Cold acclimation in *Peromyscus*: individual variation and sex effects in maximum and daily metabolism, organ mass and body composition

Enrico L. Rezende[1],*, Kimberly A. Hammond[2] and Mark A. Chappell[2]

[1]Departament de Genètica i de Microbiologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Bellaterra 08193, Spain
and [2]Department of Biology, University of California, Riverside, CA 92521, USA

*Author for correspondence (enrico.rezende@uab.cat)

SUMMARY

We studied metabolic and organ mass responses to thermal acclimation (7 weeks at 5°C or 23°C) in deer mice, *Peromyscus maniculatus*. Cold acclimation resulted in significantly higher maximal oxygen consumption in thermogenesis ($\dot{V}_{O_2max}$) and daily mean oxygen consumption ($\dot{V}_{O_2mean}$), an increase in the mass of most visceral organs, a lower absolute body fat and a marginally significant increase in hematocrit. The mass of digestive organs and body fat content differed significantly between sexes. Acclimation effects on fat content were more pronounced in females. Variation in heart and lung mass was positively correlated with $\dot{V}_{O_2max}$ and $\dot{V}_{O_2mean}$, while body fat content was negatively correlated with both traits. Nonetheless, a large fraction of the metabolic difference between cold- and warm-acclimated groups remained unexplained. Associations between traits at lower levels of biological organization measured here and whole-organism energetics remained consistent across acclimation temperatures, except for the correlation between kidney mass and $\dot{V}_{O_2mean}$, which was positive and significant in cold acclimation and negligible following warm acclimation. We conclude that: (1) $\dot{V}_{O_2max}$ and $\dot{V}_{O_2mean}$ share a common physiological basis that remains overall the same across acclimation regimes; (2) changes in these traits are associated primarily with changes in heart mass; and (3) male and female deer mice respond differently to thermal acclimation, possibly due to differences in reproductive allocation.

Key words: acclimation, body composition, maximal oxygen consumption, organ phenotypic plasticity, *Peromyscus maniculatus*.

INTRODUCTION

Complex phenotypic traits, such as behavior and organismal performance, are emergent characters that arise from multiple systems which must interact in a coherent fashion. Understanding how these systems respond to changing environmental conditions is particularly challenging, because responses encompass several levels of organization involving multiple physiological functions. For example, it is well known that aerobic performance emerges from the interaction of numerous systems that contribute to the oxygen cascade [(Weibel, 1984; Wagner, 1996; Treacher and Leach, 1998; Richardson et al., 1999; Basset and Howley, 2000) and references therein], and that cold acclimation results in increased aerobic capacity in endothermic species. Consequently, responses to thermal acclimation or seasonal acclimatization include, but are not limited to, systems involved in oxygen delivery or associated with thermogenesis [e.g. the role of brown adipose tissue and noradrenaline as determinants of non-shivering thermogenesis (Heldmaier and Jablonka, 1985; Nespolo et al., 1999; Nespolo et al., 2001)]. Other physiological functions may also have to respond in a coherent fashion; hence, it is not surprising that acclimatory effects following chronic cold exposure are present in organs that process food (e.g. Zhao et al., 1995; del Valle et al., 2004) and remove waste (e.g. Inbar et al., 1975).

How traits involved in multiple processes at different levels of organization function in a coherent fashion is a central question in physiological studies of phenotypic plasticity and evolution (Garland and Kelly, 2006). In this context, aerobic metabolism is a good system for studying integrated responses for a variety of reasons. For instance, the physiological basis underlying aerobic performance is well described, although discrepancies exist regarding how different steps in the oxygen cascade limit overall capacity (e.g. Bacigalupe and Bozinovic, 2002; Darveau et al., 2002). In addition, measurements of selection on aerobic performance in the field, and interspecific comparisons showing significant associations between performance and environmental characteristics, suggest this trait is important to fitness (Hayes and O'Connor, 1999; Rezende et al., 2004a). Ultimately, understanding the mechanistic foundations of aerobic metabolism involves determining which correlated responses occur at lower levels and the contribution of these responses to overall performance.

Studies of the functional and genetic basis of aerobic capacity, and its evolutionary relevance, can be classified into four major groups. First, there are comparative studies that attempt to correlate interspecific variation in aerobic capacity with differences in traits, such as cardiac output (Bishop, 1997). Second, selection experiments permit examination of correlated evolutionary responses to selection across multiple levels of organization (e.g. Ksiażek et al., 2004; Swallow et al., 2005; Rezende et al., 2006). Third, studies of individual variation in aerobic capacity within species or populations can disentangle the role played by different traits as determinants of overall capacity. Fourth, acclimation and acclimatization experiments can be used to understand how plastic responses at the organismal level are associated with changes at lower levels of physiological organization. These approaches can be employed in a complementary fashion. For instance, several studies have addressed how plasticity evolves in response to artificial selection [(Swallow et al., 2005; Garland and Kelly, 2006) and references therein], or how individual variation is affected by thermal

acclimation or acclimatization (e.g. Rezende et al., 2004b; Szafrańska et al., 2007).

Here we report how individual variation in mean daily energy expenditure ($\dot{V}_{O2mean}$) and maximum metabolic rate (maximum oxygen consumption; $\dot{V}_{O2max}$) in deer mice (*Peromyscus maniculatus sonoriensis* Le Conte) respond to thermal acclimation, and how these responses correlate with variations in organ mass, body composition and hematocrit. The prominent role of hypertrophy and elevated function of brown adipose tissue in cold acclimation has been well studied in a variety of mammals, including deer mice (Van Sant and Hammond, 2008). However, little is known about how other systems may change in response to thermal acclimation, and how these changes are associated with individual variation in $\dot{V}_{O2mean}$ and $\dot{V}_{O2max}$. The goals of this study were to determine: (i) how $\dot{V}_{O2mean}$ and $\dot{V}_{O2max}$ are related within and across acclimation treatments; (ii) which traits at lower levels of biological organization respond to thermal acclimation; (iii) whether there is sexual dimorphism in the response to thermal acclimation in *P. maniculatus*; and (iv) how responses at lower levels correlate with responses at the whole-organism level. *Peromyscus maniculatus* has been employed in a variety of studies on thermoregulation and altitude adaptation (e.g. Snyder et al., 1988; Chappell and Snyder, 1984; Chappell, 1985; Hayes and O'Connor, 1999; Chappell et al., 2007), and we think the approach outlined here may be useful for addressing similar questions in other study systems.

MATERIALS AND METHODS

We worked with mice from a laboratory colony of *P. m. sonoriensis* maintained in captivity for at least four generations at the University of California, Riverside (elevation 340 m above sea level). The colony founders (38 individuals) were captured in the White Mountains of eastern California at a local altitude of about 3800 m above sea level. Thirty-nine adults were separated into two groups that were subjected to contrasting ambient temperatures (T_a=5°C and 23°C). Both groups had initially been maintained at 23°C, and after subjecting one group to cold acclimation (20 individuals at T_a=5°C), body mass, food consumption, $\dot{V}_{O2max}$ and ventilatory variables were monitored every week during 7 weeks of acclimation (Rezende et al., 2004b). Deer mice were housed identically in standard plastic small mammal cages (27.5 cm L × 17 cm W × 12 cm H), and provided with bedding (wood shavings and cotton) and *ad libitum* water and rodent chow (LabDiet Rodent Diet 5001, Purina Mills, St Louis, MO, USA).

Apparent food consumption was estimated three times per week, and was calculated as the difference between food given and food remaining (crumbs were subtracted from apparent food consumption) divided by the elapsed time. Body mass remained constant in both groups during the last few weeks of acclimation. Therefore, we assumed that mice were in energetic equilibrium and used food consumption as a surrogate for daily energy expenditure. Mean daily oxygen consumption ($\dot{V}_{O2mean}$) was calculated assuming a food caloric content of 16.7 kJ g^{-1}, a digestibility of 89% (Mueller and Diamond, 2001), and the 0.0497 ml O$_2$/J conversion. Although changes in gut morphology following acclimation might affect digestibility and consequently $\dot{V}_{O2mean}$ estimates, most studies suggest that acclimation affects primarily food consumption and gut capacity while digestibility remains relatively constant (e.g. Zhao et al., 1995; del Valle et al., 2004).

Rates of oxygen consumption ($\dot{V}_{O2}$) during cold exposure were measured with open-flow respirometry in an atmosphere of heliox (79% He, 21% O$_2$). Heliox is severalfold more conductive than air and elicits maximal thermogenesis at relatively moderate temperatures (Rosenmann and Morrison, 1974; Chappell and Bachman, 1995). Briefly, mice were weighed and placed in the metabolism chamber at –5°C; the chamber was flushed with heliox for 1 min and $\dot{V}_{O2}$ was subsequently recorded for approximately 15 min. $\dot{V}_{O2max}$ was estimated as the highest continuous 60 s average of $\dot{V}_{O2}$ [measurements are described in detail in Rezende et al. (Rezende et al., 2004b)]. We also calculated the ratio between daily $\dot{V}_{O2mean}$ and $\dot{V}_{O2max}$ ($\dot{V}_{O2mean}/\dot{V}_{O2max}$), to assess how much energy individuals spent on average with respect to their maximum aerobic capacity.

One day after their last metabolic trial, individuals were killed and dissected (mice were between 386 and 1031 days old, with a mean body mass ± s.e. of 24.5±0.5 g). We anesthetized mice (0.07 ml of 65 mg ml^{-1} sodium pentobarbitol injected i.p.) and obtained replicate blood samples (approximately 150 µl) with two heparinized microhematocrit tubes *via* retro-orbital puncture. Samples were centrifuged for 10 min at 1000 *g*, and hematocrit was calculated as the mean proportion of packed cells relative to the total sample volume. After the mice had been killed (additional 0.1 ml of 65 mg ml^{-1} sodium pentobarbitol injected i.p.), we dissected out the visceral organs, removed all gut contents and measured the wet and dry mass of the heart, lungs, stomach, large and small intestines, cecum, liver, kidneys, spleen and carcass (i.e. head, feet, tail, musculoskeletal system and skin). Organs and carcass were dried for at least 48 h at 60°C and weighed again, and subsequently fat was extracted using petroleum ether (Kerr et al., 1982) in a Goldfische apparatus. Absolute fat content was estimated by subtraction (dry mass before extraction minus dry mass after extraction), and relative fat content was computed as a percentage of wet body mass.

All procedures were approved by the UC Riverside Institutional Animal Care and Use Committee (protocol # A-M 0408026 to K.A.H.) and conform to US National Institutes of Health Guidelines (NIH publication 78-23) and US laws.

Statistical analyses

During the last week of acclimation, raw and mass-independent food consumption were significantly repeatable across days for both cold- and warm-acclimated groups using pairwise Pearson correlations (1-tailed P<0.001 and P<0.014, respectively) or the intraclass correlation coefficient calculated from repeated-measures ANOVA [P<0.001 in both cases (Hayes and Jenkins, 1997; Rezende et al., 2005)]. Similarly, $\dot{V}_{O2max}$ was highly repeatable between weeks 6 and 7 after controlling for body size and acclimation regime (Pearson R=0.695, 1-tailed P<0.001) and values did not differ between weeks (paired *t*-test, t_{38}=0.40, P=0.692). Therefore, for subsequent analyses we employed the mean of data gathered in weeks 6 and 7 as the estimate of daily $\dot{V}_{O2mean}$ and the maximum estimate of $\dot{V}_{O2max}$ as more 'reliable' estimates of individual traits (see Hayes and Jenkins, 1997; Rezende et al., 2005).

The association between organ size, body composition, $\dot{V}_{O2max}$ and daily $\dot{V}_{O2mean}$ was analyzed with multiple regressions controlling for differences in body size and acclimation regime. We employed dummy variables (0–1) to define acclimatory regime (warm *vs* cold) and sex; hence this model is mathematically analogous to a two-factor ANCOVA. We initially analyzed the effects of acclimation on organ size and body composition accounting for differences in body size and sex. Unless stated otherwise, models do not include the acclimation×sex interaction (most responses to acclimation did not differ significantly between sexes in preliminary analyses).

We applied the following rationale to test which variables are potentially associated with acclimation effects in $\dot{V}_{O2}$ estimates. After

estimating the difference in $\dot{V}_{O_2}$ between warm- and cold-acclimated animals controlling for size and sex, analyses were repeated with the addition of one (or more) other variables to the model. If the acclimation effects were no longer significant following the inclusion of an additional variable, and the added variable was a significant predictor of $\dot{V}_{O_2}$, we inferred that the variable partly accounted for the difference between acclimation groups. Conversely, if the difference between acclimation groups remained significant and its magnitude was unchanged after adding another variable to the model, then other traits probably accounted for the acclimation effects on $\dot{V}_{O_2max}$ [for similar analyses, see Rezende et al. (Rezende et al., 2006)]. The same approach was employed to determine which variables could underlie sex differences.

Subsequently, we analyzed how $\dot{V}_{O_2max}$, daily $\dot{V}_{O_2mean}$, organ size, body composition and hematocrit were correlated at the level of individual variation, accounting for the effects of body size and sex. In order to avoid statistical redundancy, we used dry carcass mass as a surrogate for body size relatively independent of other organs (see Christians, 1999; Nespolo et al., 2002). To visualize how acclimation might have affected the relationship between $\dot{V}_{O_2max}$ and $\dot{V}_{O_2mean}$ with subordinate traits, we estimated the partial correlation between these variables separately for cold- and warm-acclimated individuals, controlling for sex and dry carcass mass. The standard deviation of these partial correlations was estimated with bootstrap with replacement (1000 replicates). Briefly, a random sample of 20 individuals was obtained with replacement from the original sample, the partial correlation between variables was calculated 1000 times, and the standard deviation was obtained from this distribution.

Statistical significance was judged at $\alpha=0.05$, and P-values presented are two-tailed unless otherwise noted. We tested directional hypotheses with one-tailed tests (e.g. $\dot{V}_{O_2max}$ and food consumption were expected to increase with cold acclimation, correlations between heart and lung mass and $\dot{V}_{O_2max}$ were expected to be positive, etc.). Because multiple regression models were performed to analyze the association between each $\dot{V}_{O_2}$ estimate and traits at lower levels of organization, statistical significance in these models was assessed after controlling for false discovery rates (Type I error) with the QVALUE program run as part of the R statistical package (The R Foundation for Statistical Computing, www.r-project.org/foundation/). Results were considered significant if the false discovery rate associated with each $P<0.05$ was smaller than 5% (i.e. q-value <0.05). All morphological indices were normally distributed (Kolmogorov–Smirnov $P>0.202$ in all cases) except for relative fat content and dry carcass mass. Visual inspections showed that the deviation from normality in relative fat content was due to a long tail of disproportionately high values for warm-acclimated females, hence log-transformation was applied only for dry carcass mass. Results are reported as means ± s.e.

RESULTS
Acclimation effects

Males attained significantly higher $\dot{V}_{O_2max}$ than females (Fig. 1). In addition, total fat content and organs of the digestive system were significantly smaller in males (Table 1; Fig. 2). After controlling for body mass and sex, acclimation effects were statistically significant for $\dot{V}_{O_2max}$, $\dot{V}_{O_2mean}$, and mass of several organs including heart, digestive organs, kidney and liver (Table 1). Effects of cold acclimation were particularly apparent for $\dot{V}_{O_2max}$ and $\dot{V}_{O_2mean}$, followed by increments in heart, digestive organs, kidney and liver mass (Fig. 2). These organs
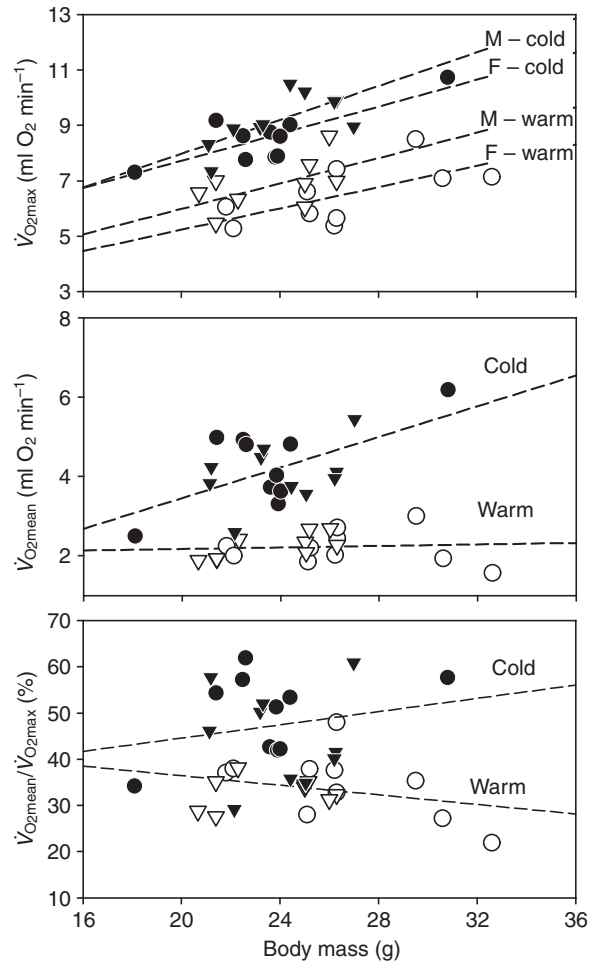

Fig. 1. Individual variation in maximum O_2 consumption ($\dot{V}_{O_2max}$), mean daily energy expenditure ($\dot{V}_{O_2mean}$) and $\dot{V}_{O_2mean}/\dot{V}_{O_2max}$ in cold- and warm-acclimated *Peromyscus maniculatus* (filled and open symbols, respectively). Circles, females (F); triangles, males (M). Dashed lines represent linear regressions plotted separately for the effects of sex or acclimation that were statistically significant (results are listed in Table 1).

increased in size with cold acclimation, concomitant with a significant reduction in absolute body fat content (Table 1). Significant acclimation×sex interactions in models without body mass show that the reduction in fat content following cold acclimation differs between sexes. In females, total fat content decreased 53% in response to cold acclimation (from 3.8 g in warm-acclimated females to 1.8 g in cold-acclimated females). The corresponding change in males was a 4% reduction (from 2.0 g to 1.9 g; acclimation×sex interaction $P=0.028$). Analyses of relative fat content yielded similar results: a 45% reduction in females (from 13.8% in warm-acclimated females to 7.6% in cold-acclimated females) and a 5% reduction in males (from 8.3% to 7.9%; $P=0.036$) (these mean estimates differ from values listed in Table 1 because of the inclusion of the acclimation×sex interaction in the model). Hematocrit increased after cold acclimation. This increase was significant ($F_{1,36}=4.37$, 2-tailed $P=0.044$) after removing body mass as a covariate, which was not significant in the full model (Table 1). Similar analyses including sex gave qualitatively similar results and the interaction was never statistically significant.

Table 1. Effects of sex, acclimation and body mass on morphological and physiological traits of *Peromyscus maniculatus*

| | Cold acclimated | | Warm acclimated | | Ratio | | | | |
	Males	Females	Males	Females	C/W	M/F	$P_{acclimation}$	P_{sex}	$P_{body\ mass}$
N	10	10	10	9					
Body mass (g)	23.17±0.80	24.33±0.80	24.60±0.83	25.75±0.80	0.94	0.95	0.135	0.226	
Carcass dry mass (g)	6.46 [5.97–7.00]	6.55 [6.06–7.07]	6.86 [6.34–7.43]	6.95 [6.42–7.53]	0.94	0.99	0.193	0.776	**<0.0001**
$\dot{V}_{O_2max}$ (ml min^{-1})	9.399±0.208	8.670±0.201	6.878±0.208	6.149±0.208	1.39	1.07	**<0.0001**	**0.004**	**<0.0001**
$\dot{V}_{O_2mean}$ (ml min^{-1})	4.241±0.189	4.239±0.183	2.157±0.189	2.155±0.189	1.97	1.00	**<0.0001**	0.993	**0.030**
Heart dry mass (g)	0.0495±0.0018	0.0483±0.0018	0.0365±0.0018	0.0353±0.0018	1.36	1.03	**<0.0001**	0.583	**0.0003**
Lung dry mass (g)[a]	0.0398±0.0012	0.0381±0.0012	0.0376±0.0012	0.0359±0.0012	1.09	1.05	0.125	0.229	**0.001**
Stomach dry mass (g)	0.1086±0.0046	0.1257±0.0045	0.0958±0.0046	0.1128±0.0046	1.13	0.86	**0.022**	**0.003**	**0.011**
Small intestine dry mass (g)	0.2244±0.0133	0.2632±0.0129	0.1655±0.0133	0.2043±0.0133	1.32	0.83	**0.001**	**0.016**	**0.017**
Large intestine dry mass (g)	0.0489±0.0032	0.0594±0.0031	0.0372±0.0032	0.0477±0.0032	1.28	0.80	**0.004**	**0.008**	**0.003**
Cecum dry mass (g)	0.0598±0.0030	0.0695±0.0029	0.0444±0.0030	0.0542±0.0030	1.32	0.84	**<0.0001**	**0.007**	**0.006**
Spleen dry mass (g)	0.0100±0.0010	0.0098±0.0010	0.0115±0.0011	0.0113±0.0010	0.87	1.02	0.236	0.896	**0.002**
Kidney dry mass (g)	0.1047±0.0032	0.1003±0.0031	0.0806±0.0032	0.0762±0.0032	1.31	1.05	**<0.0001**	0.235	**0.0004**
Liver dry mass (g)	0.4515±0.0119	0.4559±0.0115	0.3839±0.0119	0.3883±0.0119	1.17	0.99	**<0.0001**	0.750	**<0.0001**
Absolute body fat (g)	1.683±0.240	2.354±0.238	2.406±0.246	3.077±0.240	0.74	0.75	**0.014**	**0.021**	**<0.0001**
Relative body fat (%)	7.12±0.91	9.24±0.91	9.60±0.94	11.73±0.91	0.77	0.80	**0.026**	0.053	**<0.0001**
Hematocrit (%)	50.90±0.86	49.70±0.86	48.94±0.86	47.75±0.86	1.04	1.02	0.062	0.242	0.463

We report adjusted means ± s.e. for a deer mouse of 24.5 g, or 95% confidence intervals between brackets for carcass dry mass back-transformed from $\log_{10}$ values. Ratios were estimated from acclimation or sex-adjusted means controlling for all additional factors and covariates (acclimation×sex interaction not included; see Materials and methods). Values in bold are significant at α=0.05.

C, cold acclimated; W, warm acclimated; M, males; F, females.

[a]One cold-acclimated female was removed from the analyses because of measurement problems.

$\dot{V}_{O_2max}$ and daily $\dot{V}_{O_2mean}$

Individual variation of $\dot{V}_{O_2mean}$ and $\dot{V}_{O_2max}$ was positively correlated (1-tailed *P*=0.036) after controlling for acclimation, sex and body mass. This association was more pronounced in warm-acclimated animals (Fig. 3), indicated by a significant $\dot{V}_{O_2mean}$×acclimation interaction ($F_{1,33}$=7.01, *P*=0.012) in a multiple regression with $\dot{V}_{O_2max}$ as the dependent variable. Although $\dot{V}_{O_2mean}$ remained a significant predictor ($F_{1,33}$=10.9, *P*=0.002), regressions performed separately for both acclimation groups supported a significant association between $\dot{V}_{O_2max}$ and $\dot{V}_{O_2mean}$ only for warm-acclimated mice ($F_{1,15}$=11.32, *P*=0.004; for cold-acclimated animals, $F_{1,16}$=0.006, *P*=0.937).

Although both $\dot{V}_{O_2mean}$ and $\dot{V}_{O_2max}$ increased during cold acclimation (Fig. 1), cold-acclimated mice maintained daily $\dot{V}_{O_2mean}$ significantly closer to their aerobic 'ceiling' than warm-acclimated

animals ($\dot{V}_{O_2mean}$ was 47.2±1.8% and 33.7±1.9% of $\dot{V}_{O_2max}$, respectively; $F_{1,35}$=25.54, *P*<0.0001). Effects of body mass and sex were not significant (*P*>0.213 in both cases). The acclimation×body mass interaction was not statistically significant when included in the regression analysis of $\dot{V}_{O_2mean}/\dot{V}_{O_2max}$ ($F_{1,34}$=3.22, *P*=0.081).

Body mass

Dry carcass mass was the best predictor of wet body mass and explained 60% of the variance in multiple regressions ($F_{1,36}$=54.0, *P*<0.0001), accounting for 27.9±4.5% of total wet body mass and 91.5±1.9% of total dry body mass. Dry whole-body mass was significantly correlated with all variables except kidney dry mass and hematocrit, whereas carcass dry mass correlated significantly only with liver dry mass and total and relative fat content (1-tailed *P*<0.05). Correlation coefficients were smaller for carcass mass
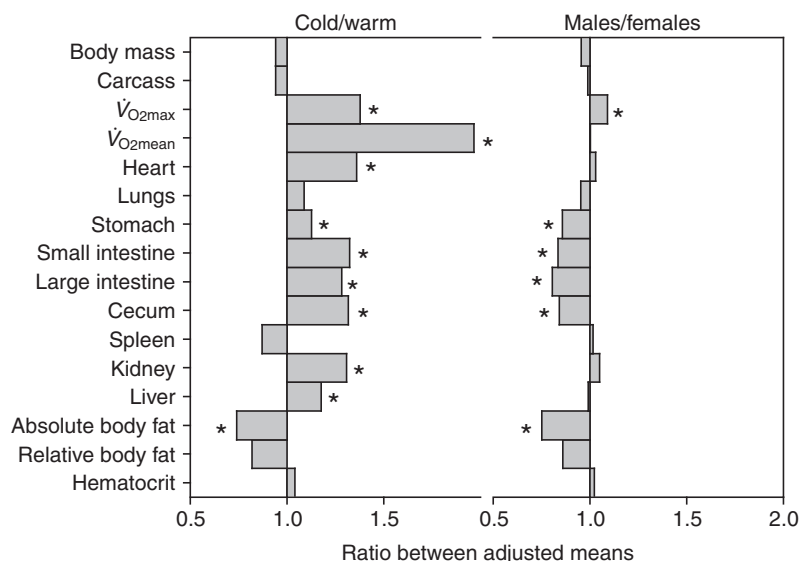

Fig. 2. Ratio between adjusted means obtained for each variable comparing cold- *vs* warm-acclimated animals, and males *vs* females. Effects of these factors were tested in a two-factorial model with acclimation and sex as fixed factors, and body mass as a covariate. Significant differences between acclimation groups or sexes are shown with asterisks (results are listed in Table 1).
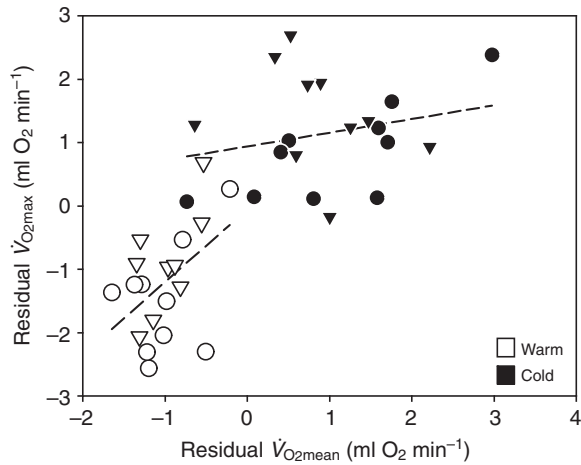
Fig. 3. Relationship between residual daily energy expenditure ($\dot{V}_{O2mean}$) and $\dot{V}_{O2max}$ after controlling for body size. Circles, females; triangles, males. Dashed lines represent linear regressions obtained separately for warm- and cold-acclimated deer mice.

(except for total and relative fat content). Accordingly regressions for $\dot{V}_{O2max}$ or $\dot{V}_{O2mean}$ against organ size used carcass dry mass as a surrogate for body size.

Organ size

After controlling for differences in acclimation regime, sex and body size, the addition of heart and lung dry mass, and total and relative fat content, significantly improved the regression model for $\dot{V}_{O2max}$ (Table 2). Differences in $\dot{V}_{O2max}$ between cold- and warm-acclimated animals decreased from 39% to 28% when heart mass was included (Table 2), although acclimation effects remained highly significant ($P<0.0001$). Conversely, sex differences in $\dot{V}_{O2max}$ decreased from 8.9% to 4.0% after total body fat was included (Table 2), and were no longer significant ($F_{1,34}=1.355$, $P=0.253$). Inclusion of all variables that significantly improved the initial regression (Table 2) failed to increase predictive power (adjusted means for cold-acclimated animals remained 28% higher than means for warm-acclimated mice), although sex differences were no longer

significant. In this model, acclimation regime, body size, heart mass and absolute body fat content remained statistically significant (1-tailed $P<0.035$ in all cases), while sex and lung dry mass did not ($P=0.175$ and 0.669, respectively).

Heart, lung, kidney and liver dry mass as well as body fat content were significantly correlated with $\dot{V}_{O2mean}$. The dry mass of the digestive tract was marginally correlated with $\dot{V}_{O2mean}$ (Table 2). Including heart mass, kidney mass, liver mass and digestive tract mass separately in the initial model resulted in the largest reduction of acclimation effects (Table 2). The inclusion of all variables that significantly improved the initial model in a single multiple regression reduced the difference in adjusted means between cold- and warm-acclimated animals to 50.2% (compared with the 90.0% difference estimated from the initial model). Nevertheless, acclimation effects were still significant ($P<0.0001$) and none of the variables remained significantly correlated with $\dot{V}_{O2mean}$ in this model ($P>0.101$ in all cases).

We tested the acclimation regime×organ size interaction in the relationship between $\dot{V}_{O2max}$ or daily $\dot{V}_{O2mean}$. The interaction was significant only for $\dot{V}_{O2mean}$ and kidney dry mass (2-tailed $P=0.043$). Regressions performed separately for the two acclimation regimes showed no relationship between kidney mass and $\dot{V}_{O2mean}$ in warm-acclimated animals (1-tailed $P=0.437$) and a significant positive correlation in cold-acclimated animals (1-tailed $P=0.041$) after accounting for body size effects and controlling for sex (Fig. 4).

DISCUSSION
Emphasis on individual variation is crucial when attempting to determine the role of physiological plasticity in performance and fitness (Hayes and Jenkins, 1997; Sears et al., 2006), and ultimately to understand how plasticity may affect population responses to selection [either natural or artificial (e.g. Garland and Kelly, 2006; Fordyce, 2006)]. For example, $\dot{V}_{O2max}$ is heritable in the rodent *Phyllotis darwini* (Nespolo et al., 2005) and under natural selection in wild populations of *P. maniculatus* (Hayes and O'Connor, 1999). In this context physiological plasticity is relevant for at least two reasons. First, differences in individual performance might not be consistent across all environmental conditions; e.g. individuals with high $\dot{V}_{O2max}$ following warm acclimation might not maintain their performance rank in the population after cold acclimation. We have previously shown that individual differences in deer mouse $\dot{V}_{O2max}$

Table 2. Analyses of covariance for $\dot{V}_{O2max}$ and daily $\dot{V}_{O2mean}$ testing for effects of acclimation, sex and different subordinate traits, controlling for differences in carcass mass (as a surrogate for body size, see Materials and methods)

Initial model (IM)	d.f.	\(\dot{V}_{O2max} \) C/W	M/F	F change	P	\(\dot{V}_{O2mean} \) C/W	M/F	F change	P
Acclimation	1,35			82.582	**<0.0001**			67.961	**<0.0001**
Sex	1,35			6.018	**0.019**			0.129	0.722
Carcass mass	1,35	1.39	1.09	13.520	**0.001 [+]**	1.90	0.97	0.099	0.755 [+]
IM + heart	1,34	1.28	1.09	9.222	**0.005 [+]**	1.61	0.97	6.789	**0.014 [+]**
IM + lungs[a]	1,33	1.37	1.07	5.441	**0.026 [+]**	1.81	0.92	11.763	**0.002 [+]**
IM + digestive tract	1,34	1.34	1.13	2.924	0.096 [+]	1.73	1.05	3.715	0.062 [+]
IM + spleen	1,34	1.40	1.09	0.861	0.360 [+]	1.96	0.98	2.848	0.101 [+]
IM + kidney	1,34	1.33	1.08	1.929	0.174 [+]	1.61	0.96	6.249	**0.017 [+]**
IM + liver	1,34	1.35	1.09	1.990	0.167 [+]	1.69	0.99	5.439	**0.026 [+]**
IM + absolute body fat	1,34	1.35	1.04	11.426	**0.002 [−]**	1.80	0.89	8.671	**0.006 [−]**
IM + relative body fat	1,34	1.38	1.07	11.053	**0.002 [−]**	1.87	0.95	4.974	**0.032 [−]**
IM + hematocrit	1,34	1.39	1.09	0.023	0.881 [+]	1.93	0.98	0.337	0.566 [−]

Signs of partial regression coefficients for carcass mass and subordinate traits are in brackets. The magnitude of acclimation and sex effects is shown with ratios between adjusted means from cold- *vs* warm-acclimated animals (C/W) and males *vs* females (M/F). Values in bold are statistically significant after accounting for false discovery rates (Materials and methods).
[a]One cold-acclimated female was removed from the analyses because of measurement problems.
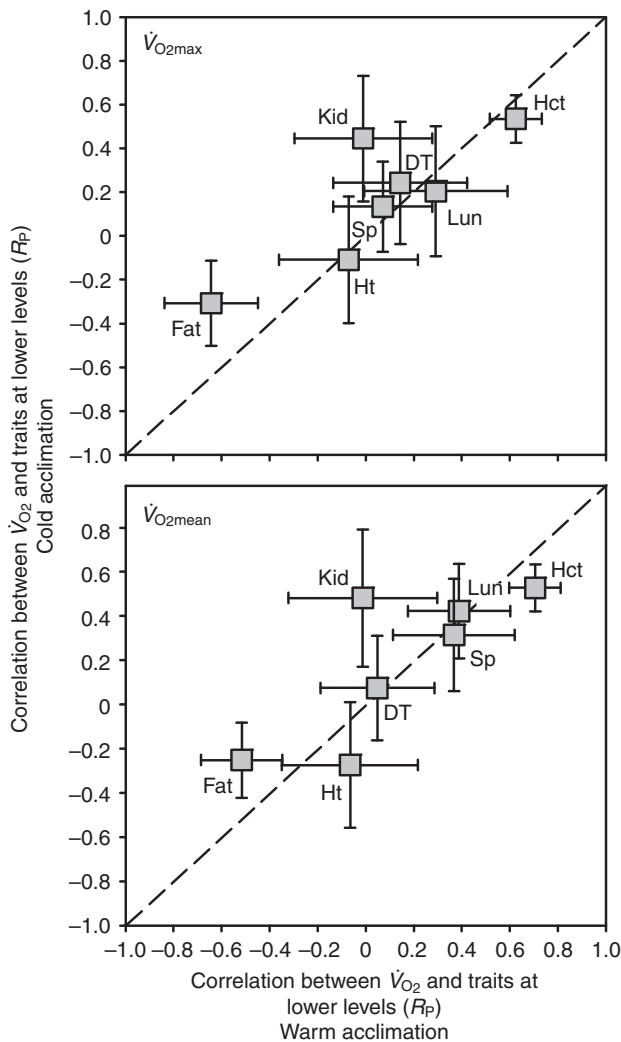
Fig. 4. Association between $\dot{V}_{O_2max}$ and $\dot{V}_{O_2mean}$ with traits at lower levels of biological organization obtained at different acclimation temperatures. Each data point represents the partial correlation (±s.d. obtained from bootstrap), controlling for dry carcass mass and sex, between $\dot{V}_{O_2}$ and lower level traits (Kid, kidney; Ht, heart; Lun, lung; Sp, spleen; Fat, total absolute fat; DT, digestive tract; Hct, hematocrit). The dashed line depicts the 1:1 relationship. Regression analyses suggest that the association between dry kidney mass and $\dot{V}_{O_2mean}$ has changed significantly with acclimation (see Results).

brown adipose tissue (Smith and Horwitz, 1969; Cannon and Nedergaard, 2003)]. Regardless of the mechanistic basis underlying this pattern, it is evident that selection on one of these traits will affect the other in a correlated fashion, although how they co-evolve will depend on the environmental conditions encountered by the animals.

Acclimation and energy balance

As reported previously (Bacigalupe et al., 2003; Rezende et al., 2004b), the occurrence and magnitude of responses to acclimation varied considerably (Fig. 2). The heart, kidney, liver and digestive organ mass increased significantly in response to cold acclimation (Table 1). Hematocrit also showed a moderate increase with cold acclimation. However, lung mass showed no response to acclimation temperature.

In general, these findings resemble results from other acclimation or acclimatization studies (Hammond et al., 2001; Russell and Chappell, 2007), though discrepancies exist regarding the plasticity of lungs and pulmonary function. Some studies reported an increase in lung mass and presumably lung capacity during cold acclimation (e.g. McDevitt and Speakman, 1994; Hammond et al., 1999; Hammond et al., 2001; Deveci and Egginton, 2002), whereas others did not (Hoppeler et al., 1995; Russell and Chappell, 2007). Even though the adult vertebrate lung seems to have limited plasticity compared with other organs (Weibel, 2000; Hsia, 2001; Henderson et al., 2002), several confounding factors possibly obscure comparisons. For instance, phenotypic plasticity during development often differs from acclimatory responses during adulthood (Spicer and Burggren, 2003; Russell et al., 2008), lung plasticity may be associated with particular environmental challenges [e.g. hypoxia (see Hammond et al., 2001)], and levels of plasticity may differ between species and/or populations (e.g. Bacigalupe et al., 2003).

Although several organs were plastic in response to thermal acclimation, only heart mass and fat content were consistent predictors of aerobic capacity ($\dot{V}_{O_2max}$) across acclimation conditions. Animals with larger hearts and less fat mass attained higher $\dot{V}_{O_2max}$. This is not surprising because of the relevance of cardiac output as a determinant of $\dot{V}_{O_2max}$ (Bishop, 1997; Rezende et al., 2006) and the negligible contribution of depot fat (white adipose tissue) to metabolism. Nevertheless, the combination of heart mass and fat content explained only 30% of the total increase in $\dot{V}_{O_2max}$ during cold acclimation.

Daily $\dot{V}_{O_2mean}$ showed the largest increase in response to cold acclimation (Fig. 2), and individual differences in $\dot{V}_{O_2mean}$ were significantly correlated with the mass of several organs [heart, lung, kidney and (marginally) digestive tract mass; Table 2]. However, causation cannot be inferred from our strictly correlational approach, and some correlates of $\dot{V}_{O_2mean}$ might reflect responses of organs to varying levels of energy expenditure instead of to temperature *per se*. For instance, kidney mass may increase in cold-acclimated mice either as a cause of or a response to increased $\dot{V}_{O_2mean}$. The lack of correlation between kidney mass and $\dot{V}_{O_2max}$ supports the 'response to $\dot{V}_{O_2mean}$' hypothesis, but does not rule out a possible contribution of kidney function to thermogenesis following thermal acclimation (e.g. Inbar et al., 1975; Greenwald and Stetson, 1988; Diaz et al., 2006).

The significant negative correlation between $\dot{V}_{O_2mean}$ and fat content suggests that those individuals that eat more have less fat (Table 2). Because body mass remained constant during the weeks before these measurements [fig. 2 of Rezende et al. (Rezende et al., 2004b)], this presumably reflects individual differences in energy balance: mice with lower energy requirements, possibly due to

are repeatable across acclimation temperatures (Rezende et al., 2004b) (see also Hayes and Chappell, 1990), suggesting physiological commonality underlying $\dot{V}_{O_2max}$ in warm- and cold-acclimated animals (e.g. central support organs and – at least partially – peripheral effectors).

The second reason physiological plasticity is important is that phenotypic traits will evolve in a correlated fashion in response to selection (Feder et al., 2000; Garland, 2003), but the association between traits may vary depending on environmental conditions (e.g. Hammond et al., 2001; Russell and Chappell, 2007; Chappell et al., 2007). For example, in our mice the relationship between $\dot{V}_{O_2max}$ and daily $\dot{V}_{O_2mean}$ was less pronounced in the cold-acclimated group (Fig. 3). This result, along with the significant differences in $\dot{V}_{O_2mean}/\dot{V}_{O_2max}$ between acclimation groups, probably reflects behavioral changes in the amount of activity and/or the recruitment of additional tissues to sustain thermogenesis in the cold [presumably

increased thermoregulatory efficiency or less spontaneous activity, may retain more fat. We regressed total and relative fat content against $\dot{V}_{O2mean}/\dot{V}_{O2max}$ to test whether individuals that spend a lower fraction of their total capacity accumulate more fat (acclimation, sex, carcass mass and $\dot{V}_{O2max}$ were controlled in the model). The significant negative correlation in both regressions ($F_{1,33}$=8.17, P=0.007 and $F_{1,33}$=4.50, P=0.042 for total and relative fat content, respectively) shows that individuals with higher metabolic rates tend to be leaner, which supports a trade-off between rates of energy expenditure and fat storage.

Sex differences

Males and females differed significantly in body composition, particularly in the mass of digestive organs and fat content (Fig. 1; Table 1). Previous studies have reported similar sex differences in digestive organs in *P. maniculatus* (Hammond et al., 1999), but did not find differences in fat content (Hammond et al., 1999; Schulte-Hostedde et al., 2001; Chappell et al., 2003). However, the acclimation periods used for these studies were not as long as in the current study. Several other factors might also account for the differences, including variation in experimental protocols [e.g. wild-caught *Peromyscus* were studied by Schulte-Hostedde et al. (Schulte-Hostedde et al., 2001)] and age effects [our mice were considerably older than the animals studied by Hammond et al. (Hammond et al., 1999)]. In addition, sex differences in relative fat content tended to be larger in warm-acclimated animals (Results; Table 1), emphasizing that plasticity is an additional confounding factor.

The finding that warm-acclimated females tend to accumulate more fat than males, and that the sex differences in body mass change during the course of cold acclimation [see fig. 2 of Rezende et al. (Rezende et al., 2004b)], suggest that control of fat storage differs between the sexes in *P. maniculatus*. Heavier digestive tracts in females might be associated with higher nutrient absorption and fat accumulation, if digestibility remains constant and food consumption increases (see Materials and methods). Previous studies have suggested that differences in body composition reflect differences in reproductive roles between sexes, and that females accumulate more fat than males to support reproduction [(Schulte-Hostedde et al., 2001; Speakman, 2008) and references therein]. Although increased fat accumulation in females might have been favored by natural selection, it is possible they are penalized in terms of thermoregulation. Females have significantly lower $\dot{V}_{O2max}$ than males (Table 1), and differences in fat content between sexes seem to account for this pattern. Future studies should test this possibility because it is not clear to what extent the higher fat content in females might impact on insulation and/or thermogenic endurance.

Conclusions and future directions

Thermal acclimation in *P. maniculatus* resulted in responses both at the whole-animal level ($\dot{V}_{O2max}$ and $\dot{V}_{O2mean}$) and in sub-organismal traits (heart, liver, kidney and digestive organ mass). Responses differed among organs and much of the whole-animal acclimatory changes could not be explained by modulation of organ size. Our results suggest that larger hearts, presumably resulting in higher cardiac output, are partly responsible for increased maximum thermogenic capacities following cold acclimation (Table 1). In contrast, kidney and liver plasticity seems to be more strongly associated with sustained energy turnover than with thermogenic capacity. Although we found correlations in the size of some organs with $\dot{V}_{O2max}$ and $\dot{V}_{O2mean}$, many acclimatory responses probably occur at lower levels of organization (e.g. capillarization, mitochondrial concentration, enzyme or substrate dynamics, etc.).

Significant sex effects were detected for $\dot{V}_{O2max}$, body fat content and mass of digestive organs. It would be of interest to determine whether these results are associated with behavioral differences in the field. For instance, lower thermogenic capacity in females may constrain activity in extreme cold (Sears et al., 2006; Hayes and O'Connor, 1999), but their greater fat content may provide more thermogenic endurance at less stressful temperatures. Hence differences between sexes may impose (or result in) different patterns of activity and foraging behavior. Finally, previous studies have reported sporadic breeding for *P. maniculatus* during winter, with considerable variation across altitudes [Dunmire (Dunmire, 1960) and references therein]. In this context, *P. maniculatus* may provide a useful model for studying the interplay between reproductive allocation, physiological performance, behavior and the different selective pressures associated with each sex (see also Speakman, 2008).

Our understanding of physiological plasticity has improved during the last three decades, yet we have much to learn about how these responses have evolved within or between species (Garland and Kelly, 2006). Studies focusing on individual variation in thermal acclimation may provide important information on how multiple physiological systems (e.g. organs that deliver oxygen, process food, remove waste, etc.) respond coherently to accommodate changing environmental conditions. Detailed information on the plasticity of whole-organism traits and subordinate traits is crucial for a comprehensive understanding of the evolution of physiological plasticity, as well as the role of plasticity in ecological diversification.

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