University of Nevada, Reno

Quantitative genetics and metabolomics of aerobic metabolism

A dissertation submitted in partial fulfillment of the
requirements for the degree doctor of Philosophy in
Ecology, Evolution, and Conservation Biology

by

Bernard W.M. Wone

Dr. Jack P. Hayes/Dissertation Advisor

May, 2011

University of Nevada, Reno
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THE GRADUATE SCHOOL

We recommend that the dissertation
prepared under our supervision by

BERNARD WONE

entitled

Quantitative Genetics And Metabolomics Of Aerobic Metabolism

be accepted in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

Jack P. Hayes, Ph.D., Advisor

David W. Zeh, Ph.D., Committee Member

Stephen H. Jenkins, Ph.D., Committee Member

Matthew L. Forister, Ph.D., Committee Member

John C. Cushman. Ph.D., Graduate School Representative

Marsha H. Read, Ph. D., Associate Dean, Graduate School

May, 2011

ABSTRACT

The quantitative genetic, biochemical, and physiological bases of variation in maximal aerobic metabolic rate (MMR) are important for understanding exercise physiology and the evolution of aerobic performance, but they still are not well understood. To this end, I studied three aspects of MMR. First, I estimated the genetic variances and covariances of MMR and basal metabolic rate (BMR). Second, I identified how membrane fatty acid (FA) composition changed in response to selection for increased MMR. Third, I measured metabolite expressions in organs primarily responsible for MMR and BMR. Such an approach allowed me to better understand the mechanistic connection (e.g., shared organs) between MMR and BMR and the evolution of aerobic energy metabolism.

In my first chapter, I determined the genetic variances and covariances of MMR and BMR. The genetic variances and covariances of metabolic traits must be known to predict how they respond to selection and how covariances among them might affect their evolutionary trajectories. To this end, I used the animal model to estimate the genetic variances and covariances of MMR and BMR in a genetically heterogeneous stock of laboratory mice. Narrow-sense heritability (h^2) was approximately 0.38+0.08 for body mass, 0.24+0.07 for whole-animal MMR, 0.26+0.08 for whole-animal BMR, 0.16+0.06 for mass-independent MMR, and 0.19+0.07 for mass-independent BMR. All h^2 estimates were significantly different from zero. The phenotypic correlation of whole animal MMR and BMR was 0.56+0.02, and the corresponding genetic correlation was 0.79+0.12. The phenotypic correlation of mass-independent MMR and BMR was

0.13+0.03, and the corresponding genetic correlation was 0.72+0.03. The genetic correlations of metabolic rates were significantly different from zero, but not significantly different from one. The genetic correlation is not so high as to preclude independent evolution of MMR and BMR.

For second chapter of my dissertation, I tested how selection for increased MMR changes membrane fatty acid (FA) composition in a genetically heterogeneous stock of laboratory mice. The membrane pacemaker hypothesis predicts that the unsaturation index (UI) of membrane FAs is positively linked to the high BMR in endotherms. To test this hypothesis, I examined the membrane FA composition of liver and gastrocnemius muscle in mice after 7 generations of selection for increased MMR (high-MMR). Although mass-independent BMR was 3.5% higher in high-MMR mice, the liver UI was not higher than in control mice. Concentration of 16:0 and 18:0 FAs were lower in the liver of high-MMR mice, whereas a greater concentration of 18:1 n-7 FA was found in the gastrocnmeius muscle of high-MMR mice. Moreover, individual variation in UI had no influence on either BMR or MMR. However, concentration of 16:1 n-7, 18:1 n-9, and 22:5 n-3 FAs in the gastrocnemius were significant predictors of BMR, but none of the liver FAs were significant predictors of BMR. In both muscle and liver 20:4 n-6 FA was a significant predictor of MMR and in liver 20:3 n-6 FA was another significant predictor of MMR. The findings did not support the prediction that UI is positively correlated with BMR, but more broadly MMR and BMR were linked to membrane FA composition changes in the skeletal muscle and liver.

For third chapter of my dissertation, I examined how 7 generations of selection for high MMR changes metabolite expression of the organs primarily responsible for

resting metabolic rate (i.e., the liver) and of organs primarily responsible for MMR (i.e., skeletal muscle as represented by the gastrocnemius and plantaris muscles). One of the pivotal challenges in evolutionary physiology is elucidating the functional connection between MMR and BMR because the main contributors to MMR are skeletal muscles wheras the main contributors to BMR are visceral organs. To this end, I used an untargeted global metabolomic analysis of the gastrocnemius and plantaris muscles and of the liver during resting metabolism to reveal adaptive metabolic responses to selection for increased MMR in a genetically heterogeneous stock of laboratory mice. In the plantaris muscle, metabolic profiles of high-MMR and control mice did not differ. In the liver, amino acid and tricarboxylic acid cycle (TCA cycle) metabolite amounts were lower in high-MMR mice than in controls. For the gastrocnemius muscle, amino acid and TCA cycle metabolite amounts were higher in high-MMR mice than in controls, indicating elevated amino acid and energy metabolism. Moreover, amounts of free fatty acids and triacylglycerol fatty acids in gastrocnemius muscle were lower in high-MMR mice than in controls, indicating elevated energy metabolism. Selection for increased MMR resulted in elevated amino acid and energy metabolism in the gastrocnemius muscle of high-MMR mice. These mice also exhibited a 3.5% correlated increase in mass-independent BMR. Because the untargeted metabolomic profiles were at resting metabolic rate and not at MMR, the elevated amino acid and energy metabolism in the gastrocnemius muscle of high-MMR mice may account for their correlated increase in mass-independent BMR.

This dissertation provided quantitative genetic parameter estimates on MMR and BMR, tested the membrane pacemaker hypothesis of metabolism with a manipulative

experiment using whole animals, and examined the biochemical variation between resting metabolism and increased MMR. Overall, the estimated genetic correlation between MMR and BMR is consistent with the assumption of the aerobic capacity model. In addition, the metabolic and fatty acid profiles suggest that increased MMR and BMR in high-MMR mice might be mechanistically linked via elevated amino acid and energy metabolism in the musculature. Lastly, my results add a genetic component to the already demonstrated roles of diet and exercise in determining membrane and intra-muscle fatty acid composition.

ACKNOWLEDGEMENTS

This dissertation would not be possible if not for the help and encouragement of many people. First, I would like to thank my advisor, Dr. Jack Hayes, for giving me a chance to work on the mouse project and in his lab. He was invaluable in all aspects of my degree and dissertation, which included encouragement, and moral and financial support. Most importantly, Jack let me pursue questions and ideas totally outside his area of expertise. I would like to thank my committee Drs. John Cushman, Matt Forister, Luis Gomez-Raya, Steve Jenkins, and David Zeh for their valuable feedback on my ideas, statistics, and writing. I especially want to thank John Cushman and David Zeh for giving me an opportunity to complete a rotation in their lab. I gained valuable research experiences in each of their labs.

I would also like to thank all those in the Hayes lab, which included: postdocs Robert Donovan, Marta Labocha, and Mike Sears; grad students Monica Base, and Cynthia Downs; and undergrad assistances Angie Adrian, Cherie Elliot, Amber Huleva, Andy Hicks, Elsie Huerta, Kim Mclean, and Alexandra Watson. If it wasn't for all their help this dissertation would not have come to fruition. Robert was instrumental in helping me design many aspects of my last two chapters of my dissertation, as well as helping me obtain the tissue samples.

I thank Grant Cramer, John Cushman, and Dave Shintani for letting me use their lab facilities and equipment. Many aspects of this dissertation were completed in their lab and I would not been able to pursue my ideas if not for them. In addition, their

undergrads and graduate students very helpful in showing me how to use the equipment and providing me with work space.

Most importantly, I am very grateful to my wife, Beate, for her never ending support and encouragement with this PhD endeavor of mine. She was always their whether it be moral support or to bounce off my crazy ideas. She sacrificed her many weekends to help me grind tissue samples. Beate, thank you and I love you! In addition, I would like to thank my family and Beate's family for all their support and encouragement.

The work herein was funded by grants from National Science Foundation IOS 0344994 to Jack Hayes and University of Nevada, Reno, Graduate Student Association and Ecology, Evolution, and Conservation Biology research grants to B. Wone.

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Chapter 1: Introduction

Two of the most fundamental physiological differences among vertebrates are their resting and maximal metabolic rates. For example, ectothermic vertebrates have much lower metabolic rates then endothermic vertebrates. Minimal and maximal rates of metabolism are associated with many other biological attributes (e. g., predation risks and reproduction), so energy metabolism is profoundly important to a vertebrate's biology and ecology (Brown et al. 2004; McNab 2006; Trebaticka, et al. 2007; Speakman 2008; Zera and Harshman 2001). A number of hypotheses have attempted to explain (1) the mechanistic bases for variation of metabolism among different kinds of vertebrates and (2) the evolution of endothermy. While many observational studies have addressed these hypotheses, few manipulative experimental studies have tested them, so what accounts for variation in metabolism among vertebrates and how endothermy evolved remains unclear.

Two of the most prominent models for explaining the diversity in metabolism among vertebrates are the membrane pacemaker hypothesis of metabolism (Hulbert 2007) and the aerobic capacity model (Bennett and Ruben 1979). The first of these models addresses mechanisms that might account for variation in resting metabolism, and the second addresses how natural selection might have led to the evolution of endothermy. The membrane pacemaker hypothesis suggests that 'leakiness of cell membranes' is the primary reason for the difference in resting or basal metabolic rate (BMR) between ectotherms and endotherms. The hypothesis also suggests that the increased PUFA composition of cell membranes directly or indirectly increases the

metabolic rate of organisms to compensate for this leakiness (Hulbert and Else 2005). The aerobic capacity model posits that endothermy evolved because (1) resting metabolic rate and aerobic capacity (i.e., maximal oxygen consumption during exercise or maximal metabolic rate or MMR) are inescapably correlated and (2) that natural selection for high aerobic capacity led to a correlated increase in resting metabolic rate, which led to endothermy (Bennett and Ruben 1979). The challenge is establishing the functional connection between resting metabolic rate and maximal metabolic rate and what accounts for their correlation, because the main contributors to resting metabolic rate are the liver and kidney while the main contributor to maximal metabolic rate is the musculature (Weibel et al. 2004).

The quantitative genetic, biochemical, and physiological bases of variation in maximal aerobic metabolic rate (MMR) are important for understanding exercise physiology and the evolution of aerobic performance, but they still are not well understood. To this end, I studied three aspects of MMR. First, I estimated the genetic variances and covariances of MMR and basal metabolic rate (BMR). Second, I identified how membrane fatty acid (FA) composition changed in response to selection for increased MMR. Third, I measured metabolite expressions in organs primarily responsible for MMR and BMR. Such an approach allows me to better understand the mechanistic connection (e.g., shared organs) between MMR and BMR and the evolution of aerobic energy metabolism.

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Chapter 2. Genetic Variances and Covariances of Aerobic Metabolic Rates in Laboratory Mice

Abstract - The genetic variances and covariances of traits must be known to predict how they may respond to selection and how covariances among them might affect their evolutionary trajectories. We used the animal model to estimate the genetic variances and covariances of basal metabolic rate (BMR) and maximal metabolic rate (MMR) in a genetically heterogeneous stock of laboratory mice. Narrow-sense heritability (h^2) was ~0.38±0.08 for body mass, 0.26±0.08 for whole-animal BMR, 0.24±0.07 for whole-animal MMR, 0.17±0.07 for mass-independent BMR, and 0.17±0.06 for mass-independent MMR. All h^2 estimates were significantly different from zero. The phenotypic correlation of whole animal BMR and MMR was 0.56±0.02, and the corresponding genetic correlation was 0.78±0.12. The phenotypic correlation of mass-independent BMR and MMR was 0.13±0.03, and the corresponding genetic correlation was 0.72±0.03. The genetic correlations of metabolic rates were significantly different from zero. A key assumption of the aerobic capacity model for the evolution of endothermy is that BMR and MMR are linked. The estimated genetic correlation between BMR and MMR is consistent with that assumption, but the genetic correlation is not so high as to preclude independent evolution of BMR and MMR.

Introduction

Energy metabolism, or what Max Kleiber (1961) called the fire of life, is perhaps the most important physiological attribute of an animal. Energy metabolism has intrigued biologists for more than a century (Rubner 1883; McNab 1992; Boratynski & Koteja 2009; Gebczynski & Konarzewski 2009), and a recent series of influential papers on the scaling of metabolic rate with body mass has rekindled a keen interest in energy metabolism (West *et al.* 1997; Brown *et al.* 2004; White *et al.* 2006; Makarieva *et al.* 2008). Thousands of studies have been published on energy metabolism which attests to its central biological importance for both domesticated and wild animals (Houston *et al.* 1993; Hayes & O'Connor 1999; Speakman 2008). Almost all of these studies investigated energy metabolism at the phenotypic level. Studies of the genetic underpinnings of energy metabolism are surprisingly uncommon (Konarzewski *et al.* 2005). Energy metabolism is strongly influenced by temperature, and as concern about global climate change grows, information on the genetic architecture of metabolic rates may become valuable, not only from the perspective of basic biological understanding, but also in modeling how species distributions may be impacted by climate change.

Information on the genetic architecture of aerobic metabolism is available from at least three sources: (i) direct estimates that rely on information about relatedness of individuals, (ii) interstrain comparisons, and (iii) artificial selection experiments. In terms of direct estimates the majority of the data are for rodents (e.g., mice (*Mus musculus*), leaf-eared mice (*Phyllotis darwini*), and bank voles (*Clethrionomys glareolus*)) probably because it is tractable to conduct experiments with appropriate breeding designs (Lacy & Lynch 1979; Bacigalupe *et al.* 2004; Leamy *et al.* 2005;

Nespolo *et al.* 2005; Sadowska et al. 2005). Besides data for rodents, the heritability of basal metabolic rate (BMR) has been estimated for zebra finches (*Taenopygia guttata*; Ronning *et al.* 2007), and the heritability of several measures of aerobic metabolism, including maximal metabolic rate (MMR), has been estimated for humans (Bouchard *et al.* 1998; Perusse *et al.* 2001).

Other information on the genetics of aerobic metabolism comes from comparing the exercise performance of strains of rats or mice. When raised under common environmental conditions, phenotypic differences among strains indicate genetic differences. In turn these differences in exercise performance imply differences in the genetics underlying aerobic metabolism (Barbato *et al.* 1998; Koch *et al.* 1999; Lightfoot *et al.* 2001; Lerman *et al.* 2002; Billat *et al.* 2004; Massett & Berk 2005). Besides studies related to exercise performance, interstrain comparisons of lab mice have identified presumptive genetic differences in resting metabolism and in the partitioning of energy metabolism into resting and active components (Sacher & Duffy 1979).

Artificial selection experiments also provide information on the genetic basis of metabolism (MacLaury & Johnson 1972). Metabolic rates can evolve as a result of direct or correlated responses to selection. In rodents, significant direct responses of metabolic rates to artificial selection have been reported for MMR elicited by swimming in bank voles, for endurance running in rats, and for BMR in mice (Koch & Britton 2001; Książek *et al.* 2004; Sadowska *et al.* 2005). Metabolic rates can also evolve as a correlated response to selection on another phenotypic trait, such as artificial selection for high voluntary-wheel running (Swallow *et al.* 1998; Rezende *et al.* 2005). In general,

these selection experiments suggest that aerobic metabolism is heritable, although heritability may not always be particularly high (Książek *et al.* 2004).

As suggested by the studies reported in the previous paragraphs, the emergence of evolutionary physiology as a distinct discipline has been accompanied by increased interest in the genetic architecture of metabolic traits (Feder *et al.* 2000; Henderson *et al.* 2002; Konarzewski *et al.* 2005; Gebczynski & Konarzewski 2009). Information on genetic architecture helps us understand how metabolic traits might respond to artificial or natural selection (Houle 1992; Lynch & Walsh 1998; Hansen *et al.* 2003), and how covariances among traits might affect evolutionary trajectories (Arnold 1987; Sadowska *et al.* 2008). Another reason to be interested in genetic covariances and their evolutionary effects is that a pervasive genetic correlation between minimal and maximal aerobic metabolism is an implicit feature of one model for the evolution of endothermy (Bennett & Ruben 1979; Hayes & Garland 1995). Herein, we used quantitative genetics analyses to estimate the genetic variance of BMR and MMR and their covariance in a large sample of mice (*Mus musculus*) from an artificial selection study. We tested the hypotheses that BMR and MMR were heritable and hence able to respond to selection. We also tested the hypothesis that the genetic covariance between BMR and MMR was significant and hence could influence the joint evolution of these traits. Our study is noteworthy for its large sample size, for obtaining rigorous measures of MMR during treadmill exercise, for obtaining robust estimates of the genetic variances and covariances of important physiological traits, and for its potential relevance to the aerobic capacity model for the evolution of endothermy.

Materials and Methods

(a) *Study organism*

We used the laboratory house mouse, *Mus musculus*, because of the extensive background information on its physiology, morphology, and life history and because it is a feasible organism in which to conduct the large-scale physiological measurements needed to estimate quantitative genetic parameters. Data presented here represent mice from the base population and control groups of a larger artificial selection experiment on aerobic metabolism. The starting population comprised 49 male and 49 female mice representing 35 families from a random-bred HS/IBG (heterogeneous stock/Institute of Behavioral Genetics) stock of mice obtained from University of Colorado, Boulder, CO, USA. Mice were divided in a stratified random fashion such that 12-13 mating pairs were allocated to each of 4 breeding blocks (hence four replicates) where no family was represented more than once (regardless of sex). We created four breeding blocks to accommodate the time consuming physiological measurements to be completed. The breeding and measurement of blocks were separated in time by approximately 4 weeks. The initial group of mice (from all 4 blocks) represented G_{-1}, where G stands for generation. These mice were bred to increase population size, so no metabolic measurements were made on these mice. Mice produced from these initial breeding pairs comprised G_0 (see figure S1 in the electronic supplementary material). Mice from G_0 had their MMR and BMR measured as described below. This manuscript reports data from the baseline population (G_0) and control mice through generation 6 (G_6).

In general mice were weaned at 21 days of age and housed five per cage. Cages were filled with corncob bedding. Due to logistical constraints, weaned mice were

typically caged with siblings. Food and water were available *ad libitum*. A constant 12:12 hr photoperiod and □22□ C ambient temperatures were maintained where the cages are kept. Environmental temperature control in our mouse rooms in the first few generations was not particularly good, however, so we sometimes had appreciably colder or warmer temperatures. In latter generations, environmental temperature controls and emergency backup systems were improved to provide more stable temperatures but in general mice experienced ambient room temperatures in the building, not a precisely regulated thermal environment. All mouse husbandry procedures and experimental protocols were approved by the university's Institutional Animal Care and Use Committee.

(b) *Timing of physiological measurements*

Measurement of BMR and MMR generally began when the mice from a particular block were ~ 8 wk old, but due to various logistical constraints (available analyzers, and personnel time, and so on) there was some range in when measurements were completed for each mouse (see Results for details). In almost all cases MMR was measured at least two days before BMR. All metabolic trials were completed between 0800 and 1700 hr.

(c) *Maximal metabolic rate*

We used forced exercise on a motorized treadmill with an incremental step test to measure MMR (Hayes *et al.* 1992; Swallow *et al.* 1998). Two custom built treadmills were used. Each was enclosed in an open-flow respirometry chamber. A fan was mounted at the front of the treadmill to ensure that air in the chamber was well mixed.

The internal dimensions of the treadmill chambers were approximately 45 x 11 x 5.7 cm. The metabolic rate of exercising mice can change rapidly but the excurrent air leaving an enclosed chamber does not reach steady state rapidly. Hence, we used an instantaneous correction for chamber washout to determine MMR (Bartholomew *et al.* 1981). The effective volume of the treadmill system was 2090 ml. Each motorized treadmill was independently controlled with a Dart Micro-Drive Controller (Dart Controls, Zionsville, ID, USA) that could incrementally increase the treadmill speed. Each treadmill had an electrical stimulator attached to a shocking grid at the end of the treadmill to motivate the mouse to run. Although, the stimulators were from the same manufacturer (Harvard Apparatus Co., Dover, MA, USA), they were different models (Models 340 and 343). We detected and statistically corrected for a difference between treadmills until generation 5 when we were able to replace the stimulators with identical custom-built stimulators that eliminated differences between the treadmills.

MMR was measured once for each mouse. Prior to placing a mouse into a treadmill, body mass was measured. Ambient air was dried with Drierite and filtered before being delivered to each treadmill. Air flow was regulated at 600 ml min^{-1} with a mass flow controller (Sensirion, Zurich, Switzerland) interfaced with LabVIEW 7.1 (National Instruments, Austin, TX, USA) computer software. Excurrent air was passed through a column of Drierite and Ascarite II to remove water vapor and CO_2, respectively prior to analysis. Oxygen concentration of the excurrent air was measured with a Sable Systems Oxilla II oxygen analyzer (Sable Systems, Las Vegas, NV, USA) interfaced to a LabVIEW data acquisition system. Oxygen concentration was sampled at 100 Hz, and this output averaged and recorded each second. Following a baseline measurement of the

ambient oxygen concentration, the mouse was placed in the chamber with the shocking grid off and the treadmill belt stationary. After a 2 min acclimation period the shocking grid was turned on, and after an additional 2 min the test was started with the treadmill set to 20 m min^{-1}. Every two minutes the treadmill speed was increased by 4 m min^{-1} (i.e., 20 m/min for 2 min, then 24 m/min for 2 min, etc) until either the mouse showed no increase in MMR with increased treadmill speed or refused to run. After generation 4, because the treadmill measurements were very time consuming, the step increments in speed were increased to 8 m min^{-1} every 2 min (i.e., 20 m/min then 28 m/min, etc) to reduce the duration of the trail. A test with 51 mice, each run with both methods, showed that MMR was slightly (2.3 %), and significantly ($p = 0.02$) higher when using the original test than when using the modified step increment. After the trial was completed, the incurrent air flow was diverted from the treadmill to obtain a second baseline measurement, and the mouse was removed from the treadmill. Following the trial, body mass was measured again to the nearest 0.01 g. MMR body mass was calculated as the average of the pre and post MMR mass measurements. MMR was defined as average metabolic rate during the highest 1-min period of oxygen consumption during forced exercise. We used the instantaneous MMR data for all genetic analyses.

(d) *Basal metabolic rate*

We used flow-through respirometry to measure BMR. For almost every mouse, BMR was measured at least two days after the MMR trial. Mice were fasted overnight and were measured at ~ 30□ C, which is within the thermal neutral zone for *Mus musculus* (Hart 1971). BMR was measured with a 16 chamber open-circuit system in

which up to 12 mice can be measured. Four empty chambers were used to measure baseline concentrations of oxygen in ambient air. Each chamber was 590 ml in volume and received dry air at 200 ml min^{-1} STP from upstream mass flow controllers (Sensirion, Zurich, Switzerland). Water and CO_2 were scrubbed from excurrent air with Drierite and Ascarite II, respectively. LabVIEW was used to control incurrent air flow rates and sampling order for all chambers. Excurrent air was monitored by two Oxilla II dual channel oxygen analyzers. With two analyzers and two channels per analyzer, the system could monitor 4 chambers at a time. Every 15 minutes the system switched excurrent airflows going to the analyzers to measure the next 4 chambers. BMR was measured for 6 hours giving 6 separate 15 min measurements for each mouse. Empty chambers were sampled for 5 minutes between each 15 min period to obtain initial and final baselines of ambient oxygen concentration for each measurement. Excurrent oxygen concentration was recorded at 1000 Hz and recorded as 5 second averages. BMR was calculated using equation 4 from Hill (1972). Body mass was measured to the nearest 0.01 g just before the mouse was placed in the BMR chamber and again when the animal was taken out. Body mass was calculated as the average of the two measurements. BMR for each mouse was calculated as the lowest 5 min metabolic rate from the six sampling periods.

(e) ***Genetic models***

Before genetic analyses of metabolic traits, we screened the data for each generation separately for outliers using least squares multiple regression of BMR or MMR on body mass and covariates, such as treadmill (1 or 2), age, observer (i.e., person who conducted the treadmill test), and which particular BMR chamber was used.

Statistical analyses were performed using SAS, version 9.1 (SAS Institute, Cary, NC, USA). If standardized residuals from these regressions were > |3.0|, observations were omitted from the genetic analyses. We eliminated mice with these residuals because we think they most likely resulted from measurement errors. For example, most BMR outliers were positive which probably resulted from mice that were not at rest, so that their metabolism was not basal but instead was elevated by activity within the chamber. Similarly, most MMR outliers were negative which we think probably resulted from mice that did not reach their MMR. Significant covariates were included in the genetic models as fixed effects. While results are not reported herein, our results were not appreciably influenced by including or excluding outliers, with the exception that in general residuals were normally distributed when outliers were excluded but they sometimes deviated from normality when outliers were included.

We used the animal model to estimate quantitative genetic parameters. The animal model is a mixed-model approach that uses the relatedness between all individuals in a pedigree to estimate the genetic variance components of a trait. These estimates are considered robust given a complete pedigree (Lynch and Walsh 1998). We implemented the animal model using ASReml v. 2.0 (Gilmour *et al.* 2006) a mixed-model software program that uses restricted maximum-likelihood (REML) estimation.

We used univariate animal models to calculate narrow-sense heritability (h^2) for BMR and MMR and to obtain starting values for subsequent bivariate analyses. For the univariate animal models, we started with a full model that included six variance components. These were the additive genetic variance, V_A, the common environmental variance attributable to natal cage, V_{C1}, the common environmental variance attributable

to post-weaning cage, V_{C2}, the maternal genetic variance, V_{Mg}, the maternal environmental variance, V_{Me}, and the environmental variance unique to individuals, V_E (Kruuk 2004). The common environmental variance attributable to post-weaning cage, the maternal genetic variance, and the maternal environmental variance were quite small and did not explain significant variation for either metabolic trait, and hence those results were not included in the h^2 analyses presented. Instead for simplicity, we estimated h^2 from a reduced model (i.e., *ACE*) that contained three variance components. These were the additive genetic variance, V_A, the common environmental variance attributable to natal cage, V_C, and environmental variance unique to individuals, V_E. One of the main advantages of the animal model is the ability to simultaneously include fixed and random effects in the model (Lynch & Walsh 1998). For all the univariate animal models, generation and sex were fitted as fixed effects (see table S1 in the electronic supplementary material). In addition, block by generation interaction was fitted as a random effect to account for possible generational effects across blocks. For the univariate BMR animal models, body mass, age, and chamber in which the animal was measured also were included as fixed effects. For the univariate MMR animal models, body mass, age, treadmill, observer, and stimulator also were included as fixed effects. Treadmill was included because there was a significant difference between the two motorized treadmills that we think was caused by differences in the stimulators used through generation 4. As stated earlier, after generation 4 we used identical custom built stimulators to eliminate this design flaw.

We estimated the significance of h^2 as the probability that the additive genetic variance component was greater than zero by using a log-likelihood ratio test (Lynch &

Walsh 1998). For example, the log-likelihood of additive genetic variance (*ACE* model) was compared to the log-likelihood of a constrained model (*CE* model) with the additive genetic component set to zero. The test compares twice the difference in log-likelihoods with a one-tailed chi square (χ^2) distribution with the degrees of freedom equal to the number of parameters constrained to zero (Shaw & Geyer 1997). If the additive genetic effects (i.e., V_A) were significant in the univariate models, we constructed bivariate animal models to evaluate the significance of the covariance of the traits (i.e., genotypic correlation). We tested whether genetic correlations were significantly different from both zero and one. To test whether the genetic correlation was significantly different from one, we used an approximation suggested by A.R. Gilmour (personal communication). That is we fixed the correlation to 0.999, and then we compared the log-likelihood from that analysis with the log-likelihood of the model where the genetic correlation was unconstrained (Dingemanse *et al.* 2009). The significance test was a log-likelihood ratio test (Lynch & Walsh 1998), where twice the difference in log-likelihood was compared to a one-tailed chi square (χ^2) distribution with one degree of freedom. We performed analyses for whole-animal BMR and MMR by omitting body mass as covariate, and we also performed mass-independent analyses by including body mass as a covariate.

In addition to h^2, we computed the additive genetic coefficient of variation, CV_A, because it is a more meaningful scale-free measure of evolvability and genetic variability of a trait (Houle 1992; Hansen *et al.* 2003). This measure is based on the additive genetic variances scaled by the trait mean and is reported as a percentage. The CV_A is thus computed as the square root of additive genetic variance scaled by the trait mean.

Likewise, we also reported the environmental coefficient of variation unique to individuals, CV_E, which is computed as the square root of the environmental variance unique to individuals scaled by the trait mean (Houle 1992). For these calculations, we used the additive genetic variance estimates from their corresponding *ACE* models. Similarly we used the environmental variance unique to individuals from their corresponding *ACE* models to compute the environmental coefficient of variation unique to individuals.

Results

BMR or MMR were measured in 1,642 laboratory mice (see table S2 in the electronic supplementary material). On average these mice had a mean body mass of 21.8 g (3.25 SD, range 13.4-32.2) when BMR was measured and a mean body mass of 23.7 g (3.43 SD, range 14.4-34.8) when MMR was measured. Mean BMR was 0.60 ml $O_2\,min^{-1}$ (0.11 SD; range 0.30-0.95), and mean MMR was 4.69 ml $O_2\,min^{-1}$ (0.74 SD, range 2.74-6.87). Complete pedigree information was available for all mice bred for the experiment regardless of whether metabolic trials were successfully completed or not. Measurements were completed at an average age of 69.9 days (9.81 SD, range 49-106) for BMR and 64.6 days (6.99 SD, range 52-84) for MMR. The univariate BMR animal models were based on a pedigree of 1,377 animals. The univariate MMR models were based on a pedigree of 1,585 animals. The bivariate models were based on a pedigree of 1,335 animals. Whole-animal metabolic traits varied between individuals and increased with body mass. In addition, BMR increased with MMR as expected (figure 1).

(a) ***Narrow-sense heritability***

All traits had significant additive genetic variation (table 1). Based on the *ACE* model, h^2 of body mass at the time BMR was measured was 0.40 (*ACE* vs. *CE*, χ^2 = 29.32, $p < 0.001$) and of body mass at the time MMR was measured was 0.36 (*ACE* vs. *CE*, $\chi^2 = 26.22$, $p < 0.001$). Based on the *ACE* model, h^2 for whole-animal BMR was 0.26 (*ACE* vs. *CE*, $\chi^2 = 9.80$, $p < 0.001$) and for whole-animal MMR was 0.24 (*ACE* vs. *CE*, χ^2 = 13.12, $p < 0.001$). Based on the univariate *ACE* models, h^2 for mass-independent BMR was 0.17 (*ACE* vs. *CE*, $\chi^2 = 5.22$, $p < 0.001$), and h^2 for mass-independent MMR was 0.17 (*ACE* vs. *CE*, $\chi^2 = 10.86$, $p < 0.001$). The additive genetic coefficient of variation, CV_A, showed that most traits have greater than 5% additive genetic coefficients of variation, with whole-animal BMR having the largest additive genetic coefficient of variation.

(b) ***Phenotypic and genetic correlations***

Owing to difficulties with model convergence for some bivariate models, we do not report the genetic correlations between BMR and body mass at the time BMR was measured, between MMR and body mass at the time MMR was measured, or between body mass at the time BMR was measured and body mass at the time MMR was measured. The phenotypic and genetic correlations between whole-animal BMR and whole-animal MMR, and between mass-independent BMR and mass-independent MMR were positive. The genetic correlations of these bivariate models were significantly different from zero. In contrast, the genetic correlation between whole-animal BMR and whole-animal MMR was significantly different from one but the genetic correlation

between mass-independent BMR and mass-independent MMR was not significantly different from one (table 2).

Discussion

(a) *Narrow-sense heritability*

Our results indicate significant h^2 and modest genetic variance for mass-independent BMR and MMR. Other studies of BMR in rodents have reported results ranging from low h^2 that was not statistically significant (Lacy & Lynch 1979; Dohm *et al.* 2001; Nespolo *et al.* 2003; Bacigalupe *et al.* 2004) to high h^2 that was statistically significant (Konarzewski *et al.* 2005; Sadowska *et al.* 2005). Previous estimates of h^2 for MMR were generally fairly high (≥ 0.4) although as far as we know only one previous study in mice has estimated heritability of MMR elicited by running (Dohm *et al*. 2001; reviewed in Konarzewski *et al.* (2005)). Variation in estimates of h^2 is not surprising because estimates of h^2 are expected to vary among populations, environmental conditions, and statistical methods (Lynch & Walsh 1998). Relatedly, estimates will depend on which fixed and random effects are included in the model (Wilson 2008).

Heritability was higher for whole-animal metabolism than mass-independent metabolism (table 1). These results have implications for selection on whole-animal traits versus selection on mass-independent traits. Given the greater genetic variances for mass and whole-animal metabolism than for mass-independent metabolism, selection on whole animal metabolism should progress more rapidly than selection on mass-independent metabolism. An example of this phenomenon may be Sprague-Dawley rats

selected for low and high running capacities. The running capacity of low and high selected lines differed by 70% after just three generations of divergent selection. This result suggests that selection on whole-animal traits (as opposed to mass-independent traits) may have major effects on performance, although these differences in performance may be caused largely by differences in mass (Koch & Britton 2001).

(b) ***Phenotypic and genetic correlations***

Phenotypically, mass-independent BMR and MMR were significantly positively correlated (figure 2). The mass-independent correlation of BMR or RMR with MMR (measured either by cold exposure or by strenuous exercise) has been estimated interspecifically for anurans, lizards, passerines, birds, shrews, rodents, and mammals (e.g., Taigen 1983; Koteja 1987; Bozinovic 1992; Hinds and Rice-Warner 1992; Sparti 1992; Walton 1993; Dutenhoffer & Swanson 1996; Thompson & Withers 1997; Rezende *et al.* 2002; Wiersma *et al.* 2007). Three of those correlations were negative but none was significantly different from zero. The significant positive correlations across species for anurans, birds, and rodents suggest (i) that genetic variation for the correlation (i.e., a genetic covariance) exists or used to exist, (ii) that genetic variation for each trait exists or used to exist and that evolutionary differences among species resulted from correlated selection (e.g., selection for both high BMR and high MMR), or (iii) that both those things were true (cf. Rezende *et al.* 2004).

Beside estimates of interspecific correlations, mass-independent intraspecific correlations of resting metabolism and MMR have been reported for a variety of amphibians, squamates, birds, and mammals. Variation in methodology, age, and other

factors would make a thorough discussion of these studies overly long so herein we provide a very brief overview of those studies. For endotherms, significant positive intraspecific correlations have been reported for at least three species of mammals (*Dasypus novemcinctus*, *Peromyscus maniculatus*, and *Spermophilus beldingii*) and one passerine bird, *Passer domesticus* (Hayes 1989, Chappell and Bachman 1995, Chappell *et al*. 1999; Boily 2002). Non-significant correlations (both positive and negative) have been reported for several other mammals (*Meriones unguiculatus*, *Mus domesticus*, *Myodes glareolus*, and *Phyllotis darwinii*) and for some birds (*Calidris canutus* and *Gallus gallus;* Dohm *et al.* 2001; Hammond *et al.* 2000; Nespolo *et al.* 2005; Sadowska *et al.* 2005; Vezina *et al.* 2006; Chappell *et al.* 2007; Boratyński and Koteja 2009). In ectotherms, the intraspecific correlation has been estimated for at least 9 species of squamates, and at least 21 species of amphibians (see Table 1 in Hayes and Garland 1995; Thompson and Withers 1997; Gomes *et al.* 2004), but for most species the number of individuals studied was small so power to detect correlations has generally been low. Two significant positive correlations for squamates and two significant negative correlations for anurans have been reported. The significant phenotypic correlations found in some species suggest that mass-independent resting metabolism and MMR may be genetically correlated (Cheverud 1988; Roff 1995).

While phenotypic correlations are important, our focus is the genetic correlation between BMR and MMR. Genetic correlations have been estimated between basal metabolism and at least three other measures of energy metabolism: MMR during running, MMR during cold exposure, and MMR during swimming. These three measures of MMR may be genetically correlated, but they likely represent different traits,

because within the same population responses of the traits to environmental changes may not be commensurate. For example, deer mice (*Peromyscus maniculatus*) acclimated to cold (3º C) increased their MMR during cold exposure by 31% but their MMR during running increased by only 9% (cf., Harri *et al*. 1984; Hayes & Chappell 1986).

Few studies have estimated the genetic correlation between BMR and MMR. The one previous study of the genetic correlation between BMR and MMR measured during running was performed on an outbred strain (ICR) of *Mus domesticus* (Dohm *et al.* 2001). The additive genetic correlation was significantly positive for some, but not all models that were fitted (e.g., including or excluding dominance or common, postnatal environmental effects).

The genetic correlation between BMR and MMR during cold exposure has also been estimated in the leaf-eared mouse (Nespolo *et al.* 2005). The correlation was positive ($r_A < 0.23$) but not statistically significant, despite a sample size of about 360 mice. The lack of statistical significance may be an issue of statistical power, and the results illustrate the need for very large sample sizes in such studies.

One of the largest studies to date (> 1,000 bank voles) found a positive genetic correlation between BMR and MMR during swimming (Sadowska *et al.* 2005). MMR during swimming might have included a thermoregulatory component, as indicated by modest hypothermia at the end of the test. Interestingly, BMR and MMR during cold exposure were not genetically correlated, even though there was a high genetic correlation between MMR during swimming and MMR during cold exposure. This important study showed that the correlations among various measures of metabolism and with mass may be complex.

Besides estimating genetic correlations from pedigrees, genetic correlations can be estimated from correlated responses to selection. That is, a positive correlated response to selection implies a positive genetic correlation and vice versa. Swiss-Webster mice have been selected for low and high BMR (Książek *et al.* 2004). Because the demands of achieving a response to selection for BMR were very high, that experiment was unable to use replicate lines, so the responses to selection are more challenging to interpret statistically (Henderson 1997; Książek *et al.* 2004). Mice selected for low BMR had slightly higher MMR during swimming and slightly lower MMR during cold exposure than mice selected for high BMR. However the standardized between line differences were sufficiently small that a reasonable interpretation is that there was no correlated response to selection and that the small observed differences can be accounted for by genetic drift (Książek *et al.* 2004).

(c) ***Aerobic capacity model***

Our study is potentially germane to the aerobic capacity model for the evolution of endothermy (Bennett & Ruben 1979). This model postulates that high BMR evolved as a correlated response to selection on aerobic capacity (i.e., MMR during vigorous exercise). The aerobic capacity model is difficult to test because endothermy evolved in mammals (or more likely their ancestors) more than 100 million years ago. It is not particularly common to estimate the strength of selection in extant populations. To do so in extinct populations may be impossible. Nonetheless, the aerobic capacity model argues that endothermy evolved as a correlated response to selection on MMR. Indeed it has been postulated that a positive correlation between BMR and MMR may be an

inescapable attribute of the design of all endotherms. If that postulation is true, then all endotherms ought to have a positive genetic correlation between BMR and MMR. We think that despite the challenges posed, this hypothesis, which we call the strong form of the aerobic capacity model, is testable.

The strong form of the aerobic capacity model postulates that all endotherms and their ancestors that evolved endothermy had a positive genetic correlation between BMR and MMR and that this correlation is ubiquitous in this group of organisms. In addition the hypothesis incorporates the idea that endothermy evolved as a correlated response to selection on MMR. Genetic correlations may evolve due to selection, drift, and other factors, so this is a demanding hypothesis. The notion that a genetic correlation would be ubiquitous and persist over tens of millions of years would only be plausible if it reflected a fundamental design constraint.

Even if there was a fundamental design constraint, that constraint might have existed only in the ancestors of endotherms that evolved endothermy. If the design constraint (genetic correlation) was lost subsequently, we refer to this scenario as the weak form of the aerobic capacity model. We do not think that existing methods can falsify this hypothesis.

If the strong form of the aerobic capacity model is true, then BMR and MMR during exercise should be highly genetically correlated in every extant endotherm. Finding that any extant endotherm did not have a highly positive genetic correlation would falsify the strong form of the aerobic capacity model. Indeed if this can be demonstrated, then the strong form of the aerobic capacity model would be falsified. While this argument is indirect, we argue that science operates by seeking to falsify

hypotheses. Our test of the genetic correlation does exactly that, at least for the strong form of the aerobic capacity model.

Our genetic analyses indicate that the genetic correlation between BMR and MMR is significantly different from zero. However, depending on whether the estimate is for whole-animal or mass-independent metabolic traits, the genetic correlation is either significantly different from one or not significantly different from one, respectively. Hence, direct selection on either BMR or MMR would be expected to result in a correlated response in the other trait. If the genetic correlation was significantly different from one but still very high (e.g., 0.9) then the capacity for independent evolution would be fairly limited. Our estimate of the genetic correlation for mass-independent metabolic traits (r_A = 0.72) is high and not significantly different from one, but if the true correlation is in fact 0.72 (not 1) then these traits could evolve independently because selection could act on the genetic variance unique to each trait. As such our results argue against the strong form of the aerobic capacity model *sensu stricto*. Indeed it would be surprising in the extreme if a perfect genetic correlation was ubiquitous between any two traits, so the strong form of the aerobic capacity model may be overly restrictive. Nonetheless, we think discussion of models of this sort may be a useful springboard to stimulate research into this difficult evolutionary problem. Whether our quantitative genetic results have any bearing on the evolution of endothermy, they do add to our knowledge of the genetic architecture of metabolic traits, an area in which much remains to be learned.

Conclusions

To summarize, we found significant additive genetic variance for BMR and MMR at both the whole-animal and mass-independent levels, and a strong positive genetic correlation between mass-independent BMR and MMR. This latter result supports the possibility that this correlation may be a pervasive or nearly pervasive feature of the design of tetrapods, but our analyses suggest that the correlation does not constitute an absolute genetic constraint and that these traits are capable of independent evolution to some degree. A remaining challenge is establishing the functional connection between BMR and MMR and what accounts for their correlation, because the main contributors to BMR are the liver and kidney while the main contributor to MMR is the musculature (Weibel *et al.* 2004).

Acknowledgments

The study was funded by NSF IOS grant 0344994 to J. Hayes. We thank C. Downs, E. Huerta, K. Mclean, and A. Watson for lab assistance. We also thank A. Gilmour for help with ASReml v. 2.0. We thank N. Dingemanse for ASReml coding issues. We thank L. Gomez-Raya, W. Muir, B. Walsh, and J. van der Werf for advice on using the animal model. We thank H. Schutz and three anonymous reviewers for helpful comments to improve our manuscript. Finally, we thank the College of Science and the Vice President for Research at UNR for additional financial support.

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Table 1. Variance components, heritability (h^2), and standard errors obtained from univariate animal models for laboratory mice as estimated by ASReml v. 2.0. Univariate model tested was the *ACE* model, where A is the additive genetic variance, C is the common environmental variance attributable to natal cage, and E is the environmental variance. Tests of statistical significance of h^2 for the models were determined by constraining V_A to zero and obtaining the log-likelihood (LL) of subsequent reduced models. Twice the difference in LL was compared to a one-tailed chi square (χ^2) distribution with the degrees of freedom equal to the number of parameters constrained to zero. Additive genetic coefficient of variation (CV_A) and environmental coefficient of variation unique to individuals (CV_E) of body mass and metabolic trait values presented are in percentages.

	Univariate *ACE* models				Coefficient of Variation	
Trait	h^2	V_A	V_C	V_E	CV_A	CV_E
body mass at BMR	0.40** (0.078)	1.91** (0.43)	1.23** (0.21)	1.62 (0.23)	6.38	5.86
body mass at MMR	0.36** (0.077)	1.84** (0.44)	1.41** (0.22)	1.82 (0.24)	5.72	5.69
whole-animal BMR	0.26** (0.080)	0.0020** (0.00067)	0.0011** (0.00031)	0.0047 (0.00042)	7.48	11.45
whole-animal MMR	0.24** (0.074)	0.056** (0.019)	0.058** (0.010)	0.12 (0.011)	5.06	7.45
mass-independent BMR	0.17* (0.071)	0.00082* (0.00034)	0.00025 (0.00016)	0.0036 (0.00025)	4.76	10.00
mass-independent MMR	0.17** (0.059)	0.014** (0.0052)	0.0079** (0.0029)	0.063 (0.0038)	2.54	5.37

* Indicates statistically significant at $P < 0.05$

** Indicates statistically significant at $P < 0.001$

Table 2. Additive genetic (r_A), phenotypic (r_P), and environmental (r_E) correlations and standard errors between pairs of traits from a bivariate animal model as estimated by ASReml v. 2.0. Analyses are from the *ACE* model, where *A* is the additive genetic variance, *C* is the common environment variance attributable to natal cage, and *E* is the environmental variance. Tests of statistical significance were determined by constraining the correlation to zero or 0.999 (i.e., $r_A = 1$) and obtaining the log-likelihood (*LL*) of the subsequent models. Because it is not possible to obtain estimates with the genetic correlation exactly equal to one, the test for significant difference from 1 was approximated by constraining the correlation to 0.999. Twice the difference in *LL* was compared to a one-tailed chi square (χ^2) distribution with one degree of freedom.

Pairs of traits	r_A	r_P	r_E	test of $r_A = 0$ *p*-value	test of $r_A = 1$ *p*-value
whole-animal BMR - whole-animal MMR	0.78 (0.12)	0.56 (0.023)	0.38 (0.051)	<0.001	0.03
mass-independent BMR - mass-independent MMR	0.72 (0.28)	0.13 (0.030)	0.02 (0.048)	<0.05	0.32

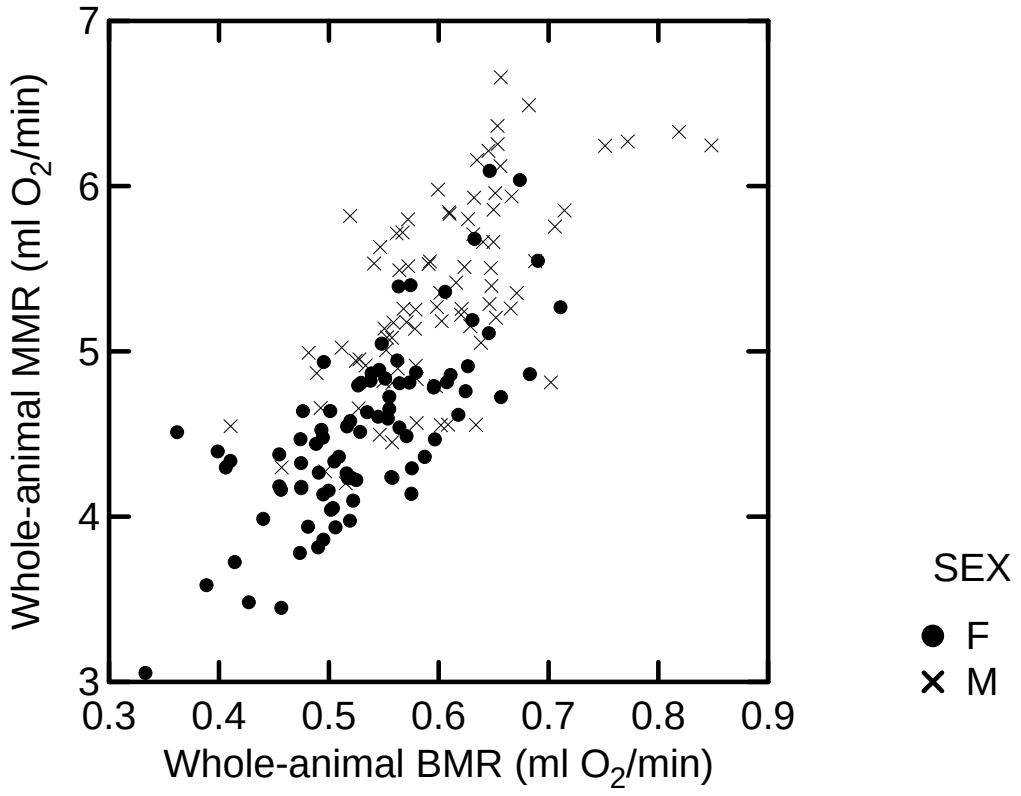

Figure 1. Scatter plot of whole-animal maximal metabolic rates (MMR) in relation to whole-animal basal metabolic rates (BMR). The figure shows data from the latest generation only (i.e., control mice of G_6) to make the data easier to visualize.

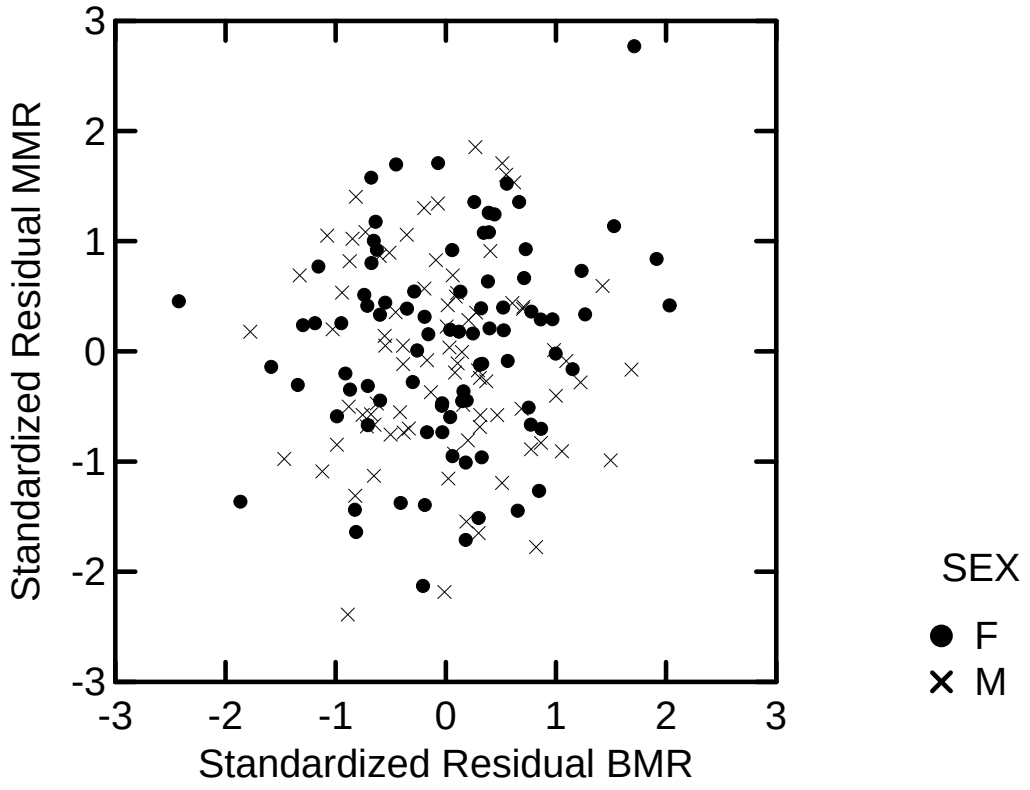

Figure 2. Scatter plot of residual basal metabolic rates (BMR) in relation to residual maximal metabolic rates (MMR). The figure shows data from the latest generation only (i.e., control mice of G_6) to make the data easier to visualize. Phenotypic correlation of the base population (Generation 0 and 1) and control mice (i.e., G_2 - G_6) is significant by conventional correlation ($r = 0.159$, $p < 0.0001$).

Charpter 3. Correlated response of membrane fatty acid composition to selection on maximal metabolic rate

Abstract - Aerobic metabolism of vertebrates varies substantially between endotherms and ectotherms, among the species in each of those groups, and among individuals within species. The membrane pacemaker hypothesis posits that the fatty acid (FA) composition of membranes accounts for variation in aerobic metabolic rates. More specifically, the hypothesis predicts that the membrane FA unsaturation index (UI) is positively correlated with resting or basal metabolic rate (BMR). Comparisons between endotherms and ectotherms support the membrane pacemaker hypothesis, but whether the hypothesis is more broadly applicable is less clear. To test if the membrane pacemaker hypothesis applies to intra-specific comparisons, we examined membrane FA composition of liver and skeletal muscle in mice after 7 generations of selection for increased maximal metabolic rate (MMR). Although mass-independent BMR was 3.5% higher in high-MMR mice, neither the liver, nor the gastrocnmenius muscle UI was higher than in control mice. Lower amounts of 16:0 and 18:0 FAs were found in the liver of high-MMR mice, whereas greater amounts of 18:1 n-7 FA were found in the gastrocnmenius muscle of high-MMR mice. Besides analyzing the effects of selection, we also analyzed the data at the individual level of variation which examines variation in metabolic rate, and fatty acid composition within treatments. Muscle 16:1 n-7, 18:1 n-9, and 22:5 n-3 FAs were significant predictors of BMR, whereas no FAs in liver were significant

predictors of BMR. In both muscle and liver, 20:4 n-6 FA was a significant predictor of MMR, and in liver 20:3 n-6 FA was also a significant predictor of MMR. Our findings did not support the prediction that UI is correlated with BMR, but more broadly BMR and MMR were linked to membrane FA composition changes in the skeletal muscle and liver. Our results suggest that the type of FAs in skeletal muscle phospholipids is associated with BMR. While diet and exercise are often considered determinants of the membrane FA composition, the correlated changes in membrane FA composition in response to selection demonstrate that there is a genetic component to membrane FA composition as well.

Introduction

Biological membranes profoundly influence the physiology of organisms ranging from vertebrates to bacteria. These physiological effects of membranes can also be important ecologically. Specifically, membrane phospholipid saturation/unsaturation (i.e., the fatty acid composition of membranes) has a marked effect on membrane fluidity, which can greatly affect cellular function, and hence the physiology and ecology of organisms (Sinensky 1974; Kogteva and Bezugov 1998; Hochachka and Somero 2002; Hoffman et al. 2009). Indeed, membrane fluidity is kept relatively constant respective to an animal's body temperature, and it reflects an animal's thermal environment (Cossins 1977; Hochachka and Somero 2002). For example, membrane fluidity of the brain is linked to the prevailing temperature in fish and other vertebrates (Hochachka and Somero 2002). In general, membrane fluidity tends to increase as the fatty acid composition

becomes more unsaturated (i.e., the number of double bond fatty acids increase). One of the pivotal physiological processes linked to membrane fatty acid composition is aerobic metabolism (Hulbert 2007).

Resting (RMR) and maximal metabolic rates (i.e., maximal oxygen consumption during exercise or MMR) vary substantially both within and among various species of vertebrates. For example, ectothermic vertebrates have lower metabolic rates (MRs) than endothermic vertebrates. Minimal and maximal rates of metabolism are associated with many other biological attributes (e. g., predation risks and reproduction), so energy metabolism is profoundly important to a vertebrate's biology and ecology (Brown et al. 2004; McNab 2006; Trebaticka et al. 2007; Speakman 2008). A number of hypotheses have attempted to explain (1) the mechanistic bases for variation of energy metabolism among different species of vertebrates and (2) the evolution of endothermy. While many observational studies have addressed these hypotheses, few manipulative experimental studies have tested them, so what accounts for variation in metabolism among vertebrates and how endothermy evolved remain unclear (Wone et al. 2009; Hayes 2010).

Two prominent models for explaining the diversity in metabolism among vertebrates are the membrane pacemaker hypothesis (Hulbert 2007) and the aerobic capacity model (Bennett and Ruben 1979). The first of these models addresses mechanisms that might account for variation in RMR. The membrane pacemaker hypothesis suggests that the differences in fatty acid composition of membrane phospholipids are the primary reason for the difference in RMR between ectotherms and endotherms (Hulbert and Else 2005). The model predicts that the membrane FA unsaturation index (UI) is positively correlated with resting or basal metabolic rate

(BMR). In particular, a higher percentage of the polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA; 22:6 n-3) is the pacemaker of BMR (Hulbert and Else 1999; 2000; 2005; Hulbert 2007). In contrast to the membrane pacemaker hypothesis, which is a proximate mechanistic hypothesis, the aerobic capacity model is an evolutionary hypothesis. The aerobic capacity model posits that endothermy evolved because (1) BMR and MMR are inescapably correlated and (2) that natural selection for high MMR led to a correlated increase in BMR, which led to endothermy (Bennett and Ruben 1979; Hayes and Garland 1995).

Why membrane fatty acid composition varies among vertebrates is unclear, but the proximate effects of membrane fatty acid composition changes are better studied than the evolutionary causes of this variation. Environmental factors are generally considered to be the most important factors affecting muscle membrane fatty acid variation. These environmental factors include diet and exercise training. The fatty acid composition of muscle membranes reflects an individual's diet (Ayre et al. 1996; Ranallo and Rhodes 1998; Andersson et al. 2002). Exercise training is also known to modulate muscle membrane fatty acid desaturation (Andersson et al. 1998; Helge et al. 1999; 2001; Nikolaidis et al. 2004; Petridou et al. 2005). In humans, there is much research in this area because there is a link between membrane fatty acid desaturation and insulin sensitivity in the muscle (Storlien et al. 1991; 1996; Borkman et al. 1993; Vessby et al. 1994; Manco et al. 2000; Bouzakri et al. 2005).

This study used an experimental mouse model derived via artificial selection to quantify associations between fatty acid composition and metabolic rates. Our selection experiment enabled us to test for effects at two levels: (1) effects of artificial selection on

metabolism and (2) effects of individual variation that included variation of mice within selection treatments. More specifically, we artificially selected on mass-independent MMR (i.e., high-MMR mice) and tested whether selection for increased MMR altered the membrane fatty acid composition. The high-MMR mice were selected on mass-independent MMR (i.e., on residuals from regressions of MMR on body mass). We studied membrane fatty acid composition in a muscle, the gastrocnemius and a visceral organ, the liver. In addition, we anticipated that selection of high MMR would lead to a correlated increase in BMR. Accordingly, we tested for differences in the UI between selection treatments for associations between the UI and individual fatty acid concentrations with BMR at the individual level of variation.

Materials and Methods

Study Organism and Metabolic Rates Measurements

We studied mice that were derived from an artificial selection experiment on aerobic metabolism (Wone et al. 2009). The base population for that selection experiment was HS/IBG mice (Heterogeneous Stock/Institute of Behavioral Genetics, University of Colorado, Boulder, CO, USA). There were three selection regimes (i.e., treatments): (1) control, (2) directional selection for increased mass-independent MMR (high-MMR), and (3) antagonistic selection for mass-independent BMR and MMR. Each treatment was replicated four times (i.e., there were four blocks) such that there were 12 lines of mice altogether (4 control lines, 4 high-MMR, and 4 antagonistically selected lines). Herein we studied only the control and high-MMR lines. The mice we studied

were offspring resulting from 7 generations of selection (high MMR) or from 7 generations of random breeding (controls). Our mice were not exercised and standard laboratory rat chow was available *ad libitum.* We measured both BMR and MMR. The metabolic rate measurements have been previously described in detail (Wone et al. 2009).

In brief, MMR was measured once using an incremental step test during forced exercise on a motorized treadmill contained within a flow-through respirometry chamber. For the incremental step test, the treadmill rate was increased 2 m min^{-1} every 8 min. A shocker grid at the rear of the treadmill was used to motivate the mouse to run. When the mouse did not move off the grid, this was an indication that the mouse was exhausted and the trial was ended.

BMR was measured at least two days after MMR with a flow-through respirometry system with 16 chambers. Twelve chambers were used to measure individual BMR and 4 chambers were used to record baseline concentrations of oxygen in ambient air. Mice were monitored during a 6 hr period consisting of 6 cycles of 1 hr each. Each mouse was monitored for 16 min out of every hour with an ambient oxygen baseline taking immediately before and immediately after each mouse measurement. BMR measurements were completed under post-absorptive conditions and were within the animal's thermal neutral zone of ~ 32° C. All mouse procedures and experimental protocols were approved by the University of Nevada, Reno Institutional Animal Care and Use Committee.

Tissue collection and phospholipid extraction/separation

We studied gastrocnemius muscle and liver membrane fatty acid composition from 80 mice. Gastrocnemius muscle and liver tissues were collected from 10 male mice of each line, four control and four selected (high-MMR) lines. We did not sample female mice because of potential confounding fatty acid profiles resulting from pre- and post-breeding conditions. Mice were injected subcutaneously with a 0.3-ml mixture of Dormitor (10%; medetomidine hydrochloride; Orion Corp, Espoo, Finland), Ketaset (10%; ketamine hydrochloride; Fort Dodge Animal Health, Fort Dodge, Iowa, USA), and sterile water (80%), and tissue collection was performed after cervical dislocation. The liver and kidney were rapidly dissected (< 90 s post-mortem time before freezing), snap frozen in liquid nitrogen, and stored at -80° C until extraction.

Approximately 50 mg of tissue was pulverized with a BioPulverizer (BioSpec Products Inc., Bartlesville, OK, USA) under dry ice and liquid nitrogen. Lipids were then extracted from tissues using methanol-chloroform (Le belle *et al.* 2002; Atherton *et al.* 2006). Ice cold methanol-chloroform (2:1, 600 µl) was added and tissue samples were placed in a sonicating bath for 15 min. After sonication of the tissue samples, chloroform-water (1:1) was added (198 µl of each). Tissue samples were centrifuged at 13,500 *x g* for 20 min. The aqueous layer was discarded, and the organic layers (i.e., extracted lipids) were stored at -80° C until PLs and neutral lipids (i.e., triacylglycerides or TA) were separated.

From the organic layer, 150 µl were transferred to activated silica Sep-Pak cartridges (Waters Corp, Milford, MA, USA) to separate into PLs and TAs. Sep-Pak cartridges were activated with 1 ml of chloroform with 0.01% butylated hydroxtoulene (BHT). Phospholipids were eluted with 2 x 1 ml of LC-MS grade methanol with 0.01%

BHT under gentle pressure. Phospholipids extracts were stored at -80° C or dried under a stream of nitrogen and derivatized. For derivation, samples were reconstituted with 750 µl of chloroform-methanol (1:1 vol/vol). We converted the PL extracts to fatty acid methyl esters (FAMEs) by incubating with 150 µl of BF_3-methanol at 80° C for 90 min. Samples were room cooled and a methylated C19 internal standard (25 mg/l) dissolved in chloroform was added. We then added 300 µl of LC-MS grade water and 600 µl of hexane (1:2 ratio) to the samples. Samples were vortex mixed for 1 min and allowed to separate over night. The organic layer was transferred into a 2 ml auto-sampler vial and condensed under a stream of nitrogen. Samples were reconstituted with 150 µl of hexane with 0.01% BHT and transferred to auto-sampler vials with 1.5 ml glass inserts for gas chromatography mass spectrometry (GC-MS) analysis.

One microliter of the FAME was injected into the Thermo Finnigan GC equipped with a HP INNOWAX column (60 m x 0.25 mm-internal diameter column, part number 1909IN-136; Agilent Technologies, Santa Clara, CA, USA). The initial column temperature was 200º C. Column temperature was increased 5º C/min to 240º C, and then the final temperature of 240º C was held for 30 min. All column effluents were introduced into a Polaris Q trap mass spectrometer (Thermo Scientific, Waltham, MA, USA) for mass analysis.

Data Analysis

For all analyses of response of metabolic rate to selection, we used a mixed-model approach in which sex was fitted as a fixed effect. In addition, block and line nested within treatment were fitted as random effects. For basal metabolic rate (BMR) analysis,

body mass, and age were included as fixed effects. For maximal metabolic rate (MMR) analysis, body mass, age, treadmill (two treadmills were used to make the measurements), and observer were included as fixed effects.

GC-MS chromatograms were analyzed using Xcalibur v.1.3 (Thermo Scientific, Waltham, MA, USA). An individual FAME peak was identified in the chromatogram by comparing mass spectra to the National Institute of Standards and Technology (NIST) Mass Spectral Library (NIST 08) (Gaithersburg, MD), the Golm Metabolite Database (Max Planck Institute of Molecular Plant Physiology, Potsdam; Germany), and the University of Nevada, Reno standards database. We used MET-IDEA for semi-quantitation of each peak in the GC-MS chromatograms (Broeckling et al. 2006). Deconvolution and semi-quantitation of peaks and overlapping peaks was achieved by directed extraction of ion intensity values based on quantifier ion-retention time for the metabolite (Broeckling et al. 2006). A 0.1-min threshold window was used for the deviation of peaks away from the predicted retention time across the data set. The FA results are presented in both concentrations and percentages of total FA detected. To determine the concentrations of individual FAs, peaks were normalized by an internal standard with the following equation.

$$\boldsymbol{A}_N = \boldsymbol{A}_O / \boldsymbol{A}_{IS},$$

where $\boldsymbol{A}_N$ - normalized area, $\boldsymbol{A}_O$ - not normalized area, $\boldsymbol{A}_{IS}$ - area of internal standard. Besides presenting concentration and percentage data for total FA, we calculated the following indices: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), n6 fatty acids, n3 fatty acids, n6/n3, unsaturated to

saturated ratio (U/S), and unsaturation index (UI; the average number of double bonds per fatty acid multiplied by 100; Hulbert et al. 2006).

Concentrations and percentages of FA were compared between high-MMR and control mice. The concentrations and percentages were compared using separate one-way analysis of variance (ANOVA), where the block and line nested within treatment were fitted as random effects in the comparison. To account for multiple ANOVA comparisons, we estimated the false discovery rate as the maximum q value (Storey 2002).

Besides analyzing the data from the perspective of the responses of the two treatments (control versus selected), we also analyzed the individual level of variation because much of the variation in metabolic rate, fatty acid composition, and UI was within treatments. These analyses were conducted by including the concentrations of individual FAs or UI as covariates in the mixed-model analyses of metabolic rates. Block and line nested within treatment were fitted as random effects in the mixed-models. For the BMR analysis, selection (effects of control versus high-MMR mice), body mass at BMR, age at BMR and chamber in which the animal was measured also were included as fixed effects. For the MMR analysis, selection (effects of control versus high-MMR mice), body mass at MMR, age at MMR, treadmill, and observer were included as fixed effects. All mixed-model statistical analyses were performed using SAS, v. 9.2 (SAS Institute, Cary, NC, USA).

Results

After 7 generations of directional selection for increased mass-independent MMR, high-MMR mice showed a 12.3% increase in mass-independent MMR and a concomitant 3.5% increase in mass-independent BMR compared to control mice (Fig. 1A). Overall, mass-adjusted MMR differed significantly between lines of control mice and lines of high-MMR mice ($n = 278$, $df = 1, 3$, $F = 48.45$, $p = 0.006$) in response to selection. Mass-adjusted MMR was lowest in the control mice and highest in the directionally selected mice (Fig. 1B). Mass-adjusted BMR differed marginally between control mice and high-MMR mice ($n = 240$, $df = 1, 3$, $F = 9.07$, $p = 0.057$).

Significant differences in the FA profile of membrane FAs were detected in both the gastrocnemius muscle and liver (Table 1) in response to selection. In the liver, the unsaturated FA with the highest percentage was 18:2 n-6 FA, and the SFA with the highest percentage was 18:0 FA. In contrast, in gastrocnemius muscle the unsaturated FA with the highest percentage was 22:6 n-3 FA, and the SFA with the highest percentage was 16:0 FA. In general, gastrocnemius muscle and liver PUFA comprised ~ 43.0% of the total FA detected, whereas gastrocnemius muscle and liver MUFA comprised ~11.0% of the total FA detected.

Directional selection for increased MMR did not result in correlated differences in UI between control and high-MMR mice for either gastrocnemius muscle or liver membranes, despite the significant differences in concentrations of individual fatty acids in response to selection. In particular, the concentration of 18:1 n-7 FA in the gastrocnemius muscle was greater in high-MMR mice than in control mice. In contrast, the concentrations of 16:0, and 20:3 n-6 FAs in the liver were greater in control mice than

in high-MMR mice. In addition the sum of the individual fatty acid concentrations was greater in the liver of control mice than in high-MMR of mice.

Individual variation in the concentrations of some FAs correlated with metabolic rates (Fig. 2). For both tissues, 20:3 n-6 and 20:4 n-6 FAs were significant predictors of MMR. For the gastrocnemius muscle, 16:1 n-7, 18:1 n-9, and 22:5 n-3 FAs were significant predictors of BMR. In contrast for the liver, no FAs were predictors of BMR. Individual variation in UI had no influence on either BMR or MMR.

Discussion

High-MMR mice responded to directional selection for increased mass-independent MMR with a small correlated increase and borderline significant increase in mass-independent BMR at generation 7. We interpret this borderline statistical significance as an indication of the lack power that is inherent to detect differences that result from selection experiments. In selection experiments the experimental unit of replication is not an individual or even a family, but a line (Henderson 1989). Recall that we had 4 lines of high-MMR mice and 4 lines of control mice, so, despite studying a substantial number of mice, our sample size for the selection experiment was effectively $n = 8$. Hence, with two treatments and four lines per treatment our error degrees of freedom for the test of responses to selection were only 3.

Overall, the liver membrane FA profile in our mice was similar to profiles reported previously for mice and rats (Couture and Hulbert 1995; Hulbert et al. 2006; Brzęk et al. 2007; Haggerty et al. 2008). In agreement with previous studies, the most

abundant SFAs in the liver of our mice were 16:0 and 18:0 FAs. In contrast, we found that the most abundant PUFA was 18:2 n-6 FA, wheras previous studies reported that either 20:4 n-6 or 18:2 n-6 FAs were the most abundant (Hulbert et al. 2006; Brzęk et al. 2007; Haggerty et al. 2008). Recall that UI is an index of the number of double bonds in an average of 100 FAs. The more unsaturated the membrane fatty acids of phospholipids, the higher the index value is expected (Hulbert et al. 2006; Hulbert 2007). Our liver UI falls in between the values of 230 and 166 reported previously for mice by Brzęk et al. (2007) and Haggerty et al. (2008).

The increase in MMR and the correlated increase in mass-independent BMR in our high-MMR mice did not result in desaturation of FAs of liver cell membranes. The liver UI was not higher in high-MMR mice than in control mice as predicted by the membrane pacemaker hypothesis (Hulbert 2003; Hulbert 2007). Most importantly, the model predicts that the percentage of 22:6 n-3 FA should be higher as BMR increases s (Hulbert and Else 1999; 2000; 2005). Mean percent of liver 22:6 n-3 FA did not differ significantly between control and high-MMR mice. The most abundant PUFA in the liver of the high-MMR mice was 20:4 n-6 FA, not 22:6 n-3. Moreover, if we treat the liver FA concentrations as fixed effects in a mixed-model analysis for BMR, none of the liver FA concentrations were significant predictors of BMR.

Our data for the FA profile of membranes from liver do not support the predictions of the membrane pacemaker hypothesis. One possible reason for this lack of support is perhaps the membrane pacemaker model applies only to inter-specific comparisons and not to intra-specific comparisons as suggested by Haggerty et al. (2008). Perhaps for inter-specific comparisons the differences in metabolic rate are due to the

unsaturation index of the membrane phospholipids, while for intra-specific comparisons the differences in metabolic rate are due to the composition of FA membrane phospholipids. If that were the case, then the membrane pacemaker hypothesis holds true for the inter-specific scenario, but not the latter. Our data suggest that BMR was linked to membrane FA composition changes in the skeletal muscle. Specifically, the type of FAs was associated with the individual variation in BMR. The current study is not the first to be inconsistent with the membrane pace maker hypothesis. Indeed, the results of a selection experiment on BMR of laboratory mice are also not consistent with the membrane pace maker hypothesis (Brzęk et al. 2007). Brzęk et al. (2007) selected for low-BMR and high-BMR. Selection resulted in a change in membrane PUFA, but not in the direction predicted by the membrane pacemaker model. Brzęk et al. (2007) reported that the unsaturation index of liver and kidney membrane FAs was significantly higher in low-BMR mice, but if the membrane pacemaker hypothesis were correct, then one would predict that the unsaturation index would be lower in mice with low BMR. Likewise, Haggerty et al. (2008) reported no association between RMR and mass-adjusted RMR and PUFA in an outbred strain (MF1) of mice. Indeed, Valencak and Ruf (2007) have suggeseted that link between membrane FA unsaturation and BMR appears to be overstated. Their phylogenetic analysis, corrected for body mass, of 42 mammalian species did not support the predictions of the model (Valencak and Ruf 2007). However, Valencak and Ruf (2007) analyzed muscle membrane FA profiles rather than liver membrane FA profiles. The liver may be more important than skeletal muscle in determining BMR, and the possible link between liver membrane FA composition desaturation and BMR remains unresolved. Although the link between liver and muscle

membrane FA compoistion and BMR is unclear, diet is known to modulate membrane FA composition (Andersson et al. 1998; Helge et al. 1999).

Changes in FA composition of membrane phospholipids observed in the current study is not likely due to the variation in lipid content of standard rodent chow. All mice were fed *ad libitum* of the standard rodent laboratory chow by Harlan Teklad, Madison, WI. Although standard rodent laboratory chow may vary in their fat content, our mouse cage housing arrangement and how mice were fed likely negated or greatly reduced the effects of variation in fat content between bags of chow. For example, bags of rodent chow were empty into a large container where different bags were likely mixed when one scoops the chow to feed the mice. Moreover, because the individual cages (4-5 mice/cage) were arbitrarily placed on the racks, no one group or treatment of mice was fed from a single bag of chow. Hence the FA composition differences observed in the mice are real and not likely a result of variation in fat content in rodent chow.

Previous studies of membrane composition reported individual FAs as percentages of total FA content rather than as concentrations (Hulbert and Else 1999; 2005; Brzęk et al. 2007; Haggerty et al. 2008). By using percentages of individual FAs, one can compare easily across different tissues. When such a comparison is done the analysis implicitly normalizes for differences in total FA content and there is no need for a reference standard in the samples. However, a large change in the concentration of one FA might alter the percentages of FAs whose concentrations have not changed, confounding the interpretation and likely missing important changes (Petridou et al. 2005). Moreover, significant changes in FA concentrations in the same direction will not be detected when comparing with percentages only (Petridou et al. 2005). Hence, in

addition to reporting percentages of the total FAs, we also report the concentrations of individual FAs. Comparisons of FA concentrations of the tissues sampled yield some interesting results. First, compared to control mice, high-MMR mice had significantly lower concentrations of 16:0 and 18:0 FAs in liver membranes. Note that 16:0 and 18:0 are saturated fatty acids. The membrane pacemaker hypothesis predicts lower levels of SFA with increasing BMR, which is what we found in for the two saturated fatty acids that are most abundant in the liver. The membrane pacemaker hypothesis predicts that saturated fatty acids in the membrane should decrease as metabolic rate increases. An alternative idea is that saturated fatty acids should increase when metabolic rate increases (Pamplona et al. 2002; Brand et al. 2003). This alternative stems from the hypothesis that membrane FA saturation reduces the oxidative effects, which PUFAs suffer, when metabolic rate is increased (Pamplona et al. 2002).

Membrane fatty acids have a central role in determining membrane properties, cell signaling, and gene expression in the skeletal muscle and other tissues such as liver (Kogteva and Bezugov 1998; Ehrenborg and Krook 2009). One interesting difference linked to possible functional implications of membrane fatty acid is that compared to control mice, high-MMR mice had significantly higher concentrations of 18:1 n-7 FA in membranes from the gastrocnemius muscle. Indeed, 18:1 n-7 FA has been reported to 1) modulate glucose oxidation, fatty acid oxidation, insulin secretion. and gene expression in rodent pancreas β and liver cells (Alstrup et al. 1999; 2004; Du et al. 2010), 2) lower fasting TAs, total cholesterol, low density lipoprotein, and non-esterified FAs in animal models of dyslipidemia (Tyburczy et al. 2009; Wang et al. 2008; 2009), and 3) positively alter inflammatory and T-cell responses in immune cells that have higher amounts of

18:1 n-7 FA in the membranes (Calder and Grimble 2002; Blewett et al. 2009). Mechanistically, it might be that elevated amounts of 18:1 n-7 FA in the gastrocnemius muscle membranes of high-MMR mice modulate the decreased amounts of free FAs and intra-muscular triacylglycerol fatty acids (Wone et al. unplubl. data). At present, exercise training is known to be a modulator of muscle membrane FA composition (Andersson et al. 1998; Helge et al. 1999; 2001; Nikolaidis et al. 2004; Petridou et al. 2005). Our mice were not trained prior to the incremental step test in the motorized treadmill. Hence, the increased concentration of 18:1 n-7 FA in the lines of high-MMR mice was a result of selection for increased MMR. We suspect that there are more biologically significant changes in membrane FA of high-MMR mice in either the gastrocnemius muscle or liver rather than the three detected. The small number of FAs detected to be statistically significantly different is likely a result the low power of selection experiments.

Our individual level analyses indicate that BMR is linked to the FA composition of gastrocnemius muscle membranes, but not to the FA composition of liver membranes or the unsaturation index. BMR and MMR are often correlated at the inter-specific level, but whether there is a functional connection responsible for this correlation is unclear. *A priori* it is difficult to envision what might cause a functional link between BMR and MMR because the main contributors to BMR are thought to be visceral organs, such the liver (Dann et al. 1990; Konarzewski and Diamond 1995), while the main contributor to MMR is the musculature (Weibel et al. 2004; Weibel and Hoppeler 2005). However, recent analyses of mammals suggest that our notions about which organs are responsible for BMR may need to be revisited because these analyses indicate that BMR correlates strongly with variation in muscle mass (Raichlen et al. 2010). In this study individual

variation in concentrations of gastrocnemius muscle FAs were significantly associated with BMR which also suggest that the musculature is a contributor to BMR. If the musculature is indeed one of the contributors to BMR in addition to the kidney and liver, then the elevated metabolic rate of muscles could be the mechanistic connection that accounts for the correlation between BMR and MMR. In the current study, 16:1 n-7, 18:1 n-9, and 22:5 n-3 FAs were significant predictors of BMR. Interestingly, 16:1 n-7 and 18:1 n-9 FAs have been implicated in the up-regulation of glucose uptake and lipid catabolism (Kien et al. 2005; Dimopoulos et al. 2006). Mechanistically, it might be that elevated BMR in our high-MMR mice is due to changes in the muscle membrane fatty acid composition (Wone et al. unpubl. data). Fatty acid concentrations were correlated across individuals, and interactions among the FAs may be complex (Table 2).

Responses to artificial selection indicate that genetic change has taken place (Brakefield 2003; Fuller et al. 2005). Hence, the response of FA concentrations response to selection for high MMR indicates FA concentrations of membranes are determined at least in part by genetic factors. These correlated responses to selection indicate that there was genetic response of FA composition to selection. This evidence for a genetic component to membrane FA composition is of interest in light of the strong effects that environmental factors, such as exercise and diet are known to play in modulating membrane FA composition (Andersson et al. 1998; Helge et al. 1999; 2001; Mitchell et al. 2004; Nikolaidis et al. 2004; Petridou et al. 2005). Insulin sensitivity has also been linked to muscle membrane FA composition (Borkman et al. 1993; Vessby et al. 1994; Manco et al. 2000). Hence, further exploration of the interplay between diet, exercise,

and metabolic rates will likely be important in understanding the genetic propensities for and environmental determinants of muscle insulin insensitivity and type 2 diabetes.

In summary, selection for increased MMR resulted in correlated responses in FA composition of membrane phospholipids from the liver and gastrocnemius muscle of mice. Our findings did not support the prediction that unsaturation index is correlated with BMR, but more broadly BMR and MMR were linked to membrane FA composition changes in the skeletal muscle and liver. Our results suggest that the type of fatty FA composition of membranes accounts for the variation in intra-specific BMR. Lastly, the correlated response to selection indicates that there is a genetic component to membrane FA compositional changes in skeletal muscles besides diet and exercise training.

Acknowledgments

This study was funded by NSF IOS 0344994 to Jack P. Hayes and UNR Graduate Student Association Research Grant to Bernard Wone. We thank Beate Wone for help with the tissue extractions. We thank John C. Cushman and Dave Shintani for the use of their lab facilities. We thank Angelica Adrian, Cynthia Downs, Amber Huleva, Andy Hicks, Else Huerta, Marta Labocha, Kim Mclean, Mike Sears, and Alexandra Watson for help with the selection experiment. The UNR Proteomics Center acknowledges support from NIH Grant P20 RR-016464 from the INBRE Program of the National Center for Research Resources.

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Table 1. Fatty acid profile of membrane phospholipids (PL) from gastrocnemius muscle and liver in control and high-MMR mice (N =40; mean ± SD).

	Gastrocnemius muscle PL		Liver PL	
Fatty acid	Control	high-MMR	Control	high-MMR
Wet mass (μmole/g)				
14:0	0.02 ± 0.02	0.03 ± 0.02	0.02 ± 0.01	0.02 ± 0.01
16:0	1.40 ± 0.58	1.44 ± 0.64	4.97 ± 1.72	4.35 ± 1.30*****
16:1 n-7	0.004 ± 0.008	0.007 ± 0.014	0.003 ± 0.002	0.003 ± 0.003
18:0	0.94 ± 0.40	1.08 ± 0.38	4.66 ± 1.53	4.15 ± 1.15******
18:1 n-9	0.48 ± 1.03	0.58 ± 0.86	0.88 ± 0.30	0.80 ± 0.34
18:1 n-7	0.10 ± 0.04	0.16 ± 0.12*****	0.48 ± 0.30	0.39 ± 0.22
18:2 n-6	0.60 ± 0.23	0.63 ± 0.29	3.86 ± 1.41	3.46 ± 1.04*
20:2 n-6	0.07 ± 0.15	0.11 ± 0.18	0.13 ± 0.13	0.11 ± 0.13
20:3 n-6	0.07 ± 0.04	0.09 ± 0.09	0.45 ± 0.17	0.38 ± 0.12*
20:4 n-6	0.41 ± 0.35	0.45 ± 0.34	3.10 ± 1.17	2.92 ± 1.32
20:5 n-3	0.04 ± 0.05	0.05 ± 0.07	0.09 ± 0.10	0.07 ± 0.06
22:5 n-3	0.23 ± 0.27	0.33 ± 0.38	0.09 ± 0.08	0.08 ± 0.090
22:6 n-3	0.73 ± 0.59	0.86 ± 0.78	1.55 ± 0.93	1.26 ± 0.64
Sum	5.09 ± 2.65	5.81 ± 2.58	20.28 ± 6.70	17.98. ± 5.04*
Percent				
14:0	0.45 ± 0.32	0.41 ± 0.26	0.07 ± 0.03	0.07 ± 0.05
16:0	26.53 ± 6.87	23.32 ± 8.88*****	22.16 ± 3.31	22.18 ± 3.27
16:1n-7	0.07 ± 0.09	0.09 ± 0.15	0.02 ± 0.07	0.02 ± 0.02
18:0	18.99 ± 6.57	20.50 ± 7.50	23.10 ± 3.40	23.31 ± 2.73
18:1n-9	8.07 ± 6.68	9.14 ± 6.75	4.58 ± 1.70	4.44 ± 1.47
18:1n-7	2.28 ± 1.16	2.73 ± 1.70*	2.37 ± 1.60	2.08 ± 0.83
18:2n-6	13.03 ± 5.91	11.48 ± 4.72	18.48 ± 2.78	18.84 ± 2.59
20:2n-6	1.41 ± 1.49	1.75 ± 2.09	0.81 ± 1.03	0.69 ± 0.79
20:3n-6	1.43 ± 0.64	1.45 ± 1.16	2.44 ± 0.80	2.24 ± 0.34
20:4n-6	7.81 ± 2.58	7.64 ± 3.19	16.15 ± 2.79	16.95 ± 4.93
20:5n-3	0.78 ± 0.92	0.97 ± 1.31	0.55 ± 0.75	0.42 ± 0.27
22:5n-3	4.60 ± 2.23	5.87 ± 6.00	0.60 ± 0.61	0.53 ± 0.48
22:6n-3	14.55 ± 8.58	14.63 ± 9.66	8.66 ± 3.76	8.25 ± 4.23
Sum	100.00	100.00	100.00	100.00
Indices				
SFA (%)	45.97 ± 8.66	44.23 ± 12.90	45.97 ± 8.66	44.23 ± 12.90
MUFA (%)	10.42 ± 7.10	11.96 ± 7.37	10.42 ± 7.10	11.96 ± 7.37
PUFA (%)	43.61 ± 6.21	43.81 ± 10.14	43.61 ± 6.21	43.81 ± 10.14
n-6 PUFA (%)	16.66 ± 6.78	15.65 ± 6.35	16.66 ± 6.78	15.65 ± 6.35
n-3 PUFA(%)	26.95 ± 10.68	28.15 ± 13.02	26.95 ± 10.68	28.15 ± 13.02

n-6/n-3	0.91 ± 0.97	0.96 ± 1.18	0.91 ± 0.97	0.96 ± 1.18
U/S	1.38 ± 1.33	1.85 ± 2.42	1.38 ± 1.33	1.85 ± 2.42
UI	196.04 ± 47.79	202.05 ± 64.97	190.81 ± 24.96	190.83 ± 25.69

*Significantly different from control ($p < 0.05$ and $q < 0.05$)

**Significantly different from control ($p < 0.01$ and $q < 0.05$)

SFA - saturated fatty acids

MUFA – monounsaturated fatty acids

PUFA – polyunsaturated fatty acids

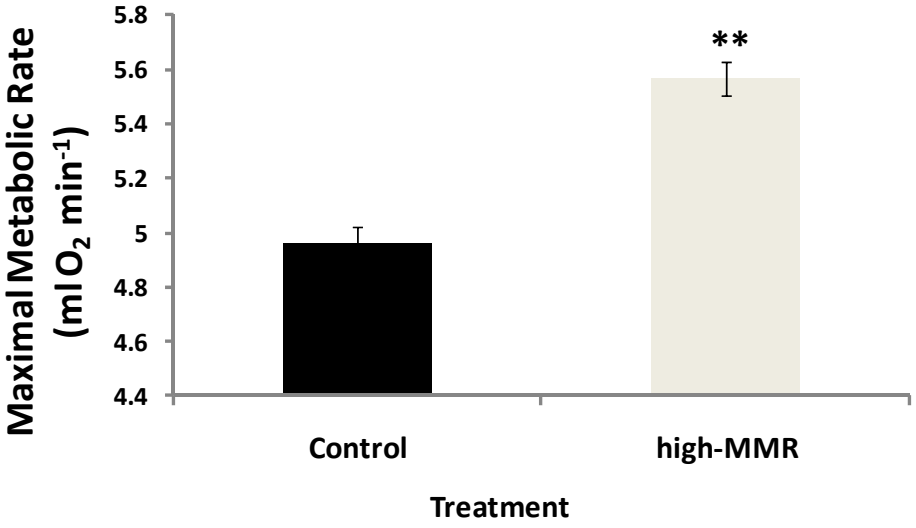
U/S – unsaturated/saturated

UI – unsaturation index

Table 2. Correlation matrix between the different concentrations of fatty acids extracted from gastrocnemius muscle (below the diagonal) and liver (above the diagonal) membranes.

	14:0	16:0	16:1 n7	18:0	18:1 n9	18:1 n7	18:2 n6	20:2 n6	20:3 n6	20:5 n3	20:4 n6	22:5 n3	22:6 n3
14:0		0.51	0.17	0.42	0.44	0.33	0.34	0.18	0.47	0.23	0.27	0.51	0.27
16:0	-0.28		-0.10	0.93	0.69	0.51	0.86	0.03	0.67	0.63	0.17	0.29	0.42
16:1 n7	0.56	-0.36		-0.05	0.07	-0.01	-0.16	0.18	0.03	-0.17	0.20	0.26	-0.06
18:0	-0.01	0.74	-0.14		0.62	0.46	0.89	-0.01	0.66	0.68	0.12	0.20	0.53
18:1 n9	0.70	-0.25	0.82	0.01		0.71	0.69	0.63	0.74	0.55	0.51	0.48	0.44
18:1 n7	0.23	0.00	0.38	0.10	0.30		0.55	0.63	0.75	0.57	0.83	0.60	0.22
18:2 n6	0.56	0.12	0.45	0.30	0.62	0.34		0.10	0.71	0.76	0.20	0.19	0.57
20:2 n6	0.64	-0.24	0.74	-0.02	0.83	0.63	0.60		0.45	0.13	0.69	0.39	0.14
20:3 n6	0.40	0.01	0.61	0.16	0.61	0.80	0.59	0.81		0.68	0.61	0.43	0.38
20:5 n3	0.42	0.32	0.51	0.43	0.70	0.20	0.46	0.59	0.48		0.33	0.25	0.37
20:4 n6	0.32	-0.26	0.57	-0.13	0.55	0.71	0.30	0.72	0.74	0.30		0.67	0.02
22:5 n3	0.46	-0.03	0.73	0.12	0.65	0.34	0.30	0.66	0.45	0.70	0.45		-0.08
22:6 n3	0.21	0.43	0.33	0.49	0.38	0.14	0.41	0.32	0.32	0.57	0.00	0.47	

a.



b.

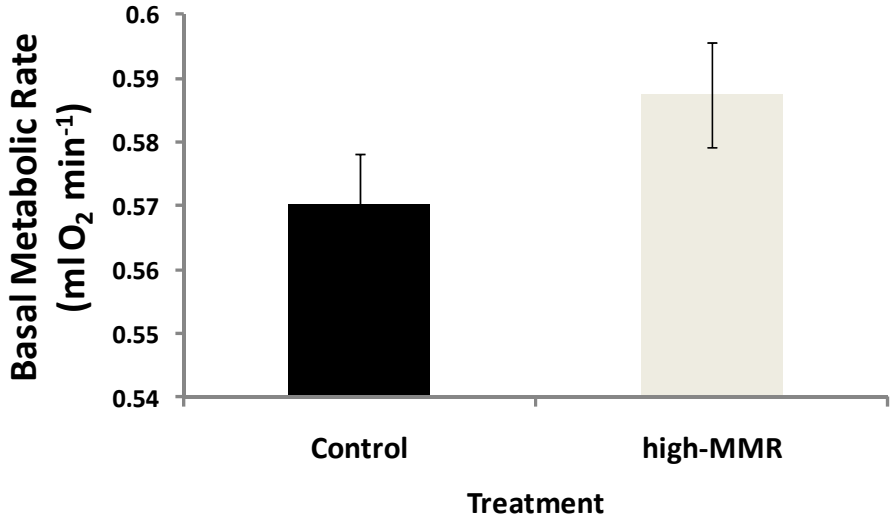
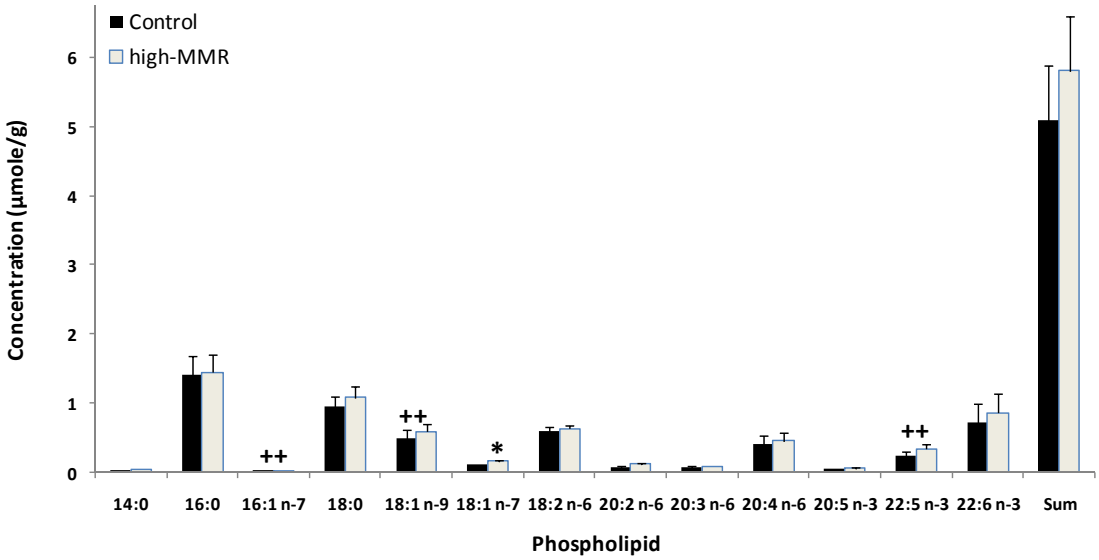


Figure 1. a. Response to selection for high maximal metabolic rate (MMR) at generation 7 (**Significantly different from control; n = 278, df = 1, 3, F =48.45, p = 0.006). b. Correlated response of mass independent basal metabolite rate (BMR) to selection for high MMR at generation 7 (n = 240, df = 1, 3, F = 9.07, p = 0.057).

a.


Control
high-MMR
Concentration (µmole/g)
0
1
2
3
4
5
6
++
++
++
*
14:0
16:0
16:1 n-7
18:0
18:1 n-9
18:1 n-7
18:2 n-6
20:2 n-6
20:3 n-6
20:4 n-6
20:5 n-3
22:5 n-3
22:6 n-3
Sum
Phospholipid

b.

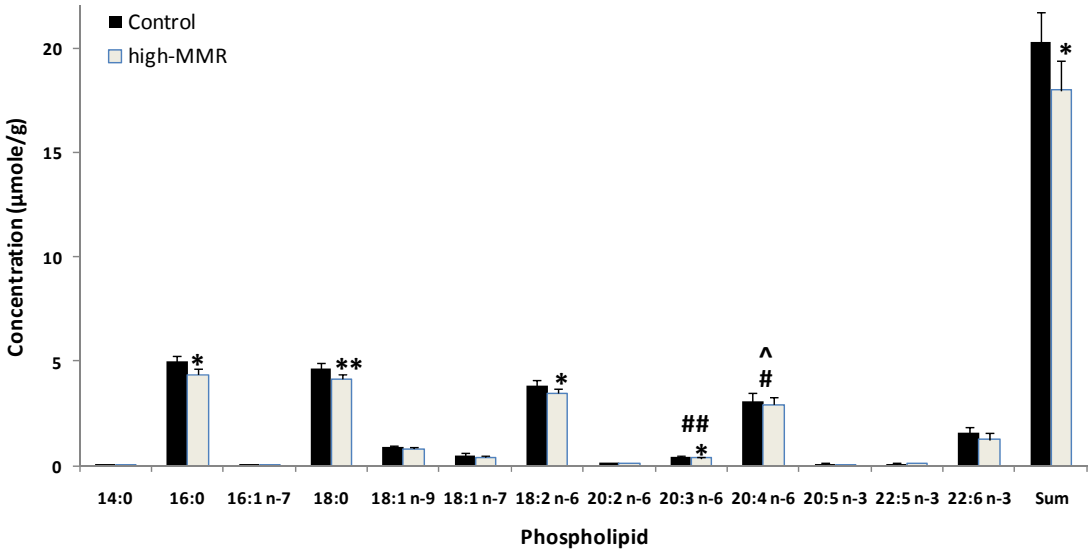


Figure 2. Membrane fatty acid composition of tissues sampled; a. gastrocnemius muscle. b. liver. Values presented are mean (± se) concentrations of phospholipid detected; *Significantly different from control ($p < 0.05$ and $q < 0.05$); **Significantly different from control ($p < 0.01$ and $q < 0.05$); ++Significant gastrocnemius muscle fatty acid predictor of BMR ($p < 0.01$); ^Significant gastrocnemius muscle fatty acid predictor of MMR ($p < 0.05$); #Significant liver fatty acid predictor of MMR ($p < 0.05$); ##Significant liver fatty acid predictor of MMR ($p < 0.01$).

Chapter 4. Metabolomics of aerobic metabolism in mice selected for increased maximal metabolic rate

Abstract - One prominent model for the evolution of endothermy proposes that elevated basal metabolic rate (BMR) evolved as a correlated response to selection on maximal aerobic metabolic rate (MMR). While a genetic covariance between BMR and MMR could account for this correlated response, how selection on MMR would mechanistically alter BMR is not clear, particularly given that the organs affecting BMR and MMR may differ. To explore possible mechanistic linkages between MMR and BMR, we artificially selected mice for high mass-independent MMR for 7 generations and then compared the metabolic profiles of skeletal muscles (gastrocnemius and plantaris) and liver during resting metabolism (RMR) in mice after 7 generations of selection for increased mass-independent MMR (i.e., high-MMR mice). Compared to controls, high-MMR mice had a 12.2% higher MMR, and a 3.5% higher mass-independent BMR. In the plantaris muscle, metabolic profiles of high-MMR and control mice did not differ. In the liver, amino acid and tricarboxylic acid cycle (TCA cycle) metabolite amounts were lower in high-MMR mice than in controls. For the gastrocnemius muscle, amino acid and TCA cycle metabolite amounts were higher in high-MMR mice than in controls, indicating elevated amino acid and energy metabolism. Moreover, gastrocnemius muscle free fatty acids and triacylglycerol fatty acids amounts were lower in high-MMR mice than in controls. Together these results suggest that selection for increased mass-independent

MMR resulted in changes in RMR of high-MMR mice. Because the untargeted metabolomic profiles were measured at RMR, or nearly so, and not at MMR, the elevated amino acid and energy metabolism in the gastrocnemius muscle of high-MMR mice may account for their correlated increase in mass-independent BMR. Collectively, the findings suggest that increased MMR and BMR in high-MMR mice may be mechanistically linked via elevated amino acid and energy metabolism in the musculature.

Introduction

A pivotal challenge in evolutionary physiology is establishing whether there is a mechanistic linkage between maximal aerobic metabolic rate (MMR) and basal metabolic rate (BMR). Indeed, MMR, also called aerobic capacity, and BMR are predictably correlated at the inter-specific level, but whether this reflects a functional connection is unclear (Koteja 1987; Hayes & Garland 1995; Bozinovic 1992; Dutenhoffer & Swanson 1996; Rezende et al. 2002). In particular, the main contributor to MMR is the musculature (Bishop 1990; Weibel et al. 2004; Weibel & Hoppeler 2005), while the main contributors to BMR are the visceral organs, such as kidneys and liver (Daan et al. 1990; Konarzewski & Diamond 1995; Even et al. 2001; Selman et al. 2001; Gębczyński 2008). More recently, among mammals BMR has been shown to correlate with muscle mass, which suggests that the prevailing thinking about which organs are most important in determining BMR may need to be revisited (Raichlen et al. 2010).

One prominent model for the evolution of endothermy proposes that elevated basal metabolic rate (BMR) evolved as a correlated response to selection on maximal aerobic metabolic rate (MMR). While a genetic covariance between BMR and MMR could account for this correlated response, how selection on MMR would mechanistically alter BMR is not clear, particularly given that the organs affecting BMR and MMR may differ. Ultimately, understanding the evolution of aerobic performance will require a better understanding of the mechanistic underpinnings of BMR and MMR. Hence to better understand the functional connection between MMR and BMR, we examined the metabolites of muscle and liver during resting metabolic rate (RMR) in lines of *Mus musculus* artificially selected for increased mass-independent MMR.

We used artificial selection to manipulate the metabolic rates of mice and then used untargeted global metabolomic profiling to test how the metabolic pathways responded to the perturbations caused by selection. For the selection experiment, we artificially selected for high mass-independent MMR (i.e., on residuals from regressions of MMR on body mass; high-MMR mice). The changes in metabolomic profiles enable one to generate hypotheses regarding physiological function. First, we artificially selected for high mass-independent MMR (i.e., high-MMR) and tested whether mass-independent BMR evolved as a correlated response. Then we compared the metabolomic profiles in the liver, the plantaris muscle, and the gastrocnemius muscle of control mice with mice resulting from 7 generations (G_7) of artificial selection for increased mass-independent MMR (i.e., high-MMR mice). The liver was chosen because it is one of the main contributors to BMR. The gastrocnemius muscle was chosen because it is one of the main contributors to MMR and one of the main locomotor

muscles. The plantaris muscle was also chosen because it is a Type I oxidative skeletal muscle, wheras the gastrocnemius muscle is a mixture of Type I oxidative and Type II glycolytic skeletal muscle.

Methods

Study Organism and Metabolic Rates Measurements

We studied mice derived from an artificial selection experiment on aerobic metabolism (Wone et al. 2009; unpubl. data). The base population for that selection experiment was HS (heterogeneous stock)/IBG (Institute of Behavioral Genetics) laboratory house mice, *Mus musculus,* obtained from the Institute of Behavioral Genetics at the University of Colorado, Boulder, CO, USA. While the larger selection experiment had three treatments, mice in this study came from only two of those treatments.Those treatments were: (1) randomly bred controls, and, (2) mice directionally selected for increased mass-independent MMR (i.e., high-MMR mice).Each treatment was replicated four times (i.e., blocks) such that there were 8 lines of mice altogether (4 control lines and, 4 high-MMR lines). Hence, we had a total of 4 blocks of mice in our experiment. Herein we studied offspring resulting from 7 generations of selection (for high MMR) or from 7 generations of random breeding (controls). The metabolic rate measurements have been previously described in detail (Wone et al. 2009). In brief, MMR was measured once using an incremental step test during forced exercise on a motorized treadmill contained within a flow-through respirometry chamber. BMR was measured at least two days after MMR. All mouse procedures and experimental protocols were

approved by the University of Nevada, Reno Institutional Animal Care and Use Committee.

Tissue collection and extraction

Tissues were collected from 10 male mice at resting metabolic rate (RMR) of each line after 7 generations of artificial selection for increased mass-independent MMR. Mice did not have access to exercise wheels, and they were collected from their home cages during the day (their inactive phase). Hence, the metabolomic profiles should be reflective of mice that were at rest or nearly so (Refinetti 2006). We did not sample female mice because of potential confounding metabolic profiles resulting from pre- and post-breeding conditions. Mice were injected subcutaneously with a 0.3-ml mixture of Dormitor (10%; medetomidine hydrochloride; Orion Corp, Espoo, Finland), Ketaset (10%; ketamine hydrochloride; Fort Dodge Animal Health, Fort Dodge, Iowa, USA), and sterile water (80%), and tissue collection was performed after cervical dislocation. The liver, gastrocnemius muscle, and plantaris muscle were rapidly dissected (< 90 s post-mortem time before freezing), snap frozen in liquid nitrogen, and stored at -80° C until extraction.

Approximately 50 mg of tissue was pulverized with a BioPulverizer (BioSpec Products Inc., Bartlesville, OK, USA) under dry ice and liquid nitrogen, with the exception of the plantaris muscle where less than 20 mg were collected. Metabolites and lipids were then extracted from the tissues using methanol-chloroform (Le Belle et al. 2002; Atherton et al. 2006). Ice cold methanol-chloroform (2:1, 600 µl) was added and tissue samples were placed in a sonicating bath for 15 min. After sonication of the

samples, chloroform-water (1:1) was added (198 µl of each) to the tissue samples. Two microliters of each of the internal standards containing 15 mg/l of phenanthrene and 25 mg/l of ribitol were also added to the samples. Samples were centrifuged at 13,500 *x g* for 20 min, and the aqueous (i.e., extracted metabolites) and organic layers (i.e., extracted lipids) were stored separately at -80° C.

Metabolomic and fatty acid profiling by GC-MS

For the aqueous layer (i.e., metabolites), 150 µl samples were transferred to a 2 ml glass auto-sampler vials and dried for 30 min at high setting in an evacuated centrifuge before derivatization (Gullberg et al. 2004; Atherton et al. 2006). To derivatize the aqueous samples, we added 40 µl of methoxyamine HCL (20 mg/ml in pyridine). The samples were vortex mixed for 1 min and then incubated at 30° C for 1 hr. Afterwards, samples were silylated at room temperature with 70 µl of N-methyl-N-trimethylsilyltrifluoroacetamid (MSTFA) for 30 min. Samples were then transferred to auto-sampler vials with 1.5 ml glass inserts for GC-MS analysis.

For the organic layer, 150 µl were transferred to activated silica Sep-Pak cartridges (Waters Corp, Milford, MA, USA) to separate into phospho- and neutral lipids. Sep-Pak cartridges were activated with 1 ml of chloroform. Neutral lipids (i.e., triacyglycerol fatty acids, or TGA) were eluted with 2 x 1 ml of ethyl acetate under gentle pressure. Neutral lipid extracts were stored at -80° C or dried under a stream of nitrogen and derivatized. The samples were reconsituted with 750 µl of chloroform-methanol (1:1 vol/vol). We converted the neutral lipid samples to fatty acid methyl esters (FAMEs) by incubating with 150 µl of BF_3-methanol at 80° C for 90 min. Samples were room cooled

and a methylated C19 internal standard (25 mg/l) dissolved in chloroform was added. We then added 300 µl of LC-MS grade water and 600 µl of hexane (1:2 ratio) to the samples. Samples were vortex mixed for 1 min and allowed to separate over night. The organic layer was transferred into a 2 ml auto-sampler vial and dried under a stream of nitrogen. Samples were reconstituted with 150 µl of hexane and transferred to auto-sampler vials with 1.5 ml glass inserts for GC-MS analysis. FAMEs analyses profile the total fatty acids found within the tissues (i.e., intramuscular TGA). To analyze the free fatty acids, we derivatized the free fatty acids and cholesterol derivatives following the derivatization protocol for the aqueous samples described earlier (Atherton et al. 2009).

One microliter of the derivatized metabolites was injected into a Thermo Finnigan gas chromatography (GC; Thermo Scientific, Waltham, MA, USA) equipped with a 30 m x 0.32 mm-internal diameter column, part number 123-3832 DB-35MS (Agilent Technologies, Santa Clara, CA, USA). The initial column temperature of 80º C was held for 2 min and then increased 5º C/min to 330º C, which was held for 6 min. Similarly, one microliter of the FAMEs was injected into the Thermo Finnigan GC equipped with a HP INNOWAX column (60 m x 0.25 mm-internal diameter column, part number 1909IN-136; Agilent Technologies, Santa Clara, CA, USA). The initial column temperature was 200º C then increased 5º C/min to 240º C, which was held for 30 min. For the derivatized free fatty acids, 2 µl was injected into the Thermo Finnigan GC equipped with a 60 m x 0.25 mm-internal diameter column, part number 122-5562 DB-5MS (Agilent Technologies, Santa Clara, CA, USA). The initial column temperature was 80º C held for 2 min and then increased 5º C/min to 315º C, which was held for 17 min.

All column effluent was introduced into a Polaris Q trap mass spectrometer (Thermo Scientific, Waltham, MA, USA) for mass analysis.

Data Analysis

For all metabolic rate analyses, we used a mixed-model approach where sex was fitted as a fixed effect. In addition, block and line nested within treatment were fitted as random effects. For basal metabolic rate (BMR) analysis, body mass, and age were included as fixed effects. For maximal metabolic rate (MMR) analysis, body mass, age, treadmill, and observer were included as fixed effects.

GC-MS chromatograms were analyzed using Xcalibur v.1.3 (Thermo Scientific, Waltham, MA, USA). An individual metabolite or FAME peak was identified in the chromatogram by comparing its mass spectra to the National Institute of Standards and Technology (NIST) Mass Spectral Library (NIST 08; Gaithersburg, MD), the Golm Metabolite Database (Max Planck Institute of Molecular Plant Physiology, Potsdam; Germany), and the University of Nevada, Reno standards databases. We used MET-IDEA for semi-quantitation of each peak in the GC-MS chromatograms (Broeckling et al. 2006). Deconvolution and semi-quantitation of peaks and overlapping peaks was achieved by directed extraction of ion intensity values based on quantifier ion-retention time for the metabolite (Broeckling et al. 2006). A 0.1-min threshold window was used for the deviation of peaks away from the predicted retention time across the data set. All peaks were normalized by an internal standard with the following equation.

$$\boldsymbol{A}_N = (\boldsymbol{A}_O) / \boldsymbol{A}_{IS},$$

where $\boldsymbol{A}_N$ - normalized area, $\boldsymbol{A}_O$ - not normalized area, $\boldsymbol{A}_{IS}$ - area of internal standard.

To visualize and model how well the metabolite signatures classified the treatments, we used partial least squares-discriminant analysis (PLS-DA; Ramadan et al. 2006; Atherton et al. 2009). We used the chemometrics software Solo (Eigenvector Research, Inc., Wenatchee, WA) to discriminate group classification of the metabolite signatures. PLS-DA is a regression extension of PCA and is considered a supervised method to model separation of independent samples and group classification (Goodacre et al. 2004). R^2X, R^2Y, and Q^2Y are used as measures for the robustness of a PLS-DA model, where R^2X is the cumulative variance explained by the metabolite signatures, and $R^2Y$is the cumulative variance explained by the PLS-DA components. Cross-validation of R^2Y estimates Q^2Y, which explains the cumulative variation predicted by the model. Thus, R^2Y and Q^2Y values indicate how well the overall model discriminates group membership in a data set. The range of these values is 0 to 1, and the more they approach 1, the better they represent good discrimination. For all PLSD-DA models, we used a 3-fold random sample cross-validation method. In addition, we used the PLS-DA class grouping to build the models (Eigenvector Research, Inc., Wenatchee, WA), where we grouped the lines of control mice as one class and the lines of high-MMR mice as the other. In addition, the identified metabolites were mapped onto general biochemical pathways according to the Kyoto Encyclopedia of Genes and Genomes (KEGG).

Concentrations of identified metabolites and fatty acids were compared between high-MMR and control mice. The individual concentrations were compared using separate one-way analysis of variance (ANOVA), where the block and line nested within

treatment are random effects. Recall that for our experimental design, the block was the unit of replication. In each block there was a treatment (i.e., high-MMR) and control lines of mice. We had a total of 4 blocks of mice in our experiment, hence there were 4 lines of high-MMR mice and 4 lines of control mice. To account for multiply ANOVA comparisons, we estimated the false discovery rate as the maximum q value (Storey 2002). All statistical analyses were performed using SAS, v. 9.2 (SAS Institute, Cary, NC, USA).

Results

After seven generations of selection, high-MMR mice had 12.3% higher mass-adjusted MMR than control mice (n = 278, df = 1, 3, F =48.45, p = 0.006; Fig 1A). Similarly, mass-adjusted BMR was also higher in high-MMR mice than controls, but that difference was only broderline (n = 240, df = 1, 3, F = 9.07, p = 0.057; Fig. 1B).

GC-MS unambiguously identified 30 metabolites in gastrocnemius muscle, 42 metabolites in liver, and 34 metabolites in plantaris muscle. PLS-DA models showed some clustering of mice as a function of selection treatment (Figure 2). These models revealed metabolic alterations in high-MMR mice compared to control mice, but only the gastrocnemius muscle gastrocnemius muscle model performed well in terms of (i.e., R^2Y or the percentage of the samples in the training set successfully classified) and prediction (i.e., Q^2Y or the percentage of the test set correctly classified by using R^2Y) abilities. The gastrocnemius muscle model model explained 58.9% (R^2Y) and predicted 27.8% (Q^2Y) of the data based on the cross-validation (Fig. 2*A*). The gastrocnemius model also explained

38.1% of the metabolite signatures (R^2X). Similarly, the liver model explained 32.7% (R^2Y) and predicted 4.1% (Q^2Y) of the data based on the cross-validation (Fig. 2*B*). The liver model also explained 41.3% of the metabolite signatures (R^2X). In contrast, the plantaris muscle model explained 27.5% (R^2Y), and predicted only 0.9% (Q^2Y) of the data based on the cross-validation (Fig. 2*C*). The plantaris model also explained 50.5% of the metabolite signatures (R^2X). Different amounts of metabolites are responsible for the differences observed in the model suggesting obvious metabolic differences between controls and high-MMR in the gastrocnemius muscle in response to selection for increased MMR.

Significant changes in metabolomic profile were detected in the gastrocnemius muscle and liver of high-MMR mice. Of the 30 metabolites identified in the gastrocnemius muscle, we detected 5 metabolites that differed significantly between high-MMR and control mice (Fig. 3; Table S1). Three of these metabolites are involved in the energy and amino acid metabolism pathways (Fig. 4*A*) and the other two are bi-products of the amino acid metabolism pathway. Of the 34 metabolites identified in the plantaris muscle, none differed significantly between high-MMR and control mice. Of the 42 metabolites identified in the liver, we detected 3 metabolites that differed significantly between high-MMR and control mice (Fig. 3; Table S1). Two of these metabolites are involved in the energy metabolism pathways and the other one is involved in the amino acid metabolism pathway (Fig. 4*B*).

The metabolomic profile indicated that glucose concentration was elevated in high-MMR lines of mice but controls and high-MMR were not significantly different. To clarify whether the elevated resting metabolic rate of high-MMR mice was associated

with fatty acid metabolism, we profiled the free fatty acids (FFA) and triacylglycerol fatty acids (TGA) of the gastrocnemius muscle. High-MMR mice had significantly less total FFA in the gastrocnemius muscle than control mice (Fig. 5). In particular, the FFA concentration of palmitic acid (16:0) was markedly decreased. Although the statistical significance was borderline ($p = 0.06$) stearic acid (18:0) concentration was also lower in high-MMR lines of mice compared to control lines of mice. High-MMR lines of mice had lower TGA concentrations in the gastrocnemius muscle (Fig. 6). Notably, TA concentrations of stearic acid, eicosadienoic acid (20:2 n-6), dihomo-gamma-linolenic acid (20:3 n-6), and docosahexaenoic acid (22:6 n-3) were decreased.

Discussion

Selection for high mass-independent MMR led to a significant increase in mass-adjusted MMR and a borderline significant ($p = 0.057$) increase in mass-adjusted BMR. Most importantly, metabolomic changes resulting from selection for high MMR were detected in the gastrocnemius muscle and liver. The overall pattern for high-MMR mice was increased energy and amino acid metabolism in the gastrocnemius muscle. Specifically, elevated TCA cycle intermediates suggest that selection for increased MMR elevated aerobic metabolism in the gastrocnemius muscle to meet the increased metabolic rates. Most TCA cycle intermediates were elevated in mice selected for high MMR compared to controls, but not all of the differences were statistically significant (Fig. 7*A*). Besides the elevated TCA cycle intermediates, many amino acids or by products of amino acid metabolism, such as creatinine derivatives, were elevated in mice selected for

high MMR compared to controls, but not all of the differences were statistically significant (Fig. 7*A*). The functional relevance of the elevated amino acid metabolism in the gastrocnemius muscle is not entirely clear, but one possible explanation is that dietary proteins are being used as an energy substrate (Cherel & Le Maho 1988; Lumeij & Remple 1991; Landys et al. 2005). Another possible explanation is that the elevated amino acid metabolism maintains the TCA cycle intermediates needed for fatty acid metabolism (Veiga et al. 1978; Dohm1986; Bauchinger & Biebach 2001).

We were only able to detect a relatively small number of statistically significant metabolite changes. We suspect that there are more biologically significant changes in the metabolites of high-MMR mice than we could statistically detect. While our artificial selection experiment offers significant advantages, one of its limitations is that selection experiment often has relatively low statistical power. The reason for this is that in selection experiments the unit of replication (i.e., the experimental unit) is line (i.e., populations) of mice not individual mice (Henderson 1989). For example, while we analyzed the metabolites of 80 mice, our effective sample size is 8 (4 line of high-MMR mice and 4 lines of control mice) and given our experimental design the error degrees of freedom for comparisons across treatments is only 3. One non-significant elevated amino acid detected in the gastrocnemius muscle was β-alanine. Elevated levels of β-alanine appear to influence maximal (sprint and endurance performance) metabolic functions. That is, β-alanine is known to increase sprint performance in humans (Derave et al. 2007; Hill et al. 2007; Thienen et al. 2009). In addition, because fatty acid oxidation is hampered by low pH levels in skeletal muscles (Derave et al. 2007; Hill et al. 2007), β-alanine, when converted to carnosine, can have a buffering effect on skeletal muscle

during aerobic metabolism of fatty acids during endurance performance (Hill et al. 2007; Baguet et al. 2010). In contrast to the gastrocnemius muscle, we detected decreased levels of amino acids in the liver.

The overall pattern for high-MMR mice was decreased energy and amino acid metabolism in the liver. That is, the decreased levels of TCA cycle metabolites suggest selection for increased MMR decreased aerobic metabolism in the liver. Most TCA cycle metabolites were decreased in mice selected for high MMR compared to controls but not all of the differences were statistically significant (Fig. 7*B*). The functional significance why there was an overall amino acid and energy metabolism decrease in the liver is not clear at his point, but it makes sense that we found elevated energy and amino acid metabolism in the gastrocnemius muscle and not the live. High MMR is the character we selected on and the liver is not thought to be strongly linked to MMR, while skeletal muscle is thought to be the main organ system contributing to MMR (Bishop 1990; Weibel et al. 2004; Weibel & Hoppeler 2005).

Our findings suggest that the metabolic composition of the gastrocnemius muscle reflects its being primed to meet the elevated metabolic capacity during MMR. Recall that high-MMR mice had a correlated increase in BMR, and that the metabolomic profiles of the high-MMR lines of mice were obtained during their inactive phase (i.e., RMR). So the metabolite changes could reflect responses caused by increased MMR, responses caused by BMR, or both. Our experimental design does not permit us to separate these effects but if the last of these is true, then perhaps skeletal muscles are the basis of the mechanistic connection (e.g., shared organs) linking MMR and BMR?

A recent inter-specific analysis of variation in muscle mass and BMR in mammals suggests that muscle mass explains more of the variation in BMR than previously thought (Raichlen et al. 2010). Perhaps variation in skeletal muscle along with variation in metabolomic profiles together can a potential mechanistic account for the correlation between MMR and BMR. As stated in the introduction, MMR and BMR are predictably correlated at the inter-specific level (Koteja 1987; Hayes & Garland 1995; Bozinovic 1992; Dutenhoffer & Swanson 1996; Rezende et al. 2002), but basis for a possible mechanistic connection between MMR and BMR remains unclear. If our metabolomic and fatty acid profiles suggest that the musculature might be an important contributor to BMR besides the visceral organs (Daan et al. 1990; Konarzewski & Diamond 1995; Even et al. 2001; Selman et al. 2001), then our findings may provide an explanation why (a) the musculature has a strong influence on BMR (Schmidt-Nielsen 1984), (b) skeletal muscle metabolism in rats accounts for nearly 50% of total tissue metabolism (Field et al. 1939), (c) muscle metabolism in humans accounts for 20% of the oxygen consumption at rest (Rolf & Brown 1997), (d) muscle mass is correlated with BMR in birds (McNab 1994; McNab & Ellis 2006), or (e) muscle mass appears to explain variation in BMR in mammals (McNab 1978; 2000; 2007; Raichlen et al. 2010) . As far as we are aware, this is the first functional approach to examine the correlation between MMR and BMR. The elevated metabolism in muscles provides a key explanation for the mechanistic connection of the correlation between MMR and BMR.

Our findings suggest that high-MMR lines of mice are utilizing fatty acids besides carbohydrates to meet their elevated metabolic capacity. Changes in the FFA and muscle TGA composition are generally linked to exercise training and to dietary fat intake

(Andersson et al. 2002; Szabo et al. 2002; Petridou et al. 2005; Dimopoulos et al. 2006; Ehrenborg & Krook 2009). Our mice were not exercised and standard laboratory rat chow was available *ad libitum*. Hence, selection for high-MMR in our lines of mice altered skeletal muscle FFA and TGA composition.

The correlated response of FFA and TGA to selection for high-MMR indicates that there is a genetic component to muscle FFA and TGA composition. Interestingly, FFA levels and TGA composition have a central role in determining membrane properties and cell signaling, and they are ligands that bind to the peroxisome proliferator-activated receptors (PPAR) α and γ in skeletal muscle and other tissues, such as liver (Borkman et al. 1993; Ehrenborg & Krook 2009). PPARs are nuclear receptor proteins that function as transcription factors regulating expression of genes for fatty acid catabolism (Ehrenborg & Krook 2009). Hence, FFA amounts and TGA composition affect skeletal muscle lipid metabolism.

Conclusions

In this study, we explored the metabolites of gastrocnemius and plantaris muscles, and of liver in inactive mice that have been selected for increased MMR. Metabolomic changes indicated elevated amino acids and aerobic metabolism in the gastrocnemius muscle, but not in the liver or plantaris muscle. Notably, FFA and TGA are also sources of fuel to meet this increase in metabolic rate. Our results also demonstrated that in addition to the effects of diet and exercise in determining intra-muscle fatty acid composition genetic factors also have a role.

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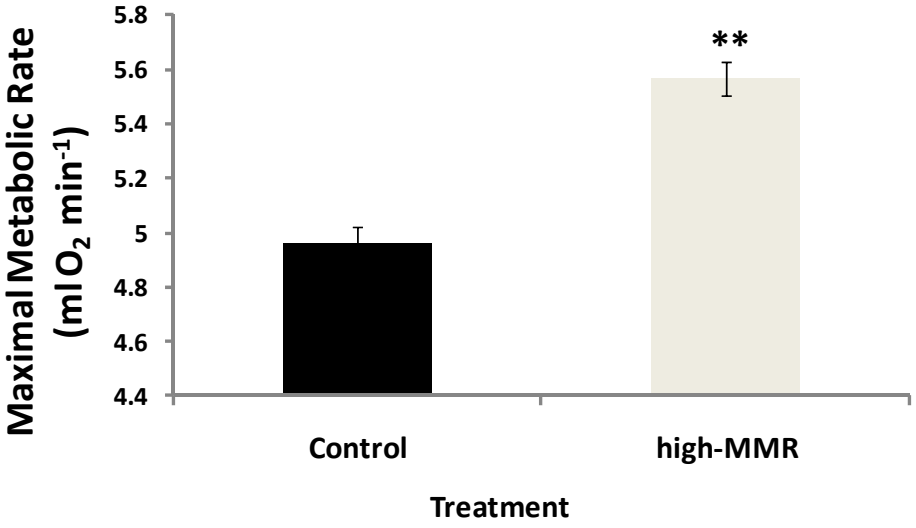
Acknowledgments

The study was funded by NSF IOS 0344994 to Jack P. Hayes and UNR Graduate Student Association Research Grant to B. Wone. We thank Beate Wone for help with the tissue extractions. We thank John Cushman and Dave Shintani for the use of their lab facilities. We thank Angelica Adrian, Cynthia Downs, Amber Huleva, Andy Hicks, Else Huerta, Marta Labocha, Kim Mclean, Mike Sears, and Alexandra Watson for help with the selection experiment. The UNR Proteomics Center acknowledges support from NIH Grant P20 RR-016464 from the INBRE Program of the National Center for Research Resources.

a.



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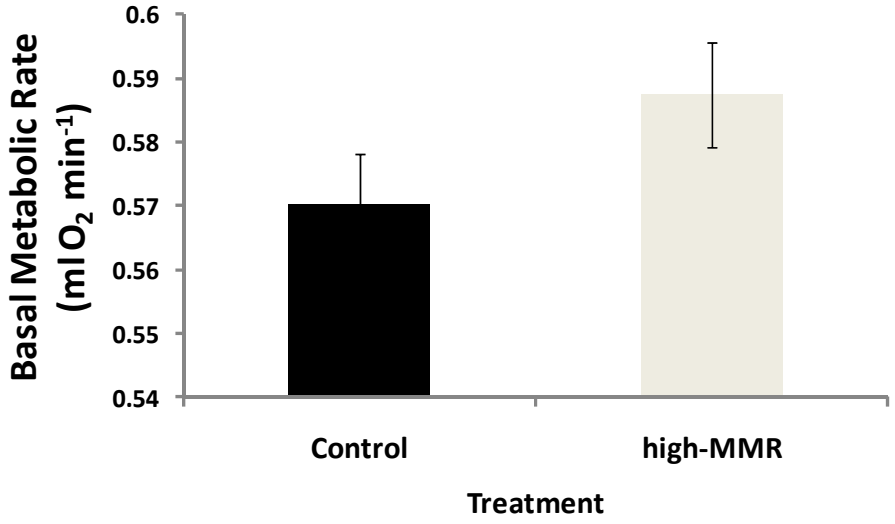
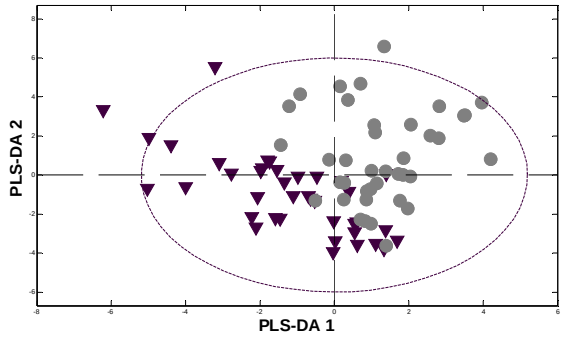


Figure 1. a. Response to selection for high maximal metabolic rate (MMR) at generation 7 (**Significantly different from control; n = 278, df = 1, 3, F =48.45, p = 0.006). b. Correlated response of mass independent basal metabolite rate (BMR) to selection for high MMR at generation 7 (n = 240, df = 1, 3, F = 9.07, p = 0.057).

A



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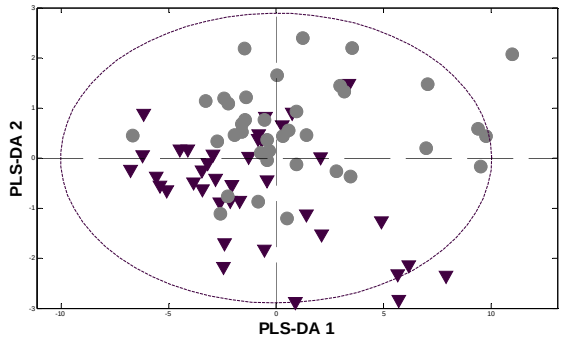

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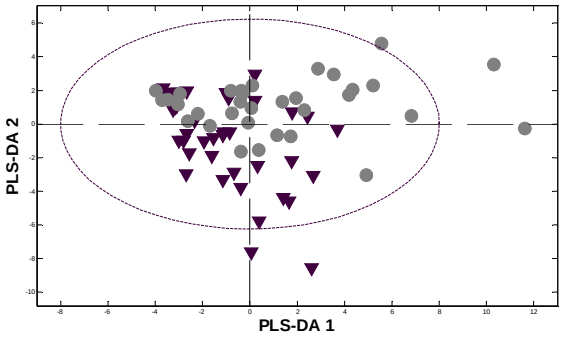

Figure 2. Global metabolite comparison of tissues sampled. The figures show the lines of control mice as one group (filled circles) and the lines of high-MMR mice another group (filled upside-down triangles) to make the data easier to visualize. (A) Gastrocnmeius muscle PLS-DA model is constructed from 30 metabolites. (B) Liver PLS-DA model is constructed from 42 metabolites. (C) Plantaris muscle PLS-

DA model is constructed from 34 metabolites. Ellipse represents the 95% confidence interval.

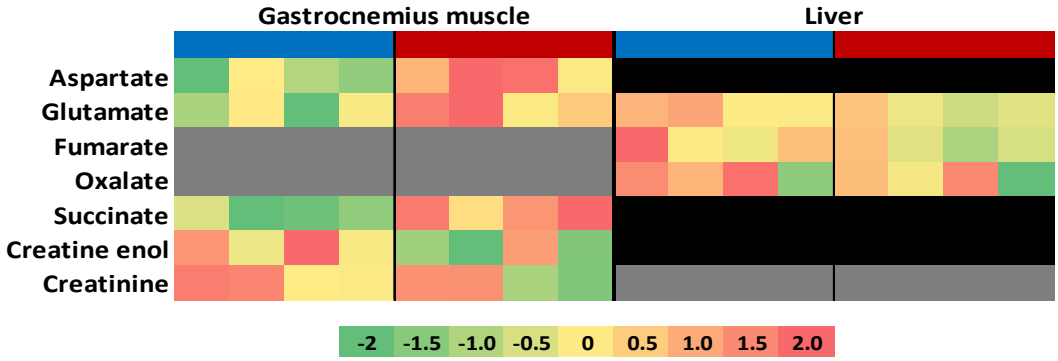

Figure 3. Heat map showing detected metabolite concentrations that were significantly different (p and q <0.05) between control and high-MMR lines of mice. Colors above the heat map denote lines of treatment: blue control, and rust high-MMR. Values presented are log2 fold change (vs. mean concentration of control lines of mice). Black color indicates metabolite levels were not significantly different between control and high-MMR lines of mice, while gray color indicates that metabolites are below detection level (not detected).

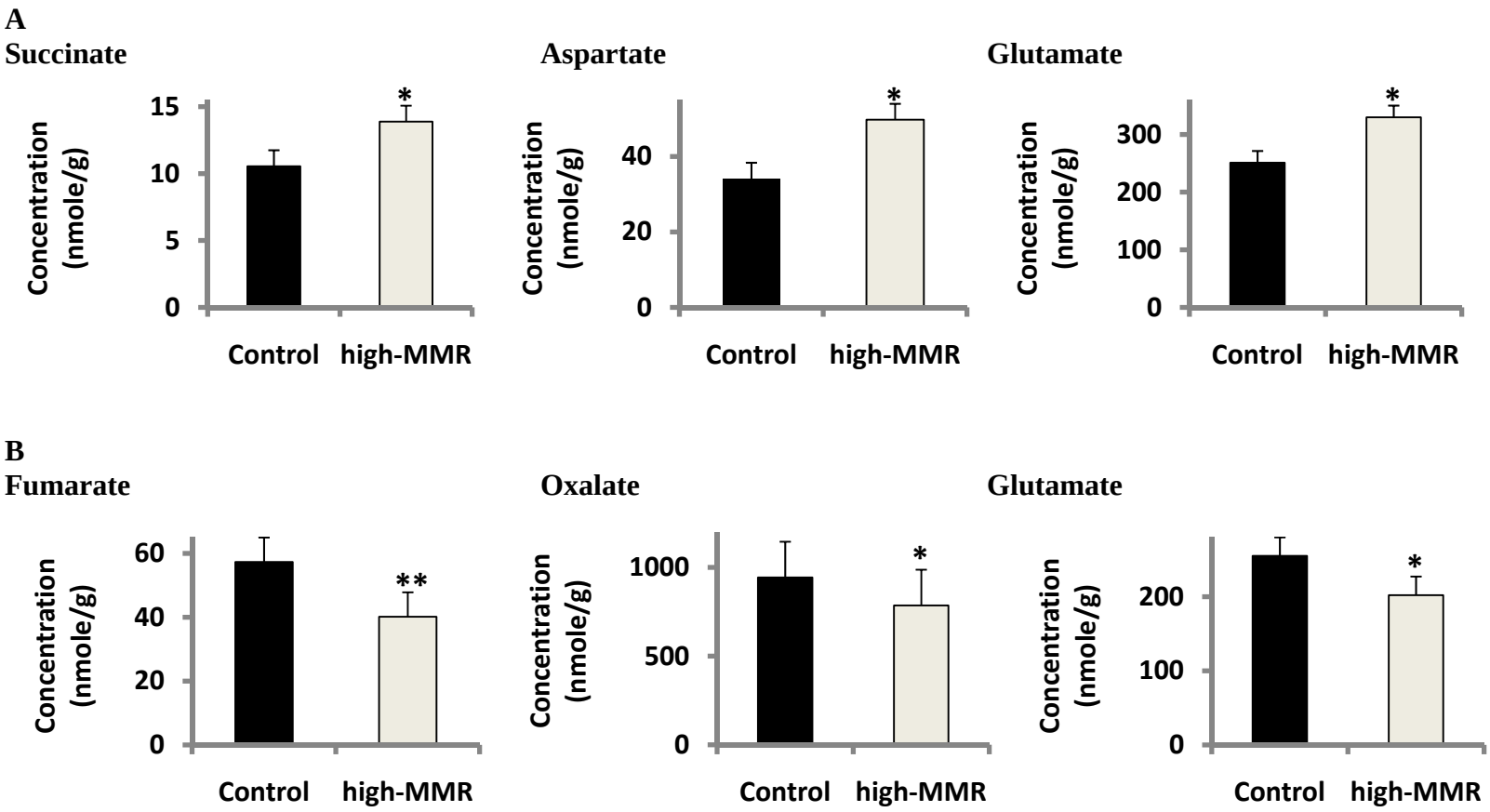

Figure 4. Significant changes in detected metabolites involved in the glycolysis/TCA cycle and amino acids metabolism; (*A*) gastrocnemius muscle, (*B*) liver. Values presented are mean (± sem) concentrations of metabolites detected. * p and $q < 0.05$. ** $p < 0.01$ and $q < 0.05$ compared to control. Filled bars control mice, open bars high-MMR mice.

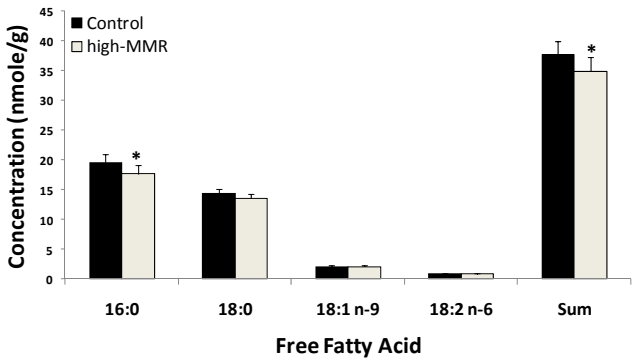

Figure 5. Free fatty acid profile of gastrocnmeius muscle. Values presented are mean (± sem) concentrations of free fatty acid detected; * p and $q < 0.05$ compared to control.

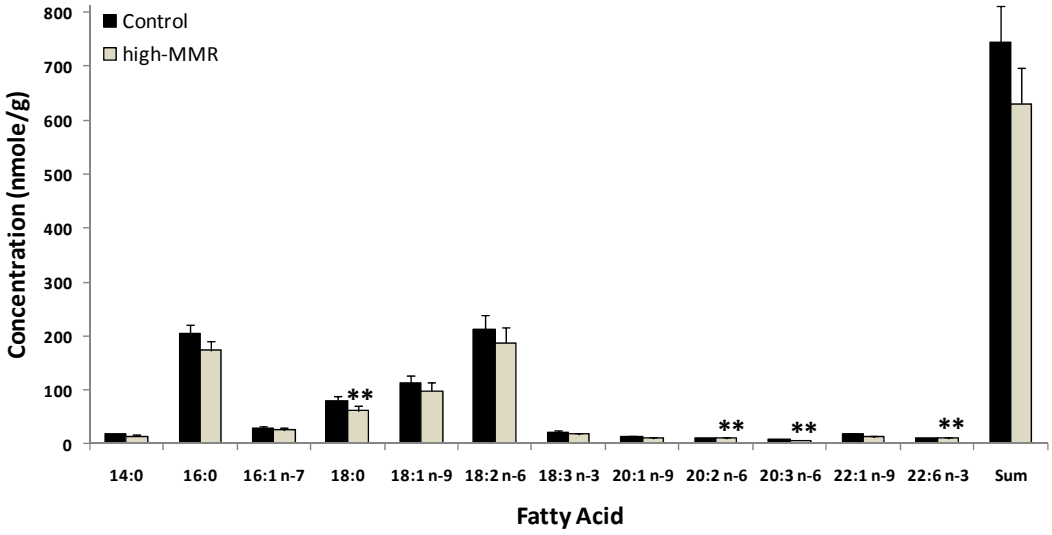

Figure 6. Triacylglyceride profile of gastrocnemius muscle. Values presented are mean (± sem) concentrations of triacylglycerides detected; * p and $q < 0.05$. ** $p < 0.01$ and $q < 0.05$ compared to control.

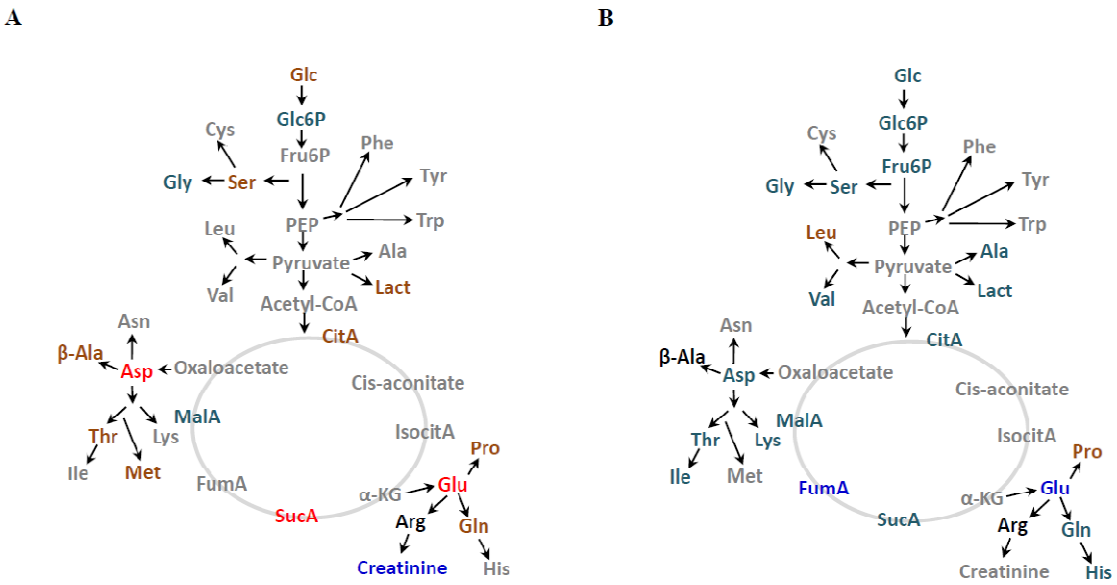

Figure 7. Differences of metabolites in glycolysis/TCA cycle and amino acids between control and high-MMR lines of mice; (A) gastrocnemius muscle; (B) liver. The metabolites in red indicate significantly higher levels in high-MMR, while the metabolites in blue indicate significantly lower levels in high-MMR. The metabolites in rust indicate non-significant higher levels in high-MMR, while the metabolites in aqua indicate non-significant lower levels in high-MMR. The metabolites in black indicate that there is no difference between control and high-MMR, while the metabolites in gray indicate that they are below detection level (not detected).

Chapter 5: Summaries, conclusions, and recommendations

The work contained herein provided quantitative genetic parameter estimates on MMR and BMR, tested the membrane pacemaker hypothesis of metabolism with a manipulative experiment using whole animals, and examined the biochemical variation between resting metabolism and increased MMR. In chapter 2, I found significant additive genetic variance for BMR and MMR at both the whole-animal and mass-independent levels, and a strong positive genetic correlation between mass-independent BMR and MMR. This latter result supports the possibility that this correlation might be a pervasive or nearly pervasive feature of the design of tetrapods, but my analyses suggest that the correlation does not constitute an absolute genetic constraint and that these traits are capable of independent evolution to some degree. A remaining challenge is establishing the functional connection between BMR and MMR and what accounts for their correlation, because the main contributors to BMR are the liver and kidney while the main contributor to MMR is the musculature (Weibel *et al.* 2004).

In chapter 3, I found that selection for increased MMR resulted in correlated responses in FA composition of cell membranes from the liver and gastrocnemius muscle of mice. No difference in the liver UI was detected despite an elevated mass-independent BMR in our high-MMR mice. This implies that the elevated mass-independent BMR is not linked to the correlated changes in the FA profile of liver membranes, and thus does not support the prediction of the membrane pacemaker hypothesis. My results suggest that the individual variation in BMR is linked to the individual variation in the FA profile of muscle membranes.

These results are important for at least two reasons. First, to my knowledge this is the second manipulative test of the membrane pacemaker model of metabolism and the results were consistent with two other intra-specific comparison studies (Brzęk et al. 2007; Haggerty et al. 2008). It's entirely likely that the model applies only to inters-specific comparisons (i.e., ectotherm vs. endotherm) and not to intra-specific comparisons (Haggerty et al. 2008). Indeed, because metabolic rates of ectotherms are vastly different compared to that of endotherms. Second, my results indicate that fatty acid composition of membrane phospholipids is more important in explaining variation in BMR intra-specifically than the unsaturation index. Because fatty acids in membranes have a central role in determining membrane properties, cell signaling, and gene expression in the skeletal muscle and other tissues such as liver (Kogteva and Bezugov 1998; Ehrenborg and Krook 2009), the type of fatty acids might have important effects on tissue metabolism at the individual level. Hence, those fatty acids that have an influence on either carbohydrate or lipid metabolism are expected to change when we alter the metabolic rate of individuals.

In chapter 4, I explored the metabolites of gastrocnemius and plantaris muscles, and liver during RMR in mice selected for increased MMR. This led to the detection of elevated amino acids, and aerobic metabolism (as indicated by metabolite changes) in the gastrocnemius muscle, but not the liver or plantaris muscle. Lastly, my metabolomic and fatty acid profile results provide a potential mechanistic account for the correlation between MMR and BMR. To my knowledge, this is the first functional approach to

understanding this correlation. The underlying mechanism seems to include elevated amino acid and energy metabolism in the muscles.

In chapter 4, I explored only a small set of metabolites given the analytical platform used (i.e., GC-MS). For a more complete analysis of the metabolome, I would need to incorporate liquid chromatography mass spectrometry (LC-MS) as well. The combined approach provides a comprehensive metabolome for the organism in question (Melvin et al. in press). In addition, to clarify whether metabolite changes reflected responses caused by increased MMR, responses caused by BMR, or both, quantitative knowledge of metabolite fluxes is required. Hence, fluxome analysis is the next step to comprehensively characterize our high-MMR mouse phenotype (Cascante and Marin 2008).

This research provided quantitative genetic parameter estimates on MMR and BMR, tested the membrane pacemaker hypothesis of metabolism with a manipulative experiment using whole animals, and examined the biochemical variation between resting metabolism and increased MMR. Overall, the estimated genetic correlation between MMR and BMR is consistent with the assumption of the aerobic capacity model. In addition, the metabolic and fatty acid profiles suggest that increased MMR and BMR in high-MMR mice might be mechanistically linked via elevated amino acid and energy metabolism in the musculature. Lastly, my results add a genetic component to the already demonstrated roles of diet and exercise in determining membrane and intra-muscle fatty acid composition.

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Appendix 1: Supporting materials for Chapters 2 & 4

Chapter 2 Supporting Materials

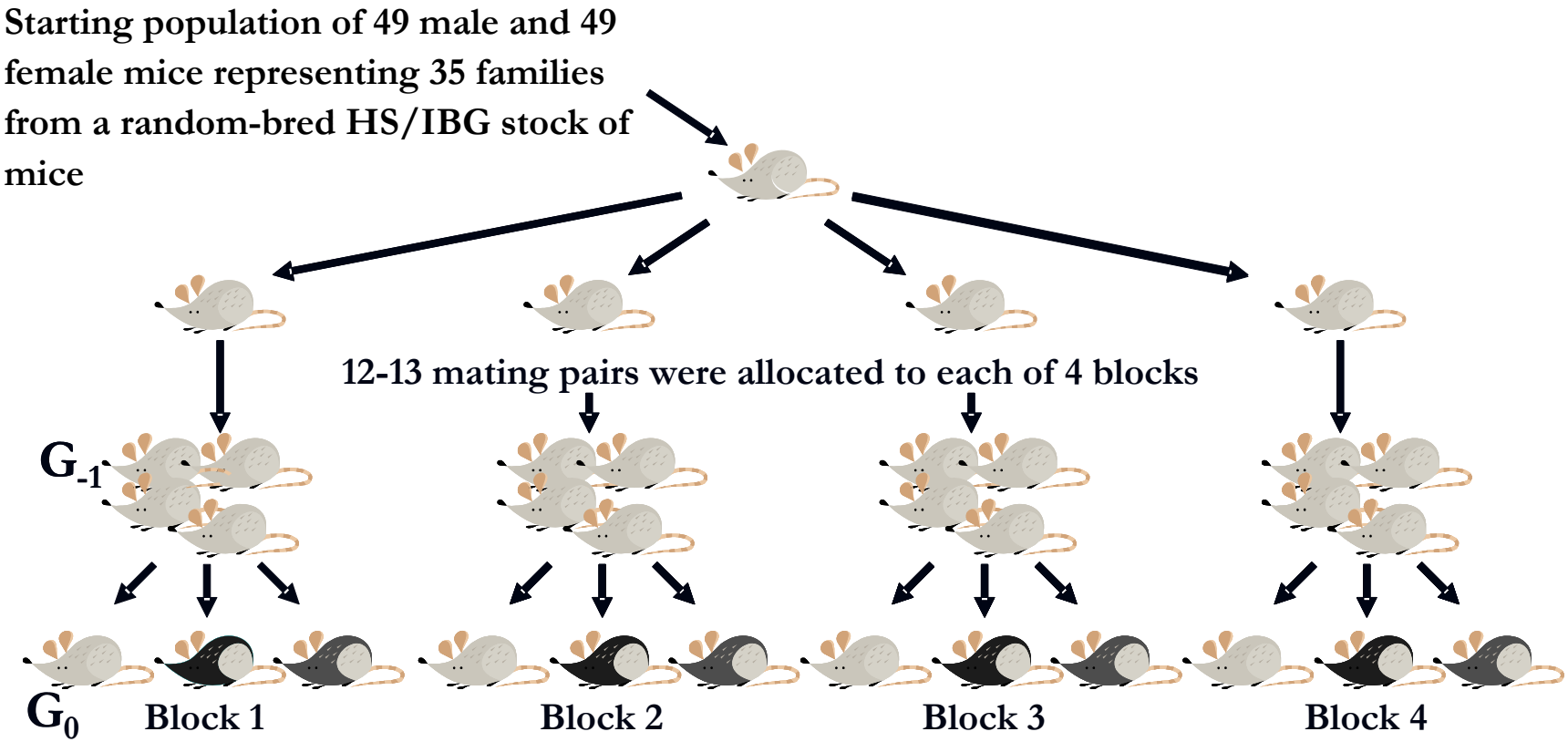

Figure S1. Breeding strategy used to test the aerobic capacity model. Four breeding blocks were created to accommodate the time consuming physiological measurements. The initial group of mice (from all 4 blocks) represented G_{-1}, where G stands for generation. Mice produced from these initial breeding pairs produced mice for G_0. In each block of G_0, mice were allocated to three treatments: control, increased maximal metabolic rate, and correlational selection. The breeding and measurement of blocks were separated in time by approximately 4 wks. All mice that comprised G_0 had MMR and BMR measured and were randomly assigned to treatment groups. All offspring that comprised G_1 also had MMR and BMR measured and selection was first imposed on mice in G_1. So the

second generation (G_2) was the first generation in which the offspring resulted from parents who were artificially selected for their metabolic rates.

Table S1. Fixed and random effects included in the univariate animal models and their associated *p*-values as estimated by ASReml 2.0. See materials and methods for description of the fixed and random effects.

Model	Fixed Effects	*P*-value	Random Effects	*P*-value
body mass at BMR	generation	NS	block	<0.001
	sex	<0.001	block x generation	NS
	age at BMR	<0.001	animal (additive genetic)	<0.001
			natal cage (common environment)	<0.001
body mass at MMR	generation	NS	block	<0.001
	sex	<0.001	block x generation	NS
	age at MMR	<0.001	animal (additive genetic)	<0.001
			natal cage (common environment)	<0.001
whole-animal BMR	generation	NS	block	<0.001
	sex	<0.001	block x generation	NS
	age at BMR	<0.001	animal (additive genetic)	<0.001
	BMR chamber	NS	natal cage (common environment)	<0.001
whole-animal MMR	generation	<0.001	block	<0.001
	sex	<0.001	block x generation	<0.001
	age at MMR	<0.001	animal (additive genetic)	<0.001
	observer	NS	natal cage (common environment)	<0.001
	treadmill	<0.001		
	stimulator	NS		
mass-independent BMR	generation	NS	block	<0.001
	sex	<0.001	block x generation	NS
	mass at BMR	<0.001	animal (additive genetic)	<0.001
	age at BMR	NS	natal cage (common environment)	<0.001
	BMR chamber	NS		
mass-independent MMR	generation	<0.001	block	<0.001
	sex	NS	block x generation	<0.001
	mass at MMR	<0.001	animal (additive genetic)	<0.001
	age at MMR	NS	natal cage (common environment)	<0.001

observer	<0.001
treadmill	<0.001
stimulator	<0.001

Table S2. Descriptive statistics for body mass and whole-animal physiological traits (i.e., BMR or MMR) obtained from Generation 0 and 1 and Control lines (G_2-G_6) of mice, where G_0 and G_1 are the base population.

	Traits						
	BMR (ml O_2/min)			Body mass (g)	MMR (ml O_2/min)			Body mass (g)
	N	Mean (SD)	Min/Max	Mean (SD)	*N*	Mean (SD)	Min/Max	Mean (SD)
G_0								
male	108	0.63 (0.11)	0.31/0.89	23.4 (2.78)	113	4.97 (0.63)	3.27/6.40	26.3 (2.91)
female	107	0.54 (0.94)	0.35/0.77	19.1 (2.38)	110	4.11 (0.52)	3.01/6.11	21.2 (2.32)
G_1								
male	238	0.68 (0.11)	0.42/0.92	24.6 (2.74)	331	4.85 (0.57)	3.06/6.44	26.1 (2.60)
female	239	0.60 (0.10)	0.30/0.91	19.7 (2.06)	340	4.02 (0.52)	2.74/5.45	21.1 (2.17)
Control (G_2)								
male	61	0.64 (0.10)	0.42/0.93	24.2 (2.73)	64	5.40 (0.57)	4.12/6.67	26.3 (2.76)
female	62	0.53 (0.09)	0.36/0.77	19.6 (1.80)	65	4.47 (0.53)	3.21/6.11	21.4 (2.06)
Control (G_3)								
male	67	0.68 (0.11)	0.47/0.95	24.1 (1.86)	66	5.51 (0.41)	4.59/6.46	26.6 (1.90)
female	57	0.57 (0.10)	0.30/0.73	19.4 (1.62)	59	4.57 (0.42)	3.35/5.41	21.4 (1.74)
Control (G_4)								
male	60	0.62 (0.07)	0.42/0.81	24.2 (2.22)	60	5.72 (0.49)	4.78/6.87	27.2 (2.28)
female	68	0.53 (0.07)	0.36/0.68	19.7 (1.98)	68	4.64 (0.50)	3.50/6.04	21.9 (2.30)
Control (G_5)								
male	66	0.60 (0.07)	0.48/0.81	23.2 (2.18)	66	5.25 (0.55)	3.71/6.53	25.8 (2.35)
female	71	0.54 (0.09)	0.35/0.77	19.4 (2.17)	72	4.57 (0.46)	3.58/5.61	21.7 (2.27)
Control (G_6)								
male	86	0.60 (0.07)	0.41/0.85	23.6 (2.10)	84	5.36 (0.57)	4.20/6.66	26.3 (2.46)
female	87	0.53 (0.07)	0.33/0.71	19.3 (2.11)	87	4.52 (0.53)	3.05/6.09	21.2 (2.21)

Chapter 4 Supporting Materials

Table S1. Metabolomic and fatty acid analyses of muscle (gastrocnemius and plantaris) and liver tissues from mice selected for increased maximal metabolic rate (high-MMR) by GC-MS.

	KEGG metabolism pathway	Name	Fold change High-MMR/Control	*p* value	*q* value
Metabolites					
Gastrocnemius muscle					
	Amino acid	acetylglutamate	1.26	0.28	0.08
	Amino acid	arginine	0.97	0.78	0.09
	Amino acid	aspartate	1.46	0.03	0.04
	Amino acid	creatine enol	0.70	0.03	0.04
	Amino acid	creatinine	0.88	0.02	0.04
	Amino acid	glutamate	1.29	0.03	0.04
	Amino acid	glutamine	1.12	0.57	0.09
	Amino acid	glycine	0.90	0.38	0.08
	Amino acid	methioine	1.04	0.69	0.09
	Amino acid	serine	1.11	0.42	0.08
	Amino acid	threonine	1.27	0.13	0.07
	Amino acid	trans-4-hydroxyproline	1.09	0.64	0.09
	Amino acid	tryptamine	0.97	0.58	0.09
	Amino acid	urea	1.26	0.34	0.08
	Amino acid	β alanine	1.74	0.23	0.07
	Carbohydrate	ascorbate	1.06	0.66	0.09
	Carbohydrate	galactose-6-phosphate	1.03	0.77	0.09
	Carbohydrate	glucose-6-phosphate (G6P)	0.92	0.71	0.09
	Carbohydrate	lactate	1.23	0.11	0.06
	Carbohydrate	trehalose	1.69	0.09	0.05
	Energy	glucose	1.12	0.46	0.08
	Energy	malate	0.78	0.11	0.06
	Energy	phosphate	0.82	0.26	0.07
	Energy	pyrophosphate	1.14	0.43	0.08

	Energy	succinate	1.32	0.01	0.03
	Lipid	cholesterol	1.05	0.81	0.09
	Lipid	glycero phosphate	0.97	0.73	0.09
	Lipid	glycerol 3-phosphate (G3P)	1.09	0.22	0.07
	Lipid	myo inositol	1.16	0.31	0.08
	Nucleotide	adenosine 5'-monophosphate (AMP)	1.12	0.38	0.08
Metabolites					
Liver					
	Amino acid	alanine	0.84	0.23	0.07
	Amino acid	arginine	0.99	0.89	0.10
	Amino acid	asparagine	0.72	0.16	0.07
	Amino acid	aspartate	0.91	0.31	0.08
	Amino acid	creatine enol	0.71	0.17	0.07
	Amino acid	glutamate	0.79	0.05	0.05
	Amino acid	glutamine	0.79	0.16	0.07
	Amino acid	glycine	0.91	0.21	0.07
	Amino acid	histidine	0.89	0.33	0.08
	Amino acid	isoleucine	0.92	0.08	0.05
	Amino acid	leucine	1.04	0.68	0.09
	Amino acid	lysine	0.92	0.09	0.05
	Amino acid	ornithine	0.83	0.33	0.08
	Amino acid	proline	1.25	0.44	0.08
	Amino acid	pyroglutamate	0.85	0.24	0.07
	Amino acid	serine	0.76	0.09	0.05
	Amino acid	threonine	0.88	0.21	0.07
	Amino acid	tyrosine	0.80	0.43	0.08
	Amino acid	urea	0.92	0.16	0.07
	Amino acid	valine	0.91	0.20	0.07
	Amino acid	β alanine	0.97	0.80	0.09
	Carbohydrate	ascorbate	0.79	0.57	0.09

	Carbohydrate	erythrose	0.87	0.68	0.09
	Carbohydrate	fructose-6-phosphate	0.90	0.59	0.09
	Carbohydrate	gluconate	0.81	0.09	0.05
	Carbohydrate	glucopyranose	0.88	0.26	0.07
	Carbohydrate	glucose	0.86	0.28	0.08
	Carbohydrate	glucose-6-phosphate (G6P)	0.83	0.14	0.07
	Carbohydrate	lactate	0.87	0.15	0.07
	Carbohydrate	oxalate	0.83	0.02	0.04
	Carbohydrate	trehalose	1.30	0.50	0.08
	Carbohydrate	α glucose	0.80	0.20	0.07
	Energy	citrate	0.89	0.59	0.09
	Energy	fumarate	0.70	0.007	0.03
	Energy	malate	0.87	0.36	0.08
	Energy	phosphate	0.81	0.09	0.05
	Energy	succinate	0.63	0.20	0.07
	Lipid	glycerol 3-phosphate (G3P)	0.86	0.36	0.08
	Lipid	myo inositol	0.87	0.32	0.08
	Nucleotide	adenosine 5'-monophosphate (AMP)	0.89	0.32	0.08
	Nucleotide	allantoin	0.77	0.17	0.07
	Nucleotide	uric acid	0.70	0.10	0.06
Metabolites					
Plantaris muscle					
	Amino acid	alanine	0.56	0.42	0.08
	Amino acid	arginine	0.75	0.50	0.08
	Amino acid	aspartate	0.64	0.38	0.08
	Amino acid	creatine enol	0.62	0.36	0.08
	Amino acid	creatinine	0.81	0.68	0.09
	Amino acid	glutamate	0.81	0.62	0.09
	Amino acid	glutamine	0.60	0.45	0.08
	Amino acid	glycine	0.60	0.41	0.08

	Amino acid	lysine	0.85	0.70	0.09
	Amino acid	serine	0.71	0.46	0.08
	Amino acid	threonine	0.65	0.46	0.08
	Amino acid	trans-4-hydroxyproline	0.55	0.42	0.08
	Amino acid	tyrosine	0.76	0.51	0.08
	Amino acid	urea	0.80	0.56	0.09
	Amino acid	valine	0.69	0.47	0.08
	Amino acid	β alanine	0.89	0.69	0.09
	Carbohydrate	fructose-6-phosphate	0.66	0.21	0.07
	Carbohydrate	glucopyranose	0.74	0.59	0.09
	Carbohydrate	glucose	1.09	0.78	0.09
	Carbohydrate	glucose-6-phosphate (G6P) Gly2P	1.12	0.77	0.09
	Carbohydrate	glyceric acid	0.77	0.65	0.09
	Carbohydrate	lactate	0.45	0.26	0.07
	Carbohydrate	oxalate	0.67	0.57	0.09
	Cofactors	gulono-1,4-lactone	0.60	0.33	0.08
	Energy	carbamate	0.80	0.63	0.09
	Energy	citrate	0.86	0.19	0.07
	Energy	fumarate	0.58	0.38	0.08
	Energy	malate	0.66	0.37	0.08
	Energy	phosphate	0.60	0.32	0.08
	Energy	succinate	0.64	0.32	0.08
	Lipid	glycerol 3-phosphate (G3P)	0.71	0.42	0.08
	Lipid	myo inositol	0.86	0.73	0.09
	Nucleotide	adenosine 5'-monophosphate (AMP)	0.83	0.69	0.09
	Nucleotide	allantoin	0.76	0.63	0.09
Free Fatty Acids Gastrocnemius muscle					
	Lipid	16:0	0.90	0.02	0.04

	Lipid	18:0	0.94	0.06	0.05
	Lipid	18:1 n-9	1.01	0.71	0.09
	Lipid	18:2 n-6	0.97	0.71	0.09
	Lipid	Cholesterol	1.14	0.06	0.05
		Sum	0.93	0.03	0.04
	Lipid	Elongase	1.04	0.06	0.05
	Lipid	Δ^9-desaturase	1.04	0.32	0.08
Triacyglycerol Fatty Acids Gastrocnemius muscle	Lipid	14:0	0.77	0.19	0.07
	Lipid	16:0	0.84	0.22	0.07
	Lipid	16:1 n-7	0.89	0.73	0.09
	Lipid	18:0	0.76	0.007	0.03
	Lipid	18:1 n-9	0.88	0.65	0.09
	Lipid	18:2 n-6	0.89	0.66	0.09
	Lipid	18:3 n-3	0.79	0.39	0.08
	Lipid	201 n-9	0.83	0.09	0.05
	Lipid	20:2 n-6	0.84	0.007	0.03
	Lipid	20:3 n-6	0.86	0.006	0.03
	Lipid	22:1 n-9	0.70	0.11	0.06
	Lipid	22:6 n-3	0.90	0.01	0.03
		Sum	0.85	0.37	0.08
	Lipid	Elongase	0.91	0.40	0.08
	Lipid	Δ^9-desaturase	1.12	0.62	0.09