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Endurance capacity of mice selectively bred for high voluntary wheel running

Thomas H. Meek, Brian P. Lonquich, Robert M. Hannon and Theodore Garland, Jr*
University of California Riverside, Riverside, CA 92521, USA
*Author for correspondence (tgarland@ucr.edu)

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SUMMARY

Mice from four lines bred for high voluntary wheel activity run ~3-fold more revolutions per day and have elevated maximal oxygen consumption during forced treadmill exercise, as compared with four unselected control (C) lines. We hypothesized that these high runner (HR) lines would have greater treadmill endurance-running capacity. Ninety-six mice from generation 49 were familiarized with running on a motorized treadmill for 3 days. On days 4 and 5, mice were given an incremental speed test (starting at 20 m min^{-1}, increased 1.5 m min^{-1} every 2 min) and endurance was measured as the total time or distance run to exhaustion. Blood samples were taken to measure glucose and lactate concentrations at rest during the photophase, during peak nightly wheel running, and immediately following the second endurance test. Individual differences in endurance time were highly repeatable between days (r=0.79), and mice tended to run longer on the second day (paired t-test, P<0.0001). Blood glucose following the treadmill test was low for all animals (~53 mg dl^{-1}) and lactate was high (~6.5 mmol l^{-1}), suggesting that exhaustion occurred. The HR lines had significantly higher endurance than the C lines (1-tailed P<0.05), whether or not body mass was used as a covariate in the analysis. The relationship between line means for wheel running and treadmill endurance differed between the sexes, reinforcing previous studies that indicate sex-specific responses to selective breeding. HR mice appear to have a higher endurance capacity than reported in the literature for inbred strains of mice or transgenics intended to enhance endurance.

Key words: artificial selection, endurance, exhaustion, experimental evolution, genetics, glucose, lactate, locomotor activity, wheel running.

INTRODUCTION

Locomotion is vital for such activities as obtaining food, escaping from predators, and locating or courting mates (see Djawdan and Garland, 1988; Garland et al., 1988; Irschick and Garland, 2001; Irschick and Le Galliard, 2008; Le Galliard and Ferriere, 2008). One aspect of locomotion that is receiving increasing attention is the correlates or determinants of individual or interspecific variation in voluntary activity levels (e.g. Thorburn and Proietto, 2000; Miles et al., 2007). Both within and among species, variation in activity levels may reflect underlying differences in 'motivation' for being active as well as physical abilities to engage in locomotion of particular intensities or for certain durations. Beyond this, activity levels may be influenced by the external environment, including physical (e.g. temperature), ecological (e.g. food availability, predator abundance) and societal factors.

Studies examining the relationship between endurance capacity and voluntary activity level provide mixed results. For example, among species of lizards, daily movement distance measured in the field is positively correlated with treadmill endurance capacity (Garland, 1999). Within one population of the lizard *Lacerta vivipara*, individuals with a low endurance at birth tended to have reduced activity in the field, reduced growth rate and high parasite load, but experienced relatively low predation risk as assessed by tail loss, whereas individuals with high endurance had high activity and growth rates, low parasite load and a high incidence of broken tails (Clobert et al., 2000). Studies of individual variation in rats (Lambert et al., 1996) and mice (Friedman et al., 1992) found no statistically significant correlation between voluntary wheel running and treadmill endurance or maximal oxygen consumption ($\dot{V}_{O2max}$). Among six inbred mouse strains, Lerman and colleagues found a positive but statistically non-significant correlation between treadmill endurance and voluntary wheel-running speed (Lerman et al., 2002). Lightfoot and coworkers compared wheel running and treadmill endurance (Lightfoot et al., 2004; Lightfoot et al., 2001) of 10 inbred mouse strains and concluded that 'the two strain distribution patterns are not concordant and thus implicate different genetic contributions to these two phenotypes'. Conversely, two lines of rats differentially selected for high and low treadmill endurance show, respectively, high and low voluntary wheel running (Waters et al., 2008).

We have used an experimental evolution approach (e.g. Garland and Kelly, 2006; Garland and Rose, 2009) with laboratory house mice to study the correlated evolution of motivation and ability during selective breeding for high daily activity levels, as measured by voluntary wheel-running behaviour on days 5 and 6 of a 6 day period of wheel access (Swallow et al., 1998a). Individuals from four replicate high runner (HR) lines run approximately three times as many revolutions per day compared with those from four non-selected control (C) lines (e.g. Girard et al., 2001; Garland, 2003; Keeney et al., 2008; Gomes et al., 2009), and also exhibit elevated home-cage activity when housed without wheel access (Malisch et al., 2008; Malisch et al., 2009). Previous studies have shown behavioural and neurobiological differences between the HR and C lines that appear to indicate altered motivation for wheel running in the HR lines (e.g. Koteja et al., 1999b; Rhodes et al., 2003; Rhodes et al., 2005; Belke and Garland, 2007).

With respect to performance abilities, mice from the HR lines exhibit higher $\dot{V}_{O2max}$ when measured during forced treadmill exercise [males from generation 10, +6% on a mass-adjusted basis (Swallow et al., 1998b); males from generation 32, +18% (Rezende et al., 2006b); females from generation 36, +13% (Rezende et al., 2006a)] and differences in various sub-organismal traits potentially associated with exercise performance, including increased hindlimb bone symmetry and larger femoral heads (Garland and Freeman,

2005; Kelly et al., 2006), increased insulin-stimulated glucose uptake by isolated extensor digitorum longus muscle (Dumke et al., 2001), and more GLUT4 in the gastrocnemius after 5 days of wheel access (Gomes et al., 2009). In addition, blood haemoglobin concentration was a positive predictor of $\dot{V}_{O2max}$ in the HR lines, but a negative predictor in the C lines (Rezende et al., 2006c). However, direct measurements of endurance-running ability have not yet been performed.

The purpose of the present study was to test the hypothesis that HR mice have greater endurance than C mice in a graded-speed treadmill test (e.g. Lerman et al., 2002; Haubold et al., 2003). We also measured blood glucose and lactate concentrations at rest, during voluntary wheel running and at the end of the endurance test. We hypothesized that wheel running would increase blood lactate concentrations relative to those at rest, and that concentrations after the endurance test would be even higher. We also hypothesized that blood glucose concentrations would be decreased following the endurance trial compared with values at rest. In human beings, endurance-trained subjects exhibit lower lactate values after exhaustive exercise (Philp et al., 2005; Phillips, 2006). Even without training (wheel access or forced exercise), HR mice exhibit several changes similar to those observed in trained athletes (or 'athletic' species). Some of these changes include increased aerobic capacity (Swallow et al., 1998b; Rezende et al., 2006a; Rezende et al., 2006b) and increased insulin-stimulated glucose uptake in some hindlimb muscles (Dumke et al., 2001). Therefore, we further hypothesized that HR mice would show a more moderate increase in lactate concentration after the endurance test, compared with C mice. Both sexes were measured because sex differences have been demonstrated for wheel-running distance and speed (Swallow et al., 1998a; Koteja et al., 1999a; Koteja et al., 1999b; Houle-Leroy et al., 2000; Swallow et al., 2001; Garland et al., 2002; Garland, 2003; Malisch et al., 2009; Rezende et al., 2006b; Rezende et al., 2009), such exercise-relevant subordinate traits as citrate synthase and hexokinase activities in mixed hindlimb muscles (Houle-Leroy et al., 2000), the home-cage activity response to a high-fat diet (Vaanholt et al., 2008), and the wheel-running response to rimonabant, a selective endocannabinoid receptor antagonist (Keeney et al., 2008). Thus, motivation and/or ability for endurance exercise might also differ between the sexes.

A notable and unexpected feature of the selection experiment has been the increase in frequency in two of the four HR lines of a small-muscle phenotype, termed mini-muscle (Garland et al., 2002). This phenotype exhibits an approximately 50% reduction in hindlimb muscle mass. Pleiotropic effects of this Mendelian recessive allele include a doubling of mass-specific aerobic capacity and hexokinase activity (Houle-Leroy et al., 2003; Rezende et al., 2006c), alterations in muscle fibre-type composition (fewer type IIb fibres) (Guderley et al., 2006; Bilodeau et al., 2009) and contractile properties (Syme et al., 2005), increased myoglobin concentration and capillarity in medial gastrocnemius (Rezende et al., 2006c; Wong et al., 2009), increased glycogen concentration in gastrocnemius (Gomes et al., 2009), and increased heart ventricle mass (Garland et al., 2002; Swallow et al., 2005). Mini-muscle individuals exhibit elevated mass-adjusted $\dot{V}_{O2max}$ in hypoxia (Rezende et al., 2006a) (but not in normoxia or hyperoxia), run faster on wheels, and run more total revolutions per day under certain conditions (Kelly et al., 2006; Gomes et al., 2009). Although the underlying gene has not yet been identified, it is known to act as a Mendelian recessive and to lie in a 2.6335 Mb interval on chromosome MMU11 (Hannon et al., 2008; Hartmann et al., 2008). Consequently, given the various unique characteristics of mice with the mini-muscle phenotype, we also compared endurance and other traits of mini-muscle individuals with those of counterparts lacking the mini-muscle phenotype.

MATERIALS AND METHODS

Experimental animals

Mice were sampled from the 49th generation of an artificial selection experiment for high voluntary wheel running (Swallow et al., 1998a; Garland, 2003; Swallow et al., 2009). The original progenitors of the selection experiment were outbred Hsd:ICR house mice (*Mus domesticus*). After two generations of random mating, 10 pairs of mice were used to create each of eight closed lines. Four were bred for high running on wheels (HR lines) and four were bred without regard to how much they run, thus serving as a control (C lines). For each generation, when the mice reach 6–8 weeks of age they are housed individually in standard cages (27 cm×17 cm×12.5 cm) attached to Wahman-type activity wheels (1.12 m circumference, 35.7 cm diameter, 10 cm wide running surface: http://www.biology.ucr.edu/people/faculty/Garland/Mice_on_Running_Wheels_by_Ted_Garland.jpg). Wheels are interfaced to a computer and revolutions are recorded in 1 min intervals, continuously for 6 days; the selection criterion is the number of revolutions run on days 5 plus 6. Within each HR family, the highest-running male and female are chosen as breeders to produce the next generation. Within C families, a male and female are chosen at random. Sibling matings are disallowed in all lines.

Ninety-six mice from generation 49 (half male, half female) were weaned at 21 days of age, then housed randomly in same-sex groups of four per cage. As in the selection routine, all mice had water and food [Harlan Teklad Laboratory Rodent Diet (W)-8604, Los Angeles, CA, USA] available *ad libitum*. Room temperature was ~73°F (~23°C) and photoperiod was 12 h:12 h, with lights on at 07:00 h.

Measurement chronology, wheel testing and endurance protocol

At an average of 72 days of age (s.d.=8.5 days, min.=58 days, max.=86 days), mice were given wheel access for 6 days in accordance with the routine selection protocol. After downloading of wheel data at the end of day 6, mice were allowed to remain with wheel access. On the seventh night of wheel access, blood samples were taken 2 h after lights-off, which corresponds to peak wheel-running activity (Girard et al., 2001; Rhodes et al., 2003; Malisch et al., 2008). Over the following 3 days, while still allowed wheel access, mice were trained to run on a two-lane, motorized treadmill for 15 min day^{-1}. Treadmill speed for each training day was 10, 14 and 18 m min^{-1}, respectively. The treadmill was set to an incline of 25 deg., which has been shown to elicit maximum O_2 consumption in mice (Kemi et al., 2002) (see also Rezende et al., 2006a). The chamber in which the mouse ran was made of clear Plexiglas with dimensions 6.5 cm width, 12.5 cm height and 44 cm length. After the 3 day training period, on each of the following 2 days mice were tested using a graded exercise endurance test, following the protocol of Lerman et al. (Lerman et al., 2002) and Haubold et al. (Haubold et al., 2003). Starting belt speed was 20 m min^{-1} and speed was increased 1.5 m min^{-1} every 2 min. An air gun and an electrical grid with a mild current (adjustable amperage, 0–12 mA) were placed at the back of the treadmill to provide motivation. Mice were judged to be exhausted when they showed an inability to maintain workload and remained on the electrical grid for 4 s (Lerman et al., 2002; Haubold et al., 2003). For both trial days, values were excluded from statistical analyses

if mice were judged to be non-cooperative. Blood samples (see below) were taken after exhaustion on the second day (Djawdan, 1993). Five days after the second treadmill test, resting blood samples were obtained during the photophase.

Although mice are active primarily at night and circadian variation in performance is known to occur in some mammals (e.g. Colquhoun, 1981), for logistical reasons we tested endurance during the photophase. So far as we are aware, possible circadian variation in the running endurance of laboratory mice has not been studied. However, in a study of maximal sprint speeds of several species of wild rodents, Djawdan and Garland [see their p. 766 (Djawdan and Garland, 1988)] stated, 'Preliminary tests indicated that animals tested at night were not significantly faster than those tested during the day...'.

Blood sampling, glucose, lactate, and organ masses

Blood samples (one 70 µl microcapillary tube) were collected as rapidly as possible from the submandibular vein using Goldenrod animal lancets (Medipoint, Mineola, NY, USA). These were immediately subsampled with 20 µl tubes for determination of glucose and lactate concentrations from whole blood in duplicate. During the final (resting) blood sample, we took an additional 70 µl sample to determine haematocrit after centrifugation.

Glucose was measured with an Ascensia Contour portable glucose monitor (Bayer, Mishawaka, IN, USA) and test strips (7098B). This instrument displays the glucose concentration ~5 s after the blood is applied to the test strip. Lactate was measured using an Accutrend/Accusport Lactate Portable Analyzer and Bm-Lactate strips (Roche Diagnostics Gmbh, Mannheim, Germany) (see Bishop, 2001). This instrument displays the lactate concentration ~60 s after the blood is applied to the test strip.

Immediately after collection of the resting blood samples (see above), mice were killed by CO_2 inhalation and the heart ventricles, lungs, liver, spleen and both triceps surae muscles (Garland et al., 2002) were dissected and weighed. All organs were then placed in a drying oven at 55°F (~13°C) for 4 days to achieve constant weight, then re-weighed.

Statistical analyses

Analyses were performed using the Mixed Procedure in SAS 9.1.3 (SAS Institute, Cary, NC, USA) to apply analysis of covariance (ANCOVA) models with Type III tests of fixed effects. Sex, mini-muscle status and line type (HR or C) were fixed factors; line was nested within line type as a random effect. The sex×line type interaction was tested relative to sex×line(line type). Covariates varied with the trait analysed and included age, time of day, bleed delay time and amount of wheel running during the 20 min prior to blood sampling (preliminary analyses indicated that wheel running over this time interval had a higher predictive ability than over 10, 30 or 40 min prior to blood sampling). Whenever possible, we used directional hypotheses, e.g. HR mice will have greater endurance.

For measures of wheel running (total revolutions per day, number of intervals with at least one revolution, mean revolutions per minute for the active intervals, highest single 1 min interval), we analysed mean values from days 5 and 6 of wheel access because those are the days used in the routine selective breeding protocol (Swallow et al., 1998a). We analysed the longer of the two endurance trials for each mouse because we were interested in the maximum performance value of each individual (e.g. Dohm et al., 1996; Swallow et al., 1998b; Rezende et al., 2006a). For glucose and lactate, which were measured on three occasions (resting, wheel running and exhaustion), values were analysed separately using age, time of day, (z-transformed time)^2 (this term allows for a curvilinear relationship between the trait and time), and bleed delay time as covariates. Prior wheel running (over 20 min) was used as an additional covariate for blood samples obtained during wheel running. To compare values at rest, during wheel running and at treadmill exhaustion, we used *a priori* contrasts in SAS Procedure Mixed for a repeated-measures ANCOVA with covariates of age and bleed delay time.

For endurance, we also tested for differences among the four replicate lines within HR or C groups, using two different approaches. First, we considered the combined analysis of HR and C lines and compared the restricted maximum likelihood (REML) of models with and without line and the sex×line (line type) interaction term. Twice the difference in the ln REML of the two models is distributed asymptotically as a χ^2 with 2 degrees of freedom. If this value exceeds 5.991, then differences among the replicate lines are statistically significant for one or both sexes. Second, we performed separate two-way ANOVAs for the HR lines and for the C lines, including sex, line and the sex×line interaction term [mini-muscle status was not included as an independent variable because it is highly confounded with HR line membership – line 3 is fixed for the mini-muscle allele (see Garland et al., 2002; Syme et al., 2005); see Introduction]. Covariates were age, time of day and (z-transformed time)^2.

Finally, we tested whether line means for wheel running could be predicted by endurance. To obtain the estimates of line means, we performed a 2-way ANCOVA of the square root of endurance-running time, with factors of line, sex and the sex×line interaction, and covariates of age, time of day and (z-transformed time)^2. To obtain estimates of line means for average wheel running on days 5 and 6 of the 6 day test, we performed a 2-way ANCOVA with factors of line, sex and the sex×line interaction, and covariates of age and wheel freeness, which is a measure of how many revolutions a wheel spins following acceleration to a standard velocity. Separately for each sex, we then regressed wheel-running line means on endurance line means and also tested whether these regression models were improved (based on adjusted R^2 and partial F-tests) by addition of dummy variables to code for line type or line 3 [which is fixed for the mini-muscle gene, see above (see also Syme et al., 2005)].

RESULTS

Voluntary wheel running

As expected from previous studies of these mice, in this sample the daily wheel-running distance of the HR lines was almost 3-fold higher than for the C lines (2-tailed P=0.0014, based on analysis of revolutions raised to the 0.9 power to improve normality of residuals), and this increase was related primarily to higher average and maximum (single highest minute) voluntary running speeds in HR *versus* C mice (P=0.0020 and P=0.0011, respectively; Table 1). Also as expected from previous studies, females ran significantly more revolutions per day than males (P=0.0473; the sex×line type interaction was not significant: P=0.3039). Females did not run significantly faster than males based on either average (P=0.2673) or maximum (P=0.1839) values, and the sex×line type interaction was not significant (P=0.2396 and P=0.2729, respectively).

Endurance

As shown in Fig. 1, endurance time was highly repeatable between days (r=0.785, P<0.0001), and on average mice ran 8.8% longer on the second test day (paired t=4.57, d.f.=73, P<0.0001). Fig. 2

Table 1. Comparison of least squares means for voluntary wheel-running traits and for ending treadmill speed during endurance test, including ratios

	Wheel *N*	Wheel distance run (m day^{-1})	Wheel mean speed (m min^{-1})	Wheel max. speed in 1 min (m min^{-1})	Endurance *N*	Treadmill ending endurance speed (m min^{-1})	Wheel mean ending/endurance speed	Wheel max. ending/endurance speed
HR male	23	8668±1125	20.3±2.04	35.3±2.46	15	44.0 (26.0, 48.5)	0.461	0.802
HR female	21	11,890±1115	24.3±2.01	40.5±2.41	22	45.5 (26.0, 50.0)	0.534	0.890
C male	26	2909±1320	10.4±2.37	21.4±2.88	26	39.5 (24.5, 42.5)	0.263	0.552
C female	26	4024±1324	10.3±2.4	22.0±2.88	25	41.0 (24.5, 44.0)	0.251	0.537

Values for wheel running are least squares means ± s.e. from SAS Procedure Mixed for averages of days 5 and 6 of a 6 day test. Values for endurance speeds are also SAS least squares means and 95% confidence intervals, based on back-transformation from the square-root scale, for the higher of two endurance tests. See text for significance levels.

C, control; HR, high runner.

shows a 'survivorship curve' based on the higher endurance time for each mouse. The HR lines had significantly higher endurance (Table 2; 1-tailed P=0.0088), running an average of 32.6 and 35.0 min on the treadmill (males and females, respectively; back-transformed least squares means), whereas the C animals ran for an average of 26.3 and 28.1 min. Results were similar when body mass (which is reduced in the HR lines) or previous wheel running (which is increased in the HR lines) were included as covariates in the model (Table 2).

Total distance run during the endurance test averaged 1060±109 and 1183±98 m for HR males and females, respectively; 794±119 and 872±119 m for C males and females, respectively (least squares means ± s.e.). The average ending speed obtained by the C animals in the endurance test was ~40 m min^{-1}, while the average ending speed of the HR lines was ~45 m min^{-1} (Table 1).

Line effects and correlation of line means

In the combined analysis of endurance for both HR and C lines, the significance level for line and the sex×line (line type) interaction term was P=0.0559, based on a χ^2 of 5.77 with 2 d.f. In the separate analysis of C lines, the effects of sex (P=0.1456), line (P=0.4518) and the sex×line interaction (P=0.4454) were not significant. In the HR lines, the sex effect was not significant (P=0.1457), line was highly significant (P=0.0044), and the sex×line interaction was marginally non-significant (P=0.0650).

Fig. 3 shows the generally positive relationship between line means for wheel-running and treadmill endurance, separately by sex. In females, the best-fitting model for predicting wheel running included both endurance-running time (P=0.0217) and line type (P=0.0185; adjusted R^2=0.888). In males, the best model for predicting wheel running included both endurance (P=0.0044) and the dummy variable for line 3 (P=0.0133; adjusted R^2=0.805).

Organ masses

As shown in numerous previous studies of these lines of mice, HR mice had a lower body mass than C, and females were smaller than males (Table 3). Controlling for variation in body mass, HR and control lines did not significantly differ for any organ mass or haematocrit, nor was the sex effect or sex×line type interaction significant (Table 3). As expected, mini-muscle mice had smaller triceps surae (P=0.0001). Mini-muscle mice also had significantly larger ventricles, lungs and liver, and marginally larger spleens (P=0.0546; Table 3).

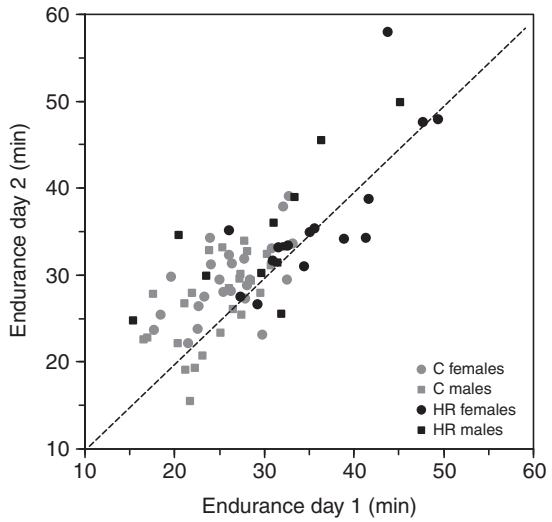

Fig. 1. Endurance time in minutes, from two separate tests. Grey circles represent control (C) females, grey squares represent control males. Black circles represent high runner (HR) females and black squares are the HR males. N=74, r=0.85, P<0.0001.

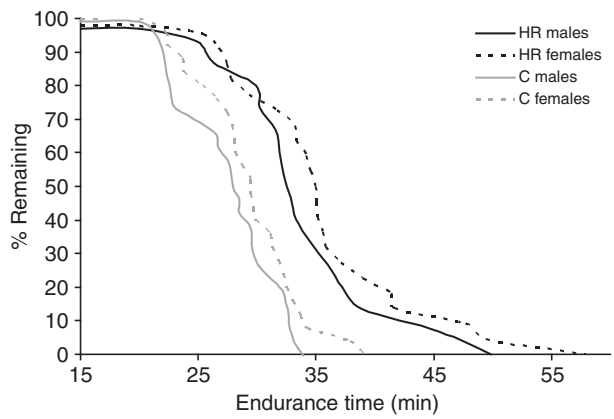

Fig. 2. The percentage of mice reaching any given time split by sex and line type. The solid black line indicates HR males (N=15), dashed black line represents HR females (N=22). The solid grey line represents the control males (N=26) with the dashed grey line for female controls (N=25). The starting belt speed was 20 m min^{-1} and increased every 2 min by 1.5 m min^{-1}. All mice lasted at least 20 min. Initial percentages are 100 for each group but lines are slightly staggered for visual clarity.

Table 2. *F*- and *P*-values from ANCOVA of endurance time with and without previous wheel running or body mass as a covariate

	F	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
HR *vs* C	10.54	1,6	**0.0088** [+]	5.94	1,6	**0.0254** [+]	5.77	1,6	**0.02655** [+]
Sex	2.01	1,6	0.2062 [+]	0.95	1,6	0.3682 [+]	0.23	1,6	0.6512 [–]
Interaction	0.01	1,6	0.9107	0.01	1,6	0.9179	0.14	1,6	0.7201
Mini-muscle	0.88	1,68	0.3517 [–]	1.53	1,67	0.2211 [–]	1.3	1,67	0.2530 [–]
Revolutions on day 5+6				2.42	1,67	0.1246			
Body mass							2.30	1,67	0.1338

Results from 2-way nested ANCOVA in SAS Procedure Mixed. Replicate line was included as random effects in analyses. Age, time of day, and (time of day)^2 were additional covariates (results not shown). + indicates direction HR>C, females>males, or mini>normal. *P* values are 2-tailed except for HR *vs* C, where values are 1-tailed. Significant values ($P<0.05$) are in bold.

Blood glucose and lactate concentrations

Glucose concentration for each blood sample in our study was measured in duplicate. During wheel running, at exhaustion and at rest the Pearson product moment correlation for the duplicates was 0.960, 0.991 and 0.960, respectively. A paired *t*-test indicated no statistical difference in mean values between the first and second duplicates ($P=0.520$, 0.268 and 0.904, respectively).

Resting blood glucose values of individual mice ranged from 72.0 to 168.5 mg dl^{-1}. As shown in Fig. 4, resting glucose did not differ statistically between HR and C mice ($P=0.3820$), but males had higher values (back-transformed least squares mean 118.5 with asymptotic 95% CI 106, 132 mg dl^{-1}) than females (98 with 95% CI 88, 108 mg dl^{-1}; $P=0.0014$; sex×line type interaction $P=0.6400$). Mini-muscle mice did not differ from normal individuals ($P=0.5366$).

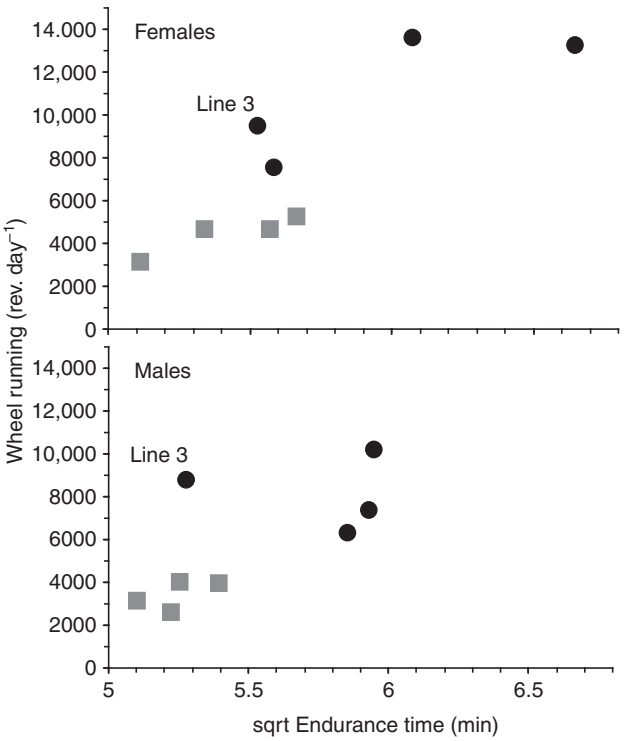

Fig. 3. Adjusted line means for endurance time (square-root transformed, sqrt) and wheel running (revolutions per day) on nights 5+6 for males and females from all eight lines. In females, the best model for predicting wheel running included both endurance-running time and line type, whereas in males it included endurance and a dummy variable coding for line 3 (i.e. line 3 males have unusually high wheel running for their endurance).

Individual glucose concentrations during peak voluntary wheel running ranged from 52.0 to 141.5 mg dl^{-1}. Males again tended to have higher concentrations than females ($P=0.0881$), but as before no effect of line type was present ($P=0.3680$; sex×line type interaction $P=0.3970$) and mini-muscle mice did not differ from normal individuals ($P=0.2300$). Wheel running during the 20 min prior to blood sampling ranged from 0 to 797 revolutions, but was not a significant predictor of glucose concentration in the full nested ANCOVA model ($P=0.9920$).

Post-endurance glucose concentrations were much lower than at rest or during wheel running, ranging from 12.0 to 120.5 mg dl^{-1} and averaging 52 mg dl^{-1} (simple mean). They did not vary significantly in relation to either sex or line type ($P=0.3318$, $P=0.5923$, respectively; sex×line type interaction $P=0.4699$), but mini-muscle individuals tended to have higher values ($P=0.0842$).

Repeated-measures ANCOVA indicated that glucose concentrations during voluntary wheel running were not significantly lower than at rest ($P=0.1738$), but values at treadmill exhaustion were lower than at rest ($P<0.0001$) or during wheel running ($P=0.0001$). Mini-muscle mice had higher glucose concentrations in general ($P=0.0140$) and showed a significant interaction with measurement condition ($P=0.0265$; Fig. 5). Glucose in normal mice decreased much more between rest (back-transformed least squares mean 105.5 mg dl^{-1}) and exhaustion (49.1 mg dl^{-1}) compared with mini-muscle individuals (112.1 to only 84.4 mg dl^{-1}, respectively; Fig. 5).

Lactate for each blood sample in our study was also measured in duplicate. During wheel running, at exhaustion, and at rest the Pearson correlation for duplicates was 0.869, 0.953 and 0.779, respectively. A paired *t*-test indicated no statistical difference in mean values between the duplicates ($P=1.00$, 0.627 and 0.051, respectively).

At rest, blood lactate concentrations ranged from 2.0 to 4.8 mmol l^{-1} and did not vary significantly in relation to sex ($P=0.3085$), line type ($P=0.1926$; sex×line type interaction $P=0.4516$) or mini-muscle status ($P=0.0535$) (Fig. 6). During wheel running, lactate ranged from 2.8 to 11.5 mmol l^{-1}, and was lower in mini-muscle individuals ($P=0.0448$) but did not vary in relation to sex or line type ($P=0.7652$ and $P=0.5914$, respectively; sex×line type interaction $P=0.6458$). Wheel running from the 20 min prior to blood sampling was not a significant predictor of lactate concentration in the full nested ANCOVA model ($P=0.6791$). At exhaustion following treadmill exercise, lactate concentrations ranged from 3.5 to 13.5 mmol l^{-1}, and did not vary in relation to mini-muscle ($P=0.2331$), sex ($P=0.3480$) or line type ($P=0.3255$; sex×line type $P=0.7570$). *A priori* contrasts in the repeated-measures ANCOVA indicated that lactate concentrations during voluntary wheel running were significantly higher than at rest ($P=0.0031$) and that values at treadmill exhaustion were higher than at rest ($P<0.0001$) or during wheel running ($P=0.0024$).

Table 3. Least squares means (back transformed from logs), 95% confidence intervals, and *P*-values for body mass, dry organ masses and haematocrit

Trait	Line type	Male	Female	$P_{line\ type}$	P_{sex}	$P_{sex \times line\ type}$	$P_{mini\text{-}muscle}$	$P_{body\ mass}$
Body mass (g)	HR	30.1 (27.89, 32.27)	22.0 (19.88, 24.17)	**0.0145**	**<0.0001**	0.6232	0.6321	–
	Control	33.8 (31.26, 36.35)	25.0 (22.44, 27.44)					
Ventricles (g)	HR	0.0337 (0.0309, 0.0367)	0.0334 (0.0301 0.0370)	0.1712	0.9065	0.3683	**0.0218**	**<0.0001**
	Control	0.0309 (0.0277, 0.0345)	0.0315 (0.0284, 0.0350)					
Lungs (g)	HR	0.0412 (0.0384, 0.0441)	0.0453 (0.0416, 0.0493)	0.1213	0.0676	0.2916	**0.0044**	**<0.0001**
	Control	0.0393 (0.0359, 0.0431)	0.0419 (0.0385, 0.0456)					
Liver (g)	HR	0.5444 (0.5093, 0.5818)	0.5246 (0.4843, 0.5682)	0.6252	0.2897	0.9780	**0.0011**	**<0.0001**
	Control	0.5359 (0.4926, 0.5831)	0.5158 (0.4765, 0.5584)					
Spleen (g)	HR	0.0234 (0.0189, 0.0291)	0.0245 (0.0186, 0.0322)	0.1484	0.3606	0.2478	0.0546	**0.0303**
	Control	0.0267 (0.0201, 0.0355)	0.0313 (0.0239, 0.0409)					
Mean triceps surae (g)	HR	0.0269 (0.0256 0.0282)	0.0262 (0.0245, 0.0279)	0.6221	0.4774	0.6172	**0.0001**	**<0.0001**
	Control	0.0271 (0.0253, 0.0289)	0.0267 (0.0251, 0.0283)					
Haematocrit (%)	HR	42.12 (39.48, 44.94)	43.18 (39.58, 47.12)	0.1694	0.3917	0.5400	0.8990	0.4563
	Control	39.64 (36.22, 43.37)	41.70 (38.56, 45.09)					
Haematocrit (%)	HR	42.37 (39.79, 45.12)	42.33 (39.95, 44.84)	0.2131	0.6211	0.5870	0.9690	–
	Control	40.34 (37.60, 43.27)	41.23 (38.47, 44.21)					

Values are back-transformed (from $\log_{10}$) least squares means from SAS Procedure Mixed, along with upper and lower 95% confidence limits in parentheses. All *P*-values are 2-tailed. $\log_{10}$ body mass was included as a covariate in analyses of dry organ masses and haematocrit; no transform was used on body mass. Hematocrit was analysed both with and without body mass as a covariate because it does not necessarily covary with body size (see Swallow et al., 2005). Significant values ($P<0.05$) are in bold. Least squares means and 95% confidence intervals for mini-muscle *vs* normal individuals are as follows: body mass, 28.04 (27.05, 29.01), 27.42 (25.01, 29.82); ventricles, 0.0344 (0.0311, 0.0380), 0.0304 (0.0291, 0.0318); lungs, 0.0444 (0.0411, 0.0480), 0.0395 (0.0382, 0.0408); spleen, 0.0302 (0.0232, 0.0392), 0.0229 (0.0206, 0.0265); mean triceps surae, 0.0204 (0.0193, 0.0216), 0.0349 (0.0341, 0.0357); haematocrit with body mass as covariate, 41.74 (38.90, 44.79), 41.53 (40.42, 42.68); haematocrit without body mass as covariate, 41.52 (38.78, 44.47), 41.59 (40.49, 42.73).

DISCUSSION

Selective breeding for high voluntary wheel-running behaviour in four replicate lines has produced mice that run almost three times more revolutions per day than four unselected control lines. Before the start of the selection experiment, it was hypothesized that the evolution of high voluntary running would entail both increases in physical capacities for exercise and changes in the brain that affect motivation, willingness to run or the reward perceived from running (e.g. Friedman et al., 1992; Garland, 2003; Swallow et al., 2009). Changes in the brain have clearly occurred and seem to indicate motivational alterations in the HR lines (e.g. Rhodes et al., 2005; Keeney et al., 2008; Rhodes and Kawecki, 2009). Previously, we reported that HR mice have elevated $\dot{V}_{O2max}$ during forced treadmill exercise (Swallow et al., 1998b; Rezende et al., 2006a; Rezende et al., 2006b). Here, we report for the first time that HR mice of both sexes have elevated treadmill endurance-running capacity compared with C mice. Thus, the results of this mouse selection experiment are consistent with findings from a selection experiment using rats in which bidirectional selection for treadmill endurance has led to corresponding divergence between the up- and down-selected lines in both $\dot{V}_{O2max}$ and voluntary wheel running (Waters et al., 2008).

The mechanistic basis of high endurance is multifactorial (Myburgh, 2003), but in the HR lines it appears to lie partly in their elevated $\dot{V}_{O2max}$ (Swallow et al., 1998b; Rezende et al., 2006a; Rezende et al., 2006b; Rezende et al., 2006c), increased insulin-stimulated glucose in extensor digitorum longus muscle (Dumke et al., 2001), and probably an elevated ability to oxidize lipids to fuel sustained exercise (Gomes et al., 2009). However, based on the present results (Table 3), it is not related to differences from the C lines with respect to haematocrit or the relative masses of heart ventricles, lungs, liver, spleen and triceps surae muscle (see also Swallow et al., 2005; Rezende et al., 2006c).

Our results suggest that both C and HR lines have higher endurance capacities than some standard inbred strains of mice (see also Lightfoot et al., 2001). In our study, with a 25 deg. incline, the average ending speed was 40.3 and 44.8 m min^{-1} for C and HR mice, respectively. One female HR mouse reached a speed of 63.5 m min^{-1} after running for 58 min. Lerman et al. (Lerman et al., 2002) and Haubold et al. (Haubold et al., 2003) used a very similar protocol to that in the present study, but with only a 7 deg. incline. The former study reported ending speeds of about 32–40 m min^{-1} for six inbred strains of mice, whereas the latter reported 28 m min^{-1} for wild-type 129 mice. Finally, using a somewhat different protocol, Massett and Berk (Massett and Berk, 2005) tested endurance in three strains of inbred mice and F1 hybrids, all of which ran substantially fewer metres than our C animals.

It is also of considerable interest to compare our values for endurance with those from recent studies using direct genetic (Tsao et al., 2001; Wang et al., 2004; Hakimi et al., 2007) or pharmacological manipulations (Narkar et al., 2008) in an attempt

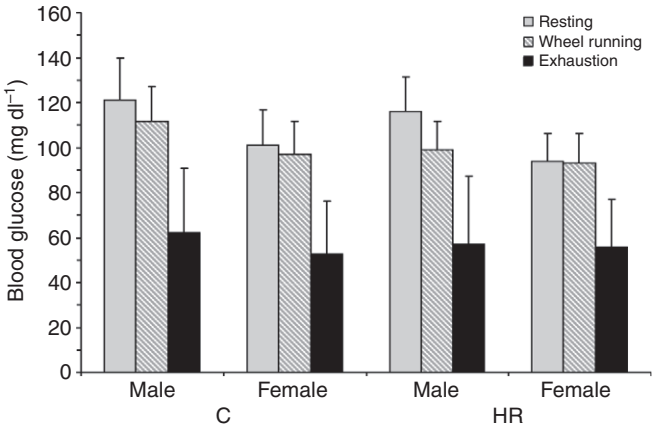

Fig. 4. Plasma glucose levels (mg dl^{-1}) at rest during the day, during nightly wheel running, and at exhaustion following a treadmill endurance test. Values are back-transformed least squares means and upper 95% confidence limits. Males have significantly higher resting glucose levels than females. Glucose levels do not differ between resting and wheel running, but post-endurance test (exhaustion) values are significantly lower than both wheel-running and resting values. Age, time of day, (time of day)^2, handling time, and prior 20 min of wheel running were all used as covariates when applicable.

to alter the endurance of mice. For the most part, their endurance protocols were much less intensive than the one employed here because of lower speeds and/or lack of an incline. In the study by Narkar and colleagues (Narkar et al., 2008), the endurance test (before some animals received 4 weeks of exercise training) involved gradually increasing treadmill speed from zero to 15 m min^{-1}, at which the speed was then maintained until exhaustion. The average endurance times for control C57BL/6J and experimental treatment groups ranged from 30 to 100 min (for untrained mice). The treadmill protocol employed by Wang and colleagues (Wang et al., 2004) used a constant speed of 10 m min^{-1} for 60 min, followed by speed increases of 1 m min^{-1} every 15 min. The final treadmill speed was 12 m min^{-1} after ~88 min of running for control mice and 17 m min^{-1} after ~145 min of running for peroxisome proliferators-activated receptor δ (PPARδ) transgenics (see figure 6A of Wang et al.). Average times for treadmill running from both studies are nearly as long as or longer than those of the mice in this study, but Narkar and colleagues (Narkar et al., 2008) and Wang and coworkers (Wang et al., 2004) used no slope and final speeds that were lower than the starting treadmill speed used in the present study. Our interpretation is that their experimentally manipulated mice (Wang et al., 2004; Narkar et al., 2008) did not have exceptionally high endurance compared with either our control lines or the HR lines. Additionally, those studies did not report any post-test physiological measure, such as blood lactate or glucose, so it is difficult to know whether their animals stopped running because of physiological exhaustion or possibly 'boredom'.

Hakimi and colleagues used three separate endurance tests to better understand the role of phosphoenolpyruvate carboxykinase (PEPCK-C) in energy metabolism (Hakimi et al., 2007). Their most comparable endurance test used a 25 deg. slope and started at 10 m min^{-1}, then increased 2 m min^{-1} every 2 min. Average ending speeds were 23.4 m min^{-1} for controls and 36.6 m min^{-1} for mice over-expressing PEPCK-C. Compared with our study, where a very similar endurance protocol was used, their PEPCK-C animals approach the speeds reached by our control animals (40 m min^{-1}). The reported blood lactate concentrations for control animals from the study by Hakimi and coworkers show a significant increase from rest to post-endurance and reach concentrations indicative of physiological fatigue (Hakimi et al., 2007). Hakimi and colleagues also report mass-specific maximal oxygen consumption obtained during forced treadmill exercise, and comparison with values in Rezende et al. (Rezende et al., 2006a; Rezende et al., 2006b) indicates that their transgenic mice have values similar to our control lines, whereas our HR lines have significantly higher values.

Additionally, Tsao and colleagues (Tsao et al., 2001) did not measure endurance but did report that mice overexpressing the glucose transporter GLUT4 ran fourfold farther on wheels than controls. However, as noted by Lightfoot and colleagues (Lightfoot

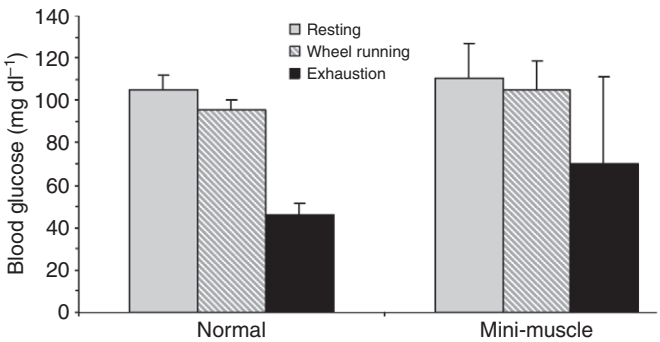

Fig. 5. Plasma glucose levels (mg dl^{-1}) at rest, during nightly wheel running and post-endurance test for mini-muscle and normal mice. Mini-muscle animals are found only in two selected lines. Mini-muscle mice have significantly higher glucose levels and show a significant interaction with measurement period. Values are back-transformed least squares means and upper 95% confidence limits. Age, time of day, (time of day)^2, handling time, and prior 20 min of wheel running were all used as covariates when applicable.

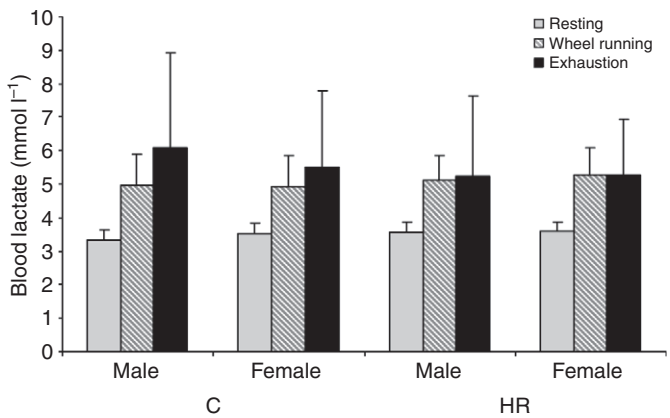

Fig. 6. Plasma lactate levels (mmol l^{-1}) at rest during the day, during nightly wheel running, and at exhaustion following a treadmill endurance test. Values are back-transformed least squares means and upper 95% confidence limits. No significant differences exist between HR and C mice or between the sexes for any measure, but mini-muscle had lower lactate concentrations during wheel running. However, *a priori* contrasts in a repeated-measures ANCOVA indicated that lactate levels during voluntary wheel running were significantly higher than at rest, and that values at treadmill exhaustion were significantly higher than at rest or during wheel running (see Results). Age, time of day, (time of day)^2, handling time, and prior 20 min of wheel running were all used as covariates when applicable.

et al., 2004), even that elevated distance would rank fourth lowest compared with the 13 inbred strains they studied. Overall, the foregoing comparisons indicate the efficacy of selective breeding (experimental evolution) as a tool to alter the performance capacities of rodents (see also Koch et al., 1998; Koch and Britton, 2001; Waters et al., 2008; Swallow et al., 2009).

Although HR lines as a group have statistically higher endurance capacity than their control lines, the two sets of lines do not show complete separation (Fig. 3). Moreover, we found that endurance capacity differs significantly among the four HR lines, but not among the four C lines. Considering the mean values for each of the eight lines [which have been genetically separated for 50 generations, including generation zero, prior to the start of selective breeding (see Swallow et al., 1998a)], we found a positive relationship between endurance capacity and amount of voluntary wheel running. This association reinforces the association demonstrated by the general differences between the HR and C lines (i.e. the former run more on wheels and have higher endurance; Table 1, Fig. 2). We also found that the line-mean relationship between endurance and wheel running differs between the sexes (Fig. 3). In females, the best-fitting model for predicting wheel running included endurance and line type, and the graphical representation shows that, relative to C females, HR females run more on the wheels than would be predicted from their treadmill endurance capacity (Fig. 3). In males, the best model included endurance and a dummy variable coding for HR line 3 (lab designation), which is fixed for the mini-muscle allele. Thus, in males, HR line 3 runs more on wheels than would be predicted from their endurance capacity (Fig. 3). This sex difference in the wheel-running endurance relationship also reinforces the sex difference in how high wheel running has evolved in general in the HR lines, i.e. females have evolved higher running distances almost exclusively by increased running speed, whereas male HR mice show a significant increase in the amount of time spent running in addition to average running speed (Garland, 2003) [Rezende et al. (Rezende et al., 2009) and references therein]. The exercise-physiological underpinnings of this sex difference in response to selective breeding are not yet known, but a recently discovered sex difference in the response to an endocannabinoid receptor antagonist points to a 'motivational' difference between HR males and females (Keeney et al., 2008).

Resting blood glucose and lactate values of both HR and C mice (Figs 4–6) are generally consistent with other studies of mice (Tsuboyama-Kasaoka et al., 1999; Banerjee et al., 2004; Pederson et al., 2005a; Pederson et al., 2005b; Ferreira et al., 2007; Svenson et al., 2007; Wende et al., 2007), rats (Pimenta et al., 2006), hamsters (Mistlberger et al., 2006) and human beings (Goldfarb et al., 1986; Rizzo et al., 2005; Scheen et al., 1998), although the lactate values are slightly higher than in some of the previous mouse studies, possibly because our study is apparently the first to measure lactate in samples obtained from the submandibular vein using lancets. The observed higher resting glucose concentrations in males are also consistent with previous studies of mice (Pederson et al., 2005a; Pederson et al., 2005b; Svenson et al., 2007). Glucose concentrations following exhaustive treadmill exercise were significantly reduced (Figs 4 and 5), but did not vary in relation to line type, sex or mini-muscle status. These low blood glucose concentrations (overall mean ~52 mg dl^{-1}) are consistent with other studies of exhaustive treadmill exercise in laboratory mice (e.g. Pederson et al., 2005a).

Voluntary wheel running increased blood lactate concentrations of all mice to values significantly greater than those at rest (Fig. 6). Exhaustive treadmill exercise further increased blood lactate concentrations of both HR and C lines, to values consistent with the literature for laboratory house mice (Pederson et al., 2005a; Pimenta et al., 2006; Hakimi et al., 2007), with these values being somewhat lower than reported for wild rodents of other species (Djawdan, 1993).

Exercise is considered a key component of body weight regulation and a cornerstone for the treatment of metabolic syndrome and other metabolic disorders (Thorburn and Proietto, 2000). As worldwide rates of obesity [and associated mortality (e.g. Allison et al., 1999)] continue to rise, understanding the determinants of voluntary physical activity is becoming increasingly important from a biomedical perspective. Diminished aerobic capacity, low expression levels of genes coding for proteins involved in oxidative phosphorylation, and mitochondrial dysfunction have been correlated with increased circulating triglyceride and glucose concentrations, insulin resistance and type II diabetes (Mootha et al., 2003; Patti et al., 2003; Bernal-Mizrachi and Semenkovich, 2006). Conversely, high aerobic capacity and associated lower-level traits may protect from components of the metabolic syndrome, and selective breeding of rats for high treadmill endurance capacity has made them resistant to the adverse effects of a high-fat diet (Noland et al., 2007). Selection for high voluntary wheel running in mice has led to correlated increases in $\dot{V}_{O2max}$ and endurance capacity, reduced body fat (Swallow et al., 2005; Nehrenberg et al., 2009), reduced circulating leptin concentrations (Girard et al., 2007) (but see Vaanholt et al., 2008), and increased circulating adiponectin concentrations [(Vaanholt et al., 2007); depending on sex and diet (Vaanholt et al., 2008)]. These characteristics, along with alterations in dopamine and endocannabinoid signalling in the brain of HR mice (Rhodes et al., 2005; Keeney et al., 2008) (cf. Stice et al., 2008), suggest that they may also show decreased susceptibility to obesity and cardiovascular disease risk factors, especially when challenged with a high-fat diet (Vaanholt et al., 2008). This hypothesis will be the focus of future research.

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