

Erythropoietin elevates $\dot{V}_{O_{2,max}}$ but not voluntary wheel running in mice

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SUMMARY

Voluntary activity is a complex trait, comprising both behavioral (motivation, reward) and anatomical/physiological (ability) elements. In the present study, oxygen transport was investigated as a possible limitation to further increases in running by four replicate lines of mice that have been selectively bred for high voluntary wheel running and have reached an apparent selection limit. To increase oxygen transport capacity, erythrocyte density was elevated by the administration of an erythropoietin (EPO) analogue. Mice were given two EPO injections, two days apart, at one of two dose levels (100 or 300 $\mu\text{g kg}^{-1}$). Hemoglobin concentration ([Hb]), maximal aerobic capacity during forced treadmill exercise ($\dot{V}_{O_{2,max}}$) and voluntary wheel running were measured. [Hb] did not differ between high runner (HR) and non-selected control (C) lines without EPO treatment. Both doses of EPO significantly ($P < 0.0001$) increased [Hb] as compared with sham-injected animals, with no difference in [Hb] between the 100 $\mu\text{g kg}^{-1}$ and 300 $\mu\text{g kg}^{-1}$ dose levels (overall mean of 4.5 g dl^{-1} increase). EPO treatment significantly increased $\dot{V}_{O_{2,max}}$ by ~5% in both the HR and C lines, with no dose \times line type interaction. However, wheel running (revolutions per day) did not increase with EPO treatment in either the HR or C lines, and in fact significantly decreased at the higher dose in both line types. These results suggest that neither [Hb] *per se* nor $\dot{V}_{O_{2,max}}$ is limiting voluntary wheel running in the HR lines. Moreover, we hypothesize that the decrease in wheel running at the higher dose of EPO may reflect direct action on the reward pathway of the brain.

Key words: artificial selection, central limitation, experimental evolution, maximum metabolic rate, oxygen transport, peripheral limitation, respiratory exchange ratio, selection limit, symmorphosis.

INTRODUCTION

Voluntary behaviors are complex traits that arise from processes in the brain ('motivation') but are ultimately limited by an organism's physical and physiological abilities to sense its surroundings, to move, to cope with environmental changes, etc. One type of voluntary behavior that is of increasing interest from both ecological and biomedical perspectives is the level of daily activity. From an ecological perspective, daily activity levels will affect encounter rates with both food and predators, can account for an important fraction of the total daily energy budget and may affect the ability of a population to persist in the face of global climate change (Rezende et al., 2009; Feder et al., 2010). From a biomedical perspective, recent decreases in activity levels in most 'first-world' countries have been associated with increases in obesity and inactivity-related morbidity from a variety of diseases, plus possible decreases in psychological quality of life (Norgan, 1993; Booth et al., 2002; Castaneda et al., 2005).

Both within and among species, we can expect that variation in voluntary activity levels reflects variation in subordinate phenotypes at many lower levels of biological organization, including cognitive, hormonal, biochemical and cellular (Thorburn and Proietto, 2000; Rhodes et al., 2005; Eisenmann and Wickel, 2009), as well as various environmental influences (e.g. Mattocks et al., 2008) and interactive effects (Dishman, 2008). Common sense and everyday experience indicates that animals have some 'excess capacity' to engage in locomotor activity above and beyond the amount (duration and/or intensity) that is typically used for routine daily activities. However, it is well known that animals often prepare themselves prior to

engaging in unusually demanding locomotor bouts, including training by human athletes and fattening or dietary selection prior to or during migration by birds (e.g. Smith et al., 2007).

The foregoing considerations lead to interesting evolutionary questions (see also Feder et al., 2010): if selection favors an increase in daily activity levels, what sorts of responses will occur? Will ability increase immediately or will motivation increase for a number of generations before abilities need to increase? Will trade-offs occur that negatively affect other aspects of organismal function? These sorts of questions can be addressed in real time through an experimental evolution approach (Garland and Rose, 2009).

Accordingly, to study the effects of selection for high voluntary activity levels, we have conducted selective breeding to increase wheel-running behavior in laboratory house mice [*Mus domesticus* (Schwarz and Schwarz, 1943)]. Mice have been bred for total wheel revolutions on days five and six of a six-day period of wheel access (Swallow et al., 1998a; Garland, 2003). After 45 generations, the four replicate lines of high runner (HR) mice ran an ~3-fold greater distance per day (Fig. 1) than four randomly bred control (C) lines. Although HR and C lines diverged quickly in the number of total revolutions, beginning at generation 16 the HR lines reached a plateau in their wheel running that has remained relatively stable despite continued selection (Fig. 1) and the emergence of a variety of correlated responses.

As discussed elsewhere, several lines of evidence indicate that the motivation for wheel running and/or the reward received from running has been altered in the HR lines (Rhodes et al., 2003; Rhodes et al., 2005; Belke and Garland, 2007). The capacity for sustained,

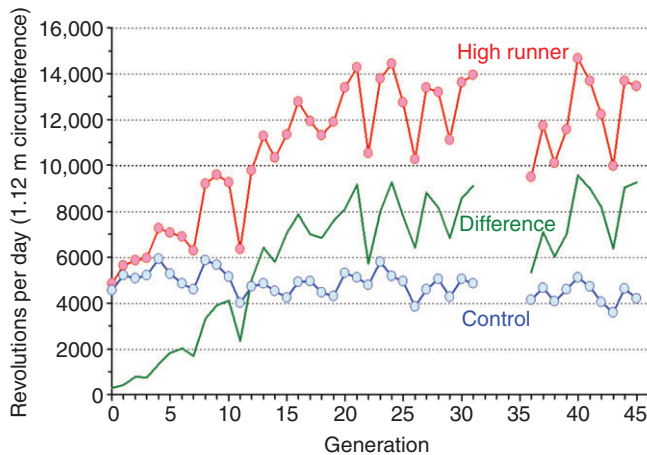


Fig. 1. Mean wheel running on days five and six of a six-day exposure to activity wheels for four replicate lines that have been selected for high running ability (high runner or HR lines) and four unselected control lines, as well as the difference between them. Mice from the HR lines reached an apparent selection limit at about generation 16 (see Garland, 2003). Wheel running was not measured, and hence selection was relaxed, for four generations while the colony was moved from the University of Wisconsin-Madison to the University of California, Riverside, CA, USA. Following resumption of selective breeding, the HR lines returned to approximately the same level.

aerobically supported exercise has also increased in the HR lines, as demonstrated by increased endurance (Meek et al., 2009) and increases maximum oxygen consumption [$\dot{V}_{O_{2,max}}$ (Swallow et al., 1998b; Rezende et al., 2005; Rezende et al., 2006a)] during forced treadmill exercise. However, the trait, or group of traits, that is limiting to even higher levels of wheel running in the HR lines remains an open question. Results to date indicate that glycogen depletion during nightly running is not responsible for the limitation (Gomes et al., 2009) nor are the HR mice limited by their ability to process enough energy to support the increased energy demands of running (Koteja et al., 1999; Koteja et al., 2001; Vaanholt et al., 2007a; Rezende et al., 2009).

One of the main predictors of endurance-running ability is whole-organism aerobic capacity (Wagner, 1996; Bassett and Howley, 2000; Lucia et al., 2001; Calbet et al., 2006; Noakes, 2007; Levine, 2008; Noakes, 2009). All else being equal, the higher the maximum aerobic capacity, the higher the maximum sustainable running speed. Maximum oxygen consumption defines the upper limits of the cardiovascular/respiratory system and of aerobic ability in general (for a review, see Levine, 2008). The ultimate determinant of organismal aerobic capacity occurs at the 'sink' of tissue oxygen consumption but all of the sequential steps of oxygen transport, from ventilatory convection in the lungs to oxygen diffusion in the peripheral tissues, contribute to $\dot{V}_{O_{2,max}}$ (Taylor and Weibel, 1981; Lindstedt et al., 1988; Wagner, 1996). One of the key steps in oxygen transport is convection through the vascular system, which is a function of blood flow and hemoglobin concentration ([Hb]). Alterations in [Hb] can have a profound effect on oxygen transport in general (Calbet et al., 2006; Thomsen et al., 2007) and on $\dot{V}_{O_{2,max}}$ in particular, as has been shown in numerous 'blood doping' studies (Vellar and Hermansen, 1971; Gledhill, 1982; Gledhill, 1985; Bassett and Howley, 2000; Wilkerson et al., 2005; Calbet et al., 2006). In mammals, the primary cardiovascular response to exercise in the short-term is to increase cardiac output (Janssen and Periasamy, 2007) and peripheral blood flow (Kingwell, 2000). One

longer-term effect of exercise is an increased [Hb] (triggered *via* tissue hypoxia). However, in some diving mammals, such as the Weddell seal (Hurford et al., 1996) and northern elephant seal (Thornton et al., 2001), and in some terrestrial mammals, including the horse (Persson et al., 1973; Thomas et al., 1981), dog (Vatner et al., 1974) and human being (Laub et al., 1993; Stewart et al., 2003), [Hb] can be increased acutely by contraction of the spleen ('autotransfusion').

In human beings, as in the HR lines of mice (see above) and in a rat line bred for high treadmill endurance (Gonzalez et al., 2006), elite endurance athletes do have elevated $\dot{V}_{O_{2,max}}$, e.g. 70–80 ml $O_2 \text{ kg}^{-1} \text{ min}^{-1}$ for professional cyclists *versus* ~40 ml $O_2 \text{ kg}^{-1} \text{ min}^{-1}$ for non-athletes (Lucia et al., 2001). However, recent studies of variation in maximal aerobic performance suggest that neurobiological attributes (Kayser, 2003; Noakes, 2007; Noakes, 2009), including focus of concentration (Rose and Parfitt, 2007) and altered perception of exertion (Baden et al., 2005), also play a role in performance ability. In sum, these recent human studies imply that at high levels of performance, ability can confer a higher aerobic 'set point' but neurobiological attributes may 'fine tune' the overall result. To use an athletic metaphor, ability gets you near the front of the pack but behavior determines whether you place first, second or third.

The purposes of the present study were to test whether one critical component of aerobic ability (blood [Hb]) is limiting to $\dot{V}_{O_{2,max}}$, and whether increases in $\dot{V}_{O_{2,max}}$ can in turn lead to increased wheel running by the HR lines. We focused on aerobic ability because HR mice, unlike C mice, sometimes run voluntarily at or near their maximal aerobic speed (Girard et al., 2001; Rezende et al., 2009) (see also Meek et al., 2009). In addition, in a study from generation 36, $\dot{V}_{O_{2,max}}$ was found to scale linearly with atmospheric partial pressure of oxygen (P_{O_2}) in HR mice but not in C mice (Rezende et al., 2006a), and there was a positive relationship between [Hb] and $\dot{V}_{O_{2,max}}$ (Rezende et al., 2006c) at the level of individual variation, suggesting that oxygen availability may underlie the higher $\dot{V}_{O_{2,max}}$ levels in HR mice.

We artificially elevated [Hb] in an attempt to increase oxygen convection in the blood and thereby increase $\dot{V}_{O_{2,max}}$. Given the positive relationship between [Hb] and $\dot{V}_{O_{2,max}}$ in HR mice and the positive effect of [Hb] on $\dot{V}_{O_{2,max}}$ in human beings (Connes et al., 2003; Wilkerson et al., 2005), we expected this elevation to occur in a dose-dependent manner. Methodologically, there are a number of ways to increase [Hb], including hypoxia exposure and blood transfusion (i.e. blood doping). However, directly administering erythropoietin (EPO) to increase erythrocyte production mimics the natural mammalian response to tissue hypoxia (for a review, see Krantz, 1991), and is both simple and potent. In this study, we used the erythropoiesis-stimulating protein darbepoetin (Aranesp®: Amgen, Inc., Irvine, CA, USA). Darbepoetin differs from endogenous EPO [and recombinant human EPO (rhEPO)] in containing two additional N-linked oligosaccharide chains. These chains result from amino acid substitutions in the peptide backbone of EPO. Functionally, the pharmacokinetic terminal half-life of darbepoetin is ~3-fold longer than rhEPO (Egrie et al., 2003), which results in a 14-fold greater potency at lower dose frequencies (Egrie and Browne, 2001).

MATERIALS AND METHODS

Selection experiment and study animals

Mice used in this study were derived from an ongoing artificial selection experiment for high voluntary wheel running (Swallow et al., 1998a; Garland, 2003; Rhodes et al., 2005). Four replicate HR

lines of mice have been bred for high wheel revolutions on days five and six of a six-day wheel testing period whereas four C (control) lines have been bred without regard to the amount of running during the test. All of the eight lines (four HR; four C) have been reproductively isolated since the inception of the selection experiment. A minimum of 10 mating pairs from each line produce litters every generation, and pups are weaned at 21 days. Mice are wheel tested at approximately 6–8 weeks of age, and within-family selection is used in choosing breeders. During wheel testing, mice are individually housed with access to Wahman-type running wheels (1.12 m circumference; Lafayette Instruments, Lafayette, IN, USA).

The current study used 96 females from generation 45. Females were used because they run a greater number of total revolutions per day and at higher speeds than males (e.g. Swallow et al., 1998a; Koteja et al., 1999; Swallow et al., 1999; Girard et al., 2001; Rezende et al., 2009), and hence may be more likely to experience a limitation related to $\dot{V}_{O_{2,max}}$. Following weaning, mice were housed in sex-specific cages in groups of four. Mice were provided food (Teklad Rodent Diet 8604, Madison, WI, USA) and water *ad libitum* and housed in temperature-controlled rooms ($\sim 22^{\circ}\text{C}$) with a light:dark cycle of 12h:12h (lights on at 07:00h).

Experimental design

Within each of the eight lines of the selection experiment (four HR; four C), 12 individuals were split into three EPO treatment levels: $0\mu\text{g kg}^{-1}$ (sham), $100\mu\text{g kg}^{-1}$ and $300\mu\text{g kg}^{-1}$ of EPO (Aranesp[®]; Amgen Inc.). To facilitate logistics, the total study sample was split into four batches with equal representation of treatment level and line within each batch. Therefore, each of the four batches contained 24 individuals (three from each of the eight lines). All batches were treated equally with regard to experimental conditions but the start date of each was staggered by two days.

Two weeks of wheel access were given to all study subjects (days 1–14), beginning at 8–10 weeks of age (mean=65 days; range=55–68 days) (Fig. 2). Wheel running was recorded for 23.5 h day^{-1} throughout this acclimation period, as previously described (Swallow et al., 1998a). Subsequently, the first of two intraperitoneal EPO injections was given to each individual, after which they were placed back into their original cage with wheel access. Every individual received an injection on day 15 and on day 17 of the experiment. Given that significant [Hb] elevation has been reported to start 5–6 days post-injection, with a maximal effect approximately 7–10 days post-injection, and multiple injections lengthen and increase the dose–response plateau of [Hb] (Breyman et al., 1996; Egrie et al., 2003; Sasu et al., 2005; Lundby et al., 2007), blood samples were obtained eight and 11 days following the second injection (day 25 and day 28, respectively). These two samples were used to verify

the expected [Hb] plateau. Wheel running was analyzed starting at seven days following the last injection to coincide with the [Hb] elevation. Wheel-running results are represented as two-day means of total wheel revolutions in the 23.5 h wheel-recording cycle. Throughout acclimation and EPO administration, wheel running was recorded in one-minute intervals. From these raw data, total revolutions per day, total number of 1-minute intervals per day with at least one revolution, mean speed (total revolutions/number of active intervals) and maximum speed in any 1-minute interval were analyzed. Wheel freeness was measured as the number of free-spinning revolutions following acceleration to a given velocity. Finally, $\dot{V}_{O_{2,max}}$ was measured (see below) over the last two consecutive days of the study (days 27 and 28).

At the conclusion of the study, mice were euthanized by inhalation of CO_2 and dissected. The lungs, liver, spleen, triceps surae muscles and ventricles of the heart were removed and weighed ($\pm 0.001\text{ g}$) as previously described (Carter et al., 1999; Swallow et al., 2005; Hannon et al., 2008). Additionally, the lungs were placed in a drying oven at 60°C for seven days, and then weighed to obtain a dry mass.

Hemoglobin assays

For each of the two samples, approximately $75\mu\text{l}$ of blood was obtained through retroorbital sinus puncture (Hoff, 2000; Malisch et al., 2007) using heparinized microhematocrit tubes (Fisher Scientific, Pittsburgh, PA, USA). This original sample was split into two duplicate $20\mu\text{l}$ samples in precalibrated microhematocrit tubes (Drummond Scientific Company, Broomall, PA, USA). Samples were combined with 5 ml of Drabkin's reagent, incubated for 30 min at room light and room temperature, and then stored at 4°C in the dark until spectrophotometric readings were taken. [Hb] was analyzed using a Beckman DU 640 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA) at 540 nm. Concentrations were interpolated from calibration curves obtained with standard hemoglobin solutions (product no. 3074; Mallinckrodt Baker, Deventer, Holland) diluted in Drabkin's reagent in the following concentrations: 0.0, 5.5, 13.8 and $17.5\text{ g }100\text{ ml}^{-1}$. The mean absorbance of the duplicate samples was used to calculate the [Hb].

$\dot{V}_{O_{2,max}}$ measurements

Forced exercise on a treadmill was used to measure $\dot{V}_{O_{2,max}}$ (Hayes and Chappell, 1990; Chappell et al., 2003; Rezende et al., 2005; Rezende et al., 2006a). The treadmill was inclined at a 15° angle to increase exertion at lower tread speeds (Kemi et al., 2002; Rezende et al., 2005; Rezende et al., 2006a). Within each batch, individuals were randomized with regards to the time of day and the order in which they were tested. A negative-pressure, open-circuit respirometry system was used to measure rates of oxygen

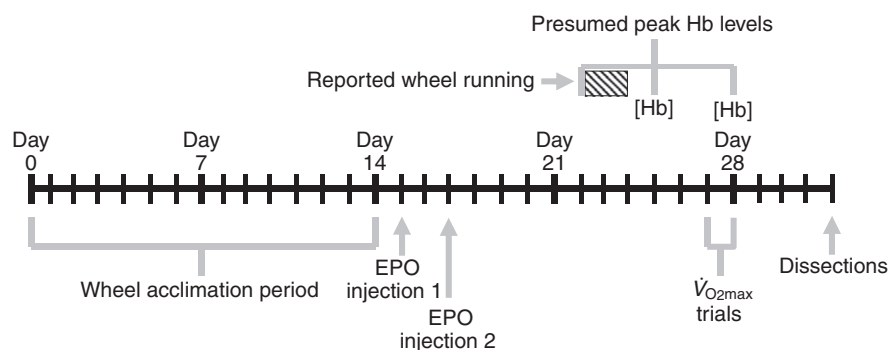


Fig. 2. Experimental design timeline, including dates of maximum oxygen consumption ($\dot{V}_{O_{2,max}}$), wheel running and hemoglobin concentration [Hb] measurements, as well as erythropoietin (EPO) administration. Mice had wheel access for the entire 32 days, the first 14 of which were considered to be an acclimation period. Wheel running was reported during the presumed [Hb] peak (7–8 days post-EPO administration).

Table 1. Significance levels for hemoglobin concentration ([Hb]), wheel running and maximum oxygen concentration ($\dot{V}_{O_{2,max}}$) traits from two-way nested ANCOVA in SAS Procedure Mixed

Trait	N	$P_{body\ mass}$	$P_{line\ type}$	P_{EPO}	$P_{line\ type \times EPO}$	P_{mini}
[Hb]						
During wheel running (g dl ⁻¹)	96		0.6358+	<0.0001+	0.4857	0.7340–
During $\dot{V}_{O_{2,max}}$ trials (g dl ⁻¹)	96		0.9923+	<0.0001+	0.4362	0.5626–
Wheel running*						
Daily revolutions (revs day ⁻¹)	92		0.0005+	0.0297–	0.2504	0.3546–
1-minute intervals run	92		0.0281+	0.0680–	0.9691	0.2267–
Mean r.p.m.	92		0.0004+	0.2231–	0.3015	0.9107+
Maximum r.p.m.	92		<0.0001+	0.6317–	0.9622	0.9473–
Respirometry						
Body mass (g)	77		0.1736–	0.9296+	0.8621	0.2184–
$\dot{V}_{O_{2,max}}$ (ml min ⁻¹)	77	<0.0001+	0.0443+	0.0316+	0.6456	0.9939+
Maximum treadmill speed (m s ⁻¹)	77	0.5544–	0.1114+	0.2620+	0.4466	0.8017–

P values were considered significant at $P < 0.05$ (in bold).

Signs following P values indicate direction of effect: + indicates high runner (HR) lines > control (C), positive effect of body mass, erythropoietin (EPO) treatment (sham injection *versus* 100 or 300 μ g kg⁻¹) or mini-muscle phenotype; – indicates HR lines < C lines, negative effect of body mass, EPO treatment, or mini-muscle phenotype.

No variables were transformed in the analyses listed above.

The following additional covariates were included in each of the analyses: age for [Hb]; age and wheel freeness for wheel-running traits; age, body mass, cooperativity score (never statistically significant; results not shown), time of day and (Z-transformed time of day)² for respirometry traits. For each of the analyses, line and batch were included as random factors (results not shown).

*Wheel-running data are means from days seven and eight post-EPO treatment.

consumption (\dot{V}_{O_2}). Tylan mass flow controllers (Billerica, MA, USA) were used to regulate gas flow (2500 ml min⁻¹) downstream from the treadmill. Approximately 100 ml min⁻¹ of excurrent air was dried (magnesium perchlorate), scrubbed of CO₂ (soda lime), redried and flowed through an Ametek/Applied Electrochemistry S-3A oxygen analyzer (Pittsburgh, PA, USA). Analyzer outputs were recorded on a Macintosh computer equipped with a National Instruments A-D converter (Austin, TX, USA) and Warthog LabHelper software (Warthog Systems, www.warthog.ucr.edu).

We calculated \dot{V}_{O_2} as:

$$\dot{V}_{O_2} = \dot{V} (FiO_2 - FeO_2) / (1 - FiO_2),$$

where \dot{V} is flow rate (STP), FiO_2 is incurrent oxygen concentration and FeO_2 is excurrent oxygen concentration. An 'instantaneous' transformation (Bartholomew et al., 1981) was applied to resolve rapid changes in respiration. The effective volume of the treadmill was 350 ml.

$\dot{V}_{O_{2,max}}$ was calculated as the highest 60 s interval during forced exercise on the motorized treadmill. At the start of each trial, individuals were placed on the treadmill and allowed to acclimate for two minutes. Following acclimation, each individual was run at increasing treadmill speeds, starting at 0.5 m s⁻¹, and raised in step increments of ~0.5 m s⁻¹ every 30 s until the mouse could no longer maintain position and \dot{V}_{O_2} no longer increased. In general, mice appeared exhausted at the end of trials. The maximum speed that each individual reached was recorded and a subjective running score [1–5; based on Swallow et al. (Swallow et al., 1998b)] was given to each trial. Trials that received a score of 1 (poor) or 2 (fair) were not used in the final analyses. Additionally, all individuals within each batch underwent $\dot{V}_{O_{2,max}}$ trials in a random order on each consecutive day of testing.

Statistical analyses

Wheel running was analyzed in a two-way, mixed model analysis of covariance (ANCOVA) with line type (HR *versus* C lines) and EPO treatment being the main factors (SAS Procedure Mixed, SAS Institute, Cary, IN, USA). Line type was treated as a fixed effect and the four replicate HR lines and the four replicate C lines were

treated as random effects nested within line type. Covariates in the wheel-running analysis included age (at expected [Hb] peak) and wheel freeness (an inverse measurement of wheel resistance). As in previous studies, presence/absence of the mini-muscle phenotype was also included as a factor (e.g. Garland et al., 2002; Swallow et al., 2005; Rezende et al., 2006b; Hannon et al., 2008). $\dot{V}_{O_{2,max}}$ was analyzed in the same manner as wheel running but with age, mini-muscle phenotype, time of day and body mass as covariates. Additionally, measurement batch was used as a random cofactor in both the wheel running and $\dot{V}_{O_{2,max}}$ analyses.

Hemoglobin concentration was also analyzed using a two-way, mixed model ANCOVA. Age (at expected [Hb] peak), mini-muscle and batch were included in the model. Repeatability between the two hemoglobin measurements was assessed *via* Pearson's product-moment correlation. Nevertheless, given the dynamic nature of the pharmacokinetic response of [Hb] to EPO treatment, [Hb] when on wheels and at $\dot{V}_{O_{2,max}}$ measurement were treated as different traits. In preliminary analyses, [Hb] on wheels (from day 25) was used as an additional covariate in the analysis of the wheel-running data, and the [Hb] at $\dot{V}_{O_{2,max}}$ (at day 28) was used in the respirometry analyses. [Hb] was never statistically significant in these analyses, and so results reported are for the model that did not include it as a covariate.

RESULTS

Hemoglobin concentration

Results in the following sections will be presented as adjusted least square means \pm standard errors derived from a two-way mixed model analysis of variance (ANOVA) with covariates (see Materials and methods). EPO treatment elevated [Hb] in both line types during wheel running and $\dot{V}_{O_{2,max}}$ trials (Table 1). The [Hb] measurements were repeatable ($R=0.971$, $N=96$, $P<0.0001$) but [Hb] was lower ($t_{95,paired}=6.409$, $P<0.0001$) at the second blood sampling ([Hb] on wheels = 19.4 g dl⁻¹ \pm 0.2 *versus* [Hb] at $\dot{V}_{O_{2,max}}$ = 19.0 g dl⁻¹ \pm 0.2). In the subsequent analyses, these two measurements were treated as separate traits. Neither line type nor the mini-muscle phenotype was a significant predictor of [Hb]. Moreover, there was no significant statistical interaction between line type and EPO treatment.

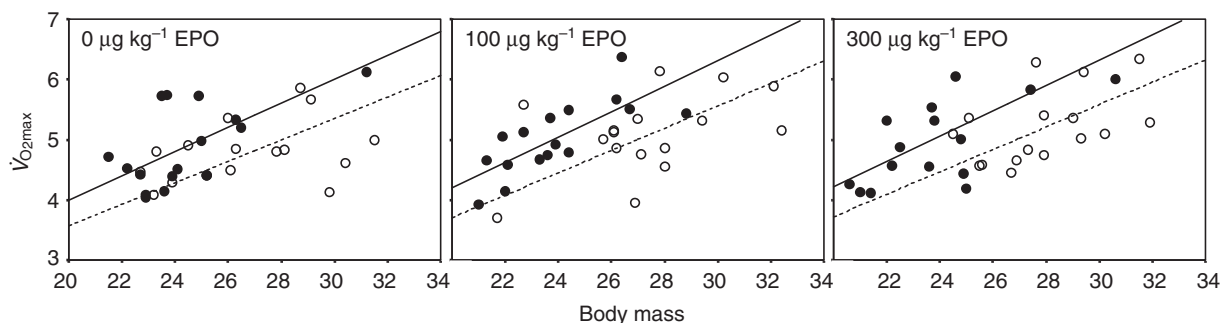


Fig. 3. Maximum oxygen consumption ($\dot{V}_{O_{2,max}}$) versus body mass for each of the three erythropoietin (EPO) dose levels (0, 100, 300 $\mu\text{g kg}^{-1}$). Filled circles are high runner (HR) mice and open circles are control (C) mice. Least-squares linear regression lines are represented as either solid (HR mice) or broken (C mice) lines. HR lines maintained an 8–10% higher $\dot{V}_{O_{2,max}}$ across treatment groups. $\dot{V}_{O_{2,max}}$ increased by ~5% in both HR and C lines in response to both the 100 and 300 $\mu\text{g kg}^{-1}$ EPO doses.

The consecutive $\dot{V}_{O_{2,max}}$ trials were repeatable ($R=0.64$, $P<0.0001$) but, on average, mice had a higher $\dot{V}_{O_{2,max}}$ on the second day of testing ($t_{93,paired}=-2.61$, $P=0.0106$). These results are consistent with previous studies (Rezende et al., 2005; Rezende et al., 2006a). HR lines had an 8–10% higher $\dot{V}_{O_{2,max}}$ than C lines, regardless of EPO dose (Fig. 3). Additionally, EPO treatment significantly increased $\dot{V}_{O_{2,max}}$ by ~5% in both HR and C lines, with no interaction between line type and dose. This difference was similar at the low (100 $\mu\text{g kg}^{-1}$) and high (300 $\mu\text{g kg}^{-1}$) EPO doses. Presence of the mini-muscle phenotype was not a significant predictor of $\dot{V}_{O_{2,max}}$. Line type was found to be a significant predictor of running score during the $\dot{V}_{O_{2,max}}$ trials ($F_{1,6}=14.37$, $P=0.0091$), with HR lines achieving higher running scores (back-transformed adjusted means, 95% C.I., lower–upper) on average (4.1, 3.6–4.6) than C lines (2.9, 1.8–3.7). However, as noted in the Materials and methods, running score was not a significant predictor of $\dot{V}_{O_{2,max}}$ in the nested ANCOVA models, and so was excluded from the results shown in Tables 1 and 2. Body mass was positively correlated with all respirometry traits, including $\dot{V}_{O_{2,max}}$, and was included as a covariate in each analysis.

Maximum treadmill speeds during $\dot{V}_{O_{2,max}}$ tests did not vary significantly in relation to line type, EPO treatment or mini-muscle status (Tables 1 and 2).

Wheel running

Wheel running analyses conducted on days 7–8 (two-day means) following EPO injections are reported here. Wheel running by individual mice varied between 360 and 22,977 revolutions per day (revs day $^{-1}$). On average, HR lines ran >3-fold more revolutions than C lines across all EPO treatment levels (Table 2). Although EPO treatment had a significant effect overall, this was predominantly due to a decrease in wheel running at the 300 $\mu\text{g kg}^{-1}$ dose, where HR and C lines both ran ~28% fewer revolutions per day as compared with the 100 $\mu\text{g kg}^{-1}$ dose (Table 2). However, this significant reduction in wheel running was associated with a 1% increase in [Hb] relative to the 100 $\mu\text{g kg}^{-1}$ dose.

Both maximum revolutions per minute (r.p.m.) and mean r.p.m. were significantly higher in HR lines across all EPO treatments. This is consistent with previous findings that HR lines run more revolutions than C lines mainly by increased speed, especially in females (e.g. Swallow et al., 1998a; Girard et al., 2001; Garland, 2003; Rezende et al., 2006; Rezende et al., 2009). There was also a marginally significant increase in time spent running by the HR lines, although this effect was not as large as the increase in speed (Table 1).

Body mass, mini-muscle and organ masses

The mean body mass (unadjusted) for HR lines was 25.2 ± 0.4 g versus 28.6 ± 0.4 g for C lines. The mean age (unadjusted) at the time of initial wheel access was 65 days for both the HR and C lines. The mini-muscle phenotype was expressed in a total of 14 mice in this study [12 from the HR line in which the trait is fixed (lab designation #3) and two from line in which the trait is polymorphic (lab designation #6)].

Body mass was a significant predictor of all organ masses, and was therefore used as a covariate in each of the organ mass analyses (along with age and time of day). Liver mass was larger in the mini-muscle phenotype, as has been reported previously (Garland et al., 2002; Swallow et al., 2005) but did not vary with line type or EPO treatment (Table 3). Ventricle mass was greater in HR lines but did not vary statistically with mini-muscle phenotype or EPO treatment. Spleen mass was larger in EPO-treated mice and showed a dose-dependent relationship. Based on back-transformed values from the least squares means shown in Table 4, spleen mass increased by ~10% from the non-treated to the 100 $\mu\text{g kg}^{-1}$ group, and by ~20% from the non-treated to the 300 $\mu\text{g kg}^{-1}$ group. Neither wet nor dry mass of the lungs was significantly affected by line type, EPO treatment or mini-muscle phenotype.

DISCUSSION

Wheel running is a voluntary activity affected by both behavioral factors (e.g. motivation/reward) and physical abilities (e.g. $\dot{V}_{O_{2,max}}$). Mice from HR lines run ~3-fold more than those from C lines on a daily basis but have reached an apparent selection limit and have not appreciably increased their running over the last 20–30 generations (Fig. 1). Wheel running is a self-rewarding behavior in rodents (for a review, see Sherwin, 1998), and previous work has indicated that enhanced motivation or reward may underlie the HR phenotype (Rhodes et al., 2003; Rhodes et al., 2005; Belke and Garland, 2007). Now that an apparent selection limit has been reached, what is preventing additional wheel running – motivation or ability? One obvious possibility is that $\dot{V}_{O_{2,max}}$ has reached an evolutionary limit (e.g. Girard et al., 2001; Gomes et al., 2009; Rezende et al., 2009). Therefore, we administered EPO to HR and C lines to test the hypothesis that aerobic capacity (i.e. an important component of endurance-running ability) may be limiting to further increase in wheel running in the HR lines. Although EPO elevated [Hb] and $\dot{V}_{O_{2,max}}$, it did not increase wheel running in either HR or C lines, and indeed caused a reduction of ~28% in wheel running in both at the higher dose (Table 2).

Table 2. Least squares means and standard errors for hemoglobin concentration ([Hb]), wheel running, body mass and maximum oxygen consumption ($\dot{V}_{O_{2,max}}$) traits from two-way nested ANCOVA in SAS Procedure Mixed (as shown in Table 1)

Trait	High-runner lines	Control lines	HR/C
Erythropoietin (EPO) ($0 \mu\text{g kg}^{-1}$)			
[Hb] while on wheels (g dl^{-1})	16.7 \pm 0.2	16.2 \pm 0.3	1.03
[Hb] at $\dot{V}_{O_{2,max}}$ (g dl^{-1})	16.3 \pm 0.3	16.0 \pm 0.3	1.02
Wheel running			
Daily revolutions (revs day^{-1})	11,214 \pm 978	3399 \pm 1162	3.30
1-minute intervals run	455 \pm 33	347 \pm 40	1.31
Mean r.p.m.	24.4 \pm 1.9	10.7 \pm 2.3	2.30
Maximal r.p.m.	41.6 \pm 2.3	20.5 \pm 2.7	2.03
Respirometry			
Body mass (g)	24.4 \pm 0.8	25.7 \pm 1.2	0.95
$\dot{V}_{O_{2,max}}$ (ml min^{-1})	4.98 \pm 0.16	4.49 \pm 0.21	1.11
Maximum treadmill speed (m s^{-1})	3.29 \pm 0.10	2.99 \pm 0.11	1.10
EPO ($100 \mu\text{g kg}^{-1}$)			
[Hb] while on wheels (g dl^{-1})	20.8 \pm 0.2	20.5 \pm 0.2	1.01
[Hb] at $\dot{V}_{O_{2,max}}$ (g dl^{-1})	20.2 \pm 0.3	20.0 \pm 0.3	1.01
Wheel running			
Daily revolutions (revs day^{-1})	12,108 \pm 980	3639 \pm 1139	3.33
1-minute intervals run	461 \pm 33	345 \pm 39	1.34
Mean r.p.m.	27.1 \pm 1.9	10.6 \pm 2.2	2.56
Maximal r.p.m.	42.2 \pm 2.3	20.5 \pm 2.6	2.06
Respirometry			
Body mass (g)	24.0 \pm 0.9	25.7 \pm 1.0	0.93
$\dot{V}_{O_{2,max}}$ (ml min^{-1})	5.28 \pm 0.17	4.78 \pm 0.19	1.10
Maximum treadmill speed (m s^{-1})	3.38 \pm 0.10	3.19 \pm 0.10	1.06
EPO ($300 \mu\text{g kg}^{-1}$)			
[Hb] while on wheels (g dl^{-1})	21.0 \pm 0.2	20.8 \pm 0.3	1.01
[Hb] at $\dot{V}_{O_{2,max}}$ (g dl^{-1})	20.6 \pm 0.3	20.7 \pm 0.3	0.99
Wheel running			
Daily revolutions (revs day^{-1})	8635 \pm 943	2664 \pm 1139	3.24
1-minute intervals run	400 \pm 32	295 \pm 39	1.36
Mean r.p.m.	21.8 \pm 1.8	10.2 \pm 2.2	2.13
Maximal r.p.m.	39.5 \pm 2.3	19.1 \pm 2.6	2.07
Respirometry			
Body mass (g)	24.1 \pm 0.8	26.1 \pm 1.1	0.92
$\dot{V}_{O_{2,max}}$ (ml min^{-1})	5.29 \pm 0.16	4.63 \pm 0.21	1.14
Maximum treadmill speed (m s^{-1})	3.19 \pm 0.09	3.13 \pm 0.11	1.02

No variables were transformed in the analyses listed above.

HR/C=ratio of means for HR/C lines.

Respirometry traits were calculated for a female weighing 25.7 g and 95 days of age.

The sham-injection ($0 \mu\text{g kg}^{-1}$ EPO) [Hb] values reported in this study fit within the normal range for laboratory mice (Halberg and Kaiser, 1976; Gödecke et al., 1999), and are consistent with a previous study of our mice from generation 14 (Swallow et al., 2005). HR lines in the present study differed only marginally (0.7%) from HR lines designated as 'sedentary' (i.e. not given wheel access) in that previous study, and were 6% lower than those designated as 'active' (i.e. mice given wheel access for eight weeks). The mice in the present study were allowed approximately three weeks of wheel access before blood sampling. Therefore, the higher [Hb] (relative to our sham-injected mice) reported in Swallow et al. (Swallow et al., 2005) for their 'active' mice most likely represents a training response to long-term wheel access. Another study, from generation 36 (Rezende et al., 2006b), found lower [Hb] values; however, those mice were not given wheel access and so are not directly comparable with animals in the present study.

The second blood sample had a lower [Hb] regardless of treatment group. Although this might represent an effect of blood loss, we think this is unlikely because only a small fraction of total blood volume was removed (Bannerman, 1983). More likely, it represents an initial decay of the pharmacokinetic response of [Hb] to EPO between the first and second blood samples. Given the

dynamic nature of this dose-response relationship, the difference between these two samples is not surprising. Nevertheless, the [Hb] measurements were repeatable between samples, and [Hb] was elevated in response to EPO. This elevation is consistent with previous EPO studies in mice (Egrie et al., 2003; Sasu et al., 2005; Imagawa et al., 2007) and humans (Connes et al., 2003; Smith et al., 2003; Wilkerson et al., 2005). However, a discrete dose-response relationship was not observed at the $300 \mu\text{g kg}^{-1}$ dose level. Although the $100 \mu\text{g kg}^{-1}$ dose increased [Hb] by ~25%, the $300 \mu\text{g kg}^{-1}$ dose elevated [Hb] by only an additional 1% (Table 2). We can offer three hypotheses to explain this result. First, the hematopoietic tissues (i.e. bone marrow) were being pushed to their maximum capacity to produce additional erythrocytes at $100 \mu\text{g kg}^{-1}$. Second, regulatory mechanisms (e.g. blood P_{O_2}) were negatively feeding back onto the erythropoietin process, dampening further red blood cell production (Krantz, 1991; Bozzini et al., 2003). Third, erythrocyte production did rise in a dose-dependent manner but the spleen was storing the additional erythrocytes; thus, regulating blood [Hb] at a constant level. Of the three possibilities, splenic storage appears to be a significant factor given the increase in spleen mass with EPO treatment (Tables 3 and 4). High blood viscosity can have deleterious effects, and these mice may have

Table 3. Significance levels for organ masses from two-way nested ANCOVA in SAS Procedure Mixed

	<i>N</i>	<i>P</i> _{line type}	<i>P</i> _{EPO}	<i>P</i> _{line type×EPO}	<i>P</i> _{mini}	<i>P</i> _{age}
Degrees of freedom		1, 6	2, 12	2, 12	1, 63	1, 63
Ventricles	95	0.0379+	0.6679–	0.6274+	0.0310+	0.9973+
Spleen	95	0.5660+	0.0046+	0.7498–	0.9564–	0.4469+
Lungs, wet mass	95	0.8993–	0.7388–	0.6341–	0.3185–	0.5070+
Lungs, dry mass	95	0.6924–	0.3835–	0.6046–	0.1863–	0.4722+
Liver	95	0.1549+	0.1374+	0.7486+	0.0001+	0.3630+

P values in bold were considered significant (*P* < 0.05).

Signs following *P* values indicate direction of effect: + indicates high runner (HR) lines > control (C), positive effect of erythropoietin (EPO), mini-muscle phenotype, or age; – indicates HR lines < C lines, negative effect of EPO treatment, mini-muscle phenotype, or age.

Organ masses were all log transformed.

log₁₀ body mass and dissection time were used as covariates.

been keeping blood viscosity low by using the spleen to store excess erythrocytes. In humans, high hematocrit levels are associated with higher risks of thrombosis (Pearson et al., 1981; Ambrus et al., 1999; Tefferi et al., 2000) and high [Hb] with high risk of cerebral infarctions (Kannel et al., 1972). In a study of transgenically-induced erythrocytosis in mice, Shibata et al. (Shibata et al., 2003) found higher mortality rates due to congestive heart failure in the transgenic animals (although thrombotic complications did not increase). It is interesting to note that for the 96 animals in this study (64 receiving EPO treatment), the mortality rate was zero. Thus, these mice may have been using their spleens to sequester excess erythrocytes, thereby regulating blood viscosity and compensating for potentially severe effects of high hematocrit levels. Regardless of the mechanism regulating further increases in [Hb], EPO treatment elevated [Hb], which in turn elevated $\dot{V}_{O_{2,max}}$.

In previous studies, ventricle mass was larger in both HR (Rezende et al., 2006b) and mini-muscle mice (Garland et al., 2002; Hannon et al., 2008), and both of those findings have been confirmed in the present study. Rezende et al. previously suggested that the increased heart size (i.e. ventricle mass) in HR lines confers an 'excess capacity' that allows for increased cardiac output during exercise (Rezende et al., 2006a). Although this could be true, C

lines appear to also have 'excess capacity' even with smaller ventricular mass, given that their $\dot{V}_{O_{2,max}}$ increased in parallel with the HR lines in response to EPO treatment (i.e. we observed no line type × EPO interaction: Table 1).

Additionally, we did not see an increase in either wet or dry lung mass. Previous work on mice has shown that inducing hyperactivity, and thereby \dot{V}_{O_2} during voluntary exercise, can result in a 23% increase in lung volume in mice and this increase in volume is supported by significant changes in the structural capacities of the pulmonary gas exchanger (e.g. increases in alveolar and capillary surface densities) (Burri et al., 1976; Hugonnaud et al., 1977). Therefore, even though lung mass did not differ with regard to either EPO treatment or line type, the structure and/or kinetics of pulmonary gas exchange may still be a significant component limiting increased wheel running. Further studies of the structure and kinetics of the lung are warranted in the HR lines.

Expressed on a mass-specific basis, the $\dot{V}_{O_{2,max}}$ values for our sham-injected mice are almost exactly in the middle of the range of values reported in previous studies on these same lines of mice (Swallow et al., 1998b; Rezende et al., 2005; Rezende et al., 2006a; Rezende et al., 2006b). EPO treatment increased [Hb] by 9.6% and $\dot{V}_{O_{2,max}}$ by 7.0% in a study of human endurance athletes (Connes et al., 2003). Our mice (HR and C) also exhibited an increase (5.5%

Table 4. Least squares means and standard errors for log₁₀ organ masses from two-way nested ANCOVA in SAS Procedure Mixed (as shown in Table 3)

Trait	High-runner lines	Control lines	HR/C
Erythropoietin (EPO) (0 µg kg ⁻¹)			
Ventricles (mg)	2.164±0.0119	2.119±0.0138	1.11
Spleen (mg)	1.932±0.0328	1.964±0.0382	0.93
Lungs, wet mass (mg)	2.383±0.0241	2.367±0.0277	1.04
Lungs, dry mass (mg)	1.693±0.0172	1.670±0.0203	1.05
Liver (mg)	3.195±0.0102	3.171±0.0117	1.06
EPO (100 µg kg ⁻¹)			
Ventricles (mg)	2.162±0.0121	2.134±0.0137	1.07
Spleen (mg)	1.972±0.0332	2.006±0.0383	0.92
Lungs, wet mass (mg)	2.381±0.0244	2.387±0.0277	0.99
Lungs, dry mass (mg)	1.697±0.0175	1.695±0.0202	1.00
Liver (mg)	3.207±0.0104	3.197±0.0116	1.02
EPO (300 µg kg ⁻¹)			
Ventricles (mg)	2.153±0.0116	2.126±0.0135	1.06
Spleen (mg)	2.024±0.0317	2.032±0.0377	0.98
Lungs, wet mass (mg)	2.376±0.0234	2.375±0.0273	1.00
Lungs, dry mass (mg)	1.697±0.0167	1.697±0.0199	1.00
Liver (mg)	3.203±0.0100	3.188±0.0115	1.04

log₁₀ body mass and dissection time of day were used as covariates.

HR/C=ratio of means for HR/C lines.

HR/C computed after antilog transformation.

and 4.3%, respectively) in $\dot{V}_{O_{2,max}}$, although it was not as large. This may be due to effects (e.g. blood viscosity) of the larger increase in [Hb] observed in this study or to differences in aerobic performance between mice and human beings. To our knowledge, this is the first study in rodents to examine the effect of [Hb] elevation on $\dot{V}_{O_{2,max}}$.

Wheel running was reduced by ~28% (revolutions per day) in both line types at the $300\mu\text{gkg}^{-1}$ dose relative to the $100\mu\text{gkg}^{-1}$ dose. As mentioned previously, there was not a significant increase in [Hb] between the $100\mu\text{gkg}^{-1}$ and $300\mu\text{gkg}^{-1}$ doses (Table 2). This striking reduction in wheel running without a measurable change in [Hb] suggests a direct physiological or motivational effect of EPO on wheel running. Traditionally, the exclusive role of EPO was thought to be erythrocyte production. However, recently EPO has been found to exert regulatory effects across a number of different tissues, including heart, kidney and reproductive tract (reviewed in Brines and Cerami, 2008). In the brain, EPO's neuroprotective effects (Sakanaka et al., 1998; Rabie and Marti, 2008) have been associated with increased neurogenesis (Wang et al., 2004; Ransome and Turnley, 2007; Xiong et al., 2008; Kadota et al., 2009) and neurotrophism (Studer et al., 2000). Additionally, EPO can be produced by glial cells (Masuda et al., 1994), and has been found to directly stimulate dopamine release in a dose-dependent manner in the striatum (Yamamoto et al., 2000). The striatum is a brain region known for regulating goal-directed behavior and reward (Khamassi et al., 2008), and has been found to be preferentially activated in our HR lines when they are deprived of wheel access following several days of wheel access (Rhodes et al., 2003). If EPO causes dopamine release, then it is possible that flooding the reward pathway with dopamine could offer a competing reward (i.e. a direct dopaminergic 'high') to the reward normally obtained through wheel running alone. Therefore, we hypothesize that EPO may be acting directly on the reward pathway by increasing dopamine release in a dose-dependent manner to reduce wheel running. Interestingly, relative to the lower dose of EPO, the HR mice exhibited a much larger absolute (but not proportional) decrease in wheel running at the high dose (12,108–8636=3472) as compared with the control mice (3639–2664=975).

In conclusion, the present results suggest that $\dot{V}_{O_{2,max}}$ is not limiting wheel running in the HR lines. Although aerobic capacity was elevated *via* enhanced oxygen delivery, it is not clear whether voluntary activity, even at the levels engaged in by the HR lines, is constrained by aerobic capacity *per se*. [Interestingly, HR mice also exhibit elevated home-cage activity when housed without access to running wheels (Malisch et al., 2008; Malisch et al., 2009)]. Instead, the reduced body fat (Swallow et al., 2001; Nehrenberg et al., 2009), reduced circulating leptin levels (Girard et al., 2007) (but see Vaanholt et al., 2008) and increased circulating adiponectin levels [(Vanholt et al., 2007b) depending on sex and diet (Vanholt et al., 2008)], suggest that lipid availability may be a limiting factor (see also Gomes et al., 2009). However, recent work in human 'ultra-endurance' athletes (Pearson, 2006) suggests that neurobiological attributes make a greater contribution to performance than has previously been acknowledged (Kayser, 2003; Baden et al., 2005; Noakes, 2007; Rose and Parfitt, 2007; Noakes, 2009): 'The brain is the oft-overlooked organ that sets ultraracers apart — they are mental freaks, not physiological ones' [T. Noakes, quoted in Pearson (Pearson, 2006); p. 1001]. Thus, it is also possible that the motivation/reward system of HR mice has reached some sort of maximum with respect to wheel running (see also Rhodes et al., 2003; Rhodes et al., 2005; Belke and Garland, 2007). Further studies

will be required to explore these alternative – and not mutually exclusive – hypotheses.

LIST OF ABBREVIATIONS

C	control lines of mice that were bred without regard to amount of wheel running
EPO	erythropoiesis-stimulating protein darbepoetin (Aranesp®)
FeO ₂	excurrent oxygen concentration
FiO ₂	incurrent oxygen concentration
Hb	hemoglobin (typically represented as [Hb]=hemoglobin concentration)
HR	high-runner lines of laboratory house mice; selectively bred for high voluntary wheel running
P _{O₂}	partial pressure of oxygen
\dot{V}	flow rate
$\dot{V}_{O_{2,max}}$	maximum oxygen consumption observed during forced exercise on a motorized treadmill (ml O ₂ min ⁻¹ , highest 1-minute mean)

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