksson, 1984). For example, endurance cyclists have lower muscle buffering capacity than other athletes that perform more short-term, high-intensity exercises (because of reduced numbers of fast glycolytic or type IIb fibers), but high-intensity, interval training can raise their muscle buffering capacity (Weston et al., 1997). This training effect is potentially caused by an increase in type IIb abundance after high-intensity training. [Type IIb fibers generate the most lactate due to their lower oxidative capacity (Baldwin et al., 1977).] In mice, Allen et al. (2001) showed that as little as 1 week of voluntary wheel running (commonly thought of as endurance training) is enough to induce a fiber-type shift in the tibialis anterior

muscle, increasing the number of type IIa fibers and reducing the number of type IIb fibers. A change in fiber type toward more oxidative fibers (type I and type IIa) may lower buffering capacity due to either lower lactate or carnosine levels (Dunnett and Harris, 1995; Dunnett et al., 1997). Future studies could explore these possibilities by direct analysis of muscle fiber types at different time points after the initiation of wheel access in HR and C lines of mice.

In conclusion, we have shown that buffering capacity is reduced in mini-muscle mice (Garland et al., 2002) as well as female mice in general. We have also provided possible explanations for these differences (e.g., potentially lower levels of carnosine in mini-muscle mice); however, it is likely that more than one process is occurring simultaneously to affect the buffering capacity of these mice. Future studies should quantify the imidazole-containing peptides in the hindlimb muscles of mini- and normal-muscled mice, as well as the amount of MCT proteins (see Introduction) present in the same muscles. Based on our current knowledge of the fiber type composition in the mini-muscle mice (see references in Introduction), we would expect lower levels of carnosine and lactate, but potentially increased concentrations of MCT1 proteins due to the lower numbers of type IIb fibers in their hindlimb muscles.

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Competing interests

Nothing to declare.

Author contributions

JCK and TG designed the experiments, analyzed the data, and drafted the manuscript. JCK collected the data; JR and EC assisted with data collection and edited the manuscript.

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Supplementary information

Supplementary information available online at xxto be added by journal

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Figure Legends

Fig. 1. Average wheel running metrics across five days (n = 92). Values are LS means ± standard errors from SAS Procedure Mixed repeated-measures ANCOVAs (see Table S1 for full results). (A) total revolutions C vs. HR. (B) revolutions per minute C vs. HR (average speed). (C) number of one-minute intervals with at least one revolution C vs. HR (running duration). (D) total revolutions of normal vs. mini-muscle (E) revolutions per minute normal vs. mini-muscle (average speed). (F) number of one-minute intervals with at least one revolution normal vs. mini-muscle (running duration). HR mice ran significantly more total revolutions per day, at higher average speeds, and for a longer duration than C mice. The day-to-day increase in distance run and average speed was significantly greater for HR than for C mice. In addition, mini-muscle individuals ran more distance and faster than normal-muscled individuals. The duration of activity varied significantly across days, such that all groups tended to run more on day 1, which probably represents a novelty effect.

Fig. 2. Average home-cage activity metrics across five days for C vs. HR mice (left column) and Normal vs. Mini-muscle mice (right column). Analyses of mice with and without wheel access were performed separately. Values are LS means ± standard errors from SAS Procedure Mixed used to implement repeated-measures ANCOVAs. (A) Total HCA (n = 191). (B) Time spent active (n = 190). (C) Mean activity per minute (n = 191). (D) Mean maximum activity in any one-minute interval (n = 191). (E) Total HCA (n = 191). (F) Time spent active (n = 190). (G) Mean activity per minute (n = 191). (H) Mean maximum activity in any one-minute interval (n = 191). HR mice had higher total activity, mean activity per minute, and maximum activity in

any one-minute interval compared to C mice when housed without access to wheels, and they were active for more minutes regardless of wheel access. Mini-muscle individuals had less total activity, less activity per minute, and less maximum activity in any one-minute interval than normal-muscled mice when housed without access to wheels. Mini-muscle mice were also active for fewer minutes than normal-muscled mice when housed with wheels.

Fig. 3. Buffering capacity (βm) of right thigh muscles. Values are LS means ± standard errors from SAS Procedure Mixed, based on $\log_{10}$-transformed data. (A) Analyses of all mice combined (n = 176) showing male and female, Control and HR mice, with or without access to running wheels, as well as normal vs. mini-muscle. Female mice had significantly lower buffering capacity than males regardless of wheel access or linetype (p = 0.0051, Table S3). Control and HR mice did not significantly differ in buffering capacity. Mini-muscle mice had lower buffering capacity than normal mice. Panels B-E represent separate analyses of one fourth of the total sample size. (B) Normal-muscled mice versus mini-muscle female mice without wheels (n = 43, p = 0.0206), (C) normal vs. mini-muscled male mice without wheels (n = 45, p = 0.0413), (D) normal vs. mini-muscled female mice with wheels (n = 46, p = 0.0084), and (E) normal vs. mini-muscled male mice with wheels (n = 42, p = 0.0004). Mini-muscle mice had significantly lower buffering capacity than normal-muscled mice regardless of sex or wheel access.