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# Cold-acclimation in *Peromyscus*: temporal effects and individual variation in maximum metabolism and ventilatory traits

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**Summary**

**Thermal acclimation in small endotherms provides an excellent model for the study of physiological plasticity, as energy requirements can be easily manipulated and the results are relevant for natural conditions. Nevertheless, how physiology changes throughout acclimation, and how individuals vary in their response to acclimation, remain poorly understood. Here we describe a high temporal-resolution study of cold acclimation in the deer mouse *Peromyscus maniculatus*. The experimental design was based on repeated measures at short intervals throughout cold acclimation, with controls (maintained at constant temperature) for measurement artifacts. We monitored body mass, maximum metabolic rate in cold exposure and ventilatory traits (respiratory frequency, tidal and minute volume and oxygen extraction) for 3 weeks at 23°C. Then, half of the individuals were held for 7 weeks at 5°C. Body mass was differently affected by cold acclimation depending on sex. Maximal metabolism ($\dot{V}_{O_2max}$) increased significantly during the first week of cold acclimation, 'overshot' after 5 weeks and dropped to a plateau about 34% above control values at week 7. Similarly, ventilatory traits increased during cold acclimation, though responses were different in their kinetics and magnitude. Body mass, maximum metabolism, and most ventilatory traits were repeatable after 7 weeks in control and cold-acclimated animals. However, repeatability tended to be lower in the cold-acclimated group, especially while animals were still acclimating. Our results show that acclimation effects may be under- and/or overestimated, depending on when trials are performed, and that different traits respond differently, and at different rates, to acclimation. Hence, future studies should be designed to ensure that animals have attained steady-state values in acclimation experiments.**

Key words: acclimation, ambient temperature, maximal oxygen consumption, physiological plasticity, *Peromyscus maniculatus*, repeatability, thermogenesis, ventilation.

## Introduction

During their lifetime, most animals can compensate for changing environmental conditions by altering functional capacities of physiological systems (physiological plasticity), by changing behavior (behavioral plasticity), or both (Garland and Carter, 1994; Huey and Berrigan, 1996). These adjustments help match performance capacity to environmental demands, and hence may be crucial ecologically. The timing and magnitude of physiological plasticity is also important in an evolutionary context, because the ability to change performance limits in concert with changing demands may have drastic consequences for fitness in a fluctuating environment (Huey and Berrigan, 1996; DeWitt et al., 1998; Wilson and Franklin, 2002).

For several reasons, thermal acclimation in small endotherms is a useful system for studying physiological plasticity. First, it can be induced simply by changing ambient temperature. Second, the response can be easily measured as maximal rates of oxygen consumption ($\dot{V}_{O_2max}$). Third, thermal acclimation is ecologically relevant in highly seasonal habitats (Rosenmann et al., 1975; Cygan, 1985; Zegers and Merritt, 1988; Hayes, 1989; Bozinovic et al., 1990; Merritt, 1995; Kronfeld-Schor et al., 2000). Fourth, there are considerable data on the mechanistic basis of thermal acclimation at different levels of organization, from organ size (e.g. McDevitt and Speakman, 1994; Speakman and McQueenie, 1996; Derting and Austin, 1998; Hammond and Kristan, 2000), to physiology and biochemistry (Golozoubova et al., 2001; Nedergaard et al., 2001; Deveci et al., 2001; Shmeeda et al., 2002), to gene expression (Jacobsson et al., 1994; Yu et al., 2002). Finally, recent studies have found significant selection on $\dot{V}_{O_2max}$ in wild populations, re-emphasizing its evolutionary and ecological relevance (Hayes and O'Connor, 1999; E. L. Rezende, F. Bozinovic and T. Garland, unpublished results).

Despite considerable study, some aspects of thermal acclimation merit additional work. There are few data on within-individual performance consistency across acclimatory events (Hayes and Chappell, 1990; Nespolo and Rosenmann, 1997). Individual consistency (repeatability) over time is a prerequisite for natural selection to affect trait variation, and it may set the upper limit on the narrow sense heritability of the

trait if certain conditions are fulfilled (Hayes and Jenkins, 1997; Dohm, 2002). Also of major interest is the time course of acclimation – the latency of response to a changed environment, and the time necessary for acclimation to reach a stable end point. Besides its biological repercussions, the time course of acclimation has practical ramifications. For comparative analyses of acclimatory responses, it is necessary to know whether the end point of a study represents completion of acclimation (i.e. a new physiological steady state) or a time when physiology is still changing in response to environmental change. For example, a brief survey of thermal acclimation studies cited in this paper (see References) revealed a sevenfold range in acclimation periods (2–4 weeks), and there were few controls on the progress or completion of acclimation (e.g. Nespolo and Rosenmann, 1997). Thus, within a single individual or a single species, it is difficult to come to conclusions about when acclimation is actually complete.

Temporal patterns may also provide clues about the mechanistic underpinnings of acclimatory responses. Presumably, acclimation requires adjustment of multiple set-points in reaction to a new thermal environment; given the complexity of potential changes at many integrative levels and the likelihood of time lags between detection and responses, we hypothesized that the kinetics of cold acclimation may include an 'overshoot' of $\dot{V}_{O_2max}$ before stable acclimation is achieved (as reported for *Abrothrix andinus;* Nespolo and Rosenmann, 1997). Accordingly, we designed a high temporal-resolution study of cold acclimation in the deer mouse *Peromyscus maniculatus*, a species with a strong acclimatory response to cold (Hayes and Chappell, 1986). The experimental design was based on repeated measures with controls for measurement artifacts, which allowed analyses of the detailed temporal pattern of acclimation and the effects of acclimation on individual consistency of body mass, $\dot{V}_{O_2max}$ and several associated ventilatory traits (the initial stages of oxygen uptake).

## Materials and methods

### *Thermal acclimation*

We used 42 adults (316–951 days old at the beginning of the experiment) from a colony of *Peromyscus maniculatus sonoriensis* Le Conte that had been maintained in constant conditions at the University of California, Riverside, USA for four generations (the colony was descended from about 35 wild deer mice captured in the White Mountains of eastern California, USA). Initial body mass ($M_b$) was 24.5±2.9 g (mean ± S.D.), with no differences between males and females ($P$=0.49). 2 weeks before measurements, animals were randomly assigned to control or cold-acclimation treatments and transferred to individual cages provided with water and food *ad libitum*. For the first 3 weeks of testing, both treatments remained under common conditions (14 h:10 h L:D photoperiod, ambient temperature $T_a$=23°C). At the beginning of the fourth week, individuals in the cold-acclimation treatment were moved to a cold room ($T_a$=5°C, 14 h:10 h L:D) for 7 weeks, whereas control animals were maintained in the same constant environment ($T_a$=23°C).

We needed repeated measurements from each mouse, but $\dot{V}_{O_2max}$ estimation by acute cold exposure may itself induce acclimation (Heimer and Morrison, 1978). To minimize this problem and control for its effects, we divided each treatment group into three subgroups ($N$=7 per group, 4 and 3 of each sex) that did not differ in age or size. (For simplicity, 'groups' will be used to make reference to the six subgroups [i.e. group effects, etc.], and 'treatments' will refer to the two different acclimatory regimes.) Each group was measured once a week, and different groups within a treatment (cold-acclimated or control) were measured every 2 days. This provided three data points per treatment per week, but each individual was measured only once per week (see Fig. 1).

Two animals died for unknown reasons during acclimation, one in each treatment. One control mouse was not included in analyses because its $M_b$ increased by 55.1% during the experiment, contrasting with the average increase in $M_b$ of 3.7±11.7% (1.2±11.5% in the cold-acclimated group and 6.2±11.9% in controls).

### *Metabolism and ventilation*

We measured $\dot{V}_{O_2max}$ in an atmosphere of heliox (79% He, 21% $O_2$), which is several-fold more conductive than air (Chappell and Bachman, 1995). The open-circuit system contained a Plexiglas metabolism chamber (volume 600 ml) supplied with heliox at 1700 ml min$^{-1}$ (maintained ±1% with a Tylan mass flow controller; Mykrolis Corporation, USA). An environmental cabinet controlled the temperature of the metabolism chamber. About 100 ml min$^{-1}$ of excurrent gas was diverted, dried and scrubbed of $CO_2$ (Drierite® and Soda lime, respectively), redried, and passed through an S-3A $O_2$ analyzer (Applied Electrochemistry; CA, USA). Flow rate, $T_a$, and $O_2$ concentration were recorded every second by a Macintosh computer running 'Labhelper' software (www.warthog.ucr.edu). Animals were placed in the metabolism chamber at a $T_a$ approx. –5°C and recording began as soon as the system was completely flushed with heliox (approx. 1 min). $T_a$ declined at a rate of approx. 0.5°C min$^{-1}$. We terminated measurements and removed animals when $V_{O_2}$ remained below initial values for more than 1 min, or did not increase as $T_a$ declined by more than 2°C. Trials lasted no longer than 15 min. Immediately after removing an animal from the chamber, body temperature $T_b$ was determined (±0.1°C) using a rectal thermocouple connected to a Bailey BAT-12 (Sensortek Inc., USA) thermometer. All $\dot{V}_{O_2max}$ tests were performed between 09:00 h and 13:00 h (local time). Oxygen consumption ($\dot{V}_{O_2}$) was calculated using equation 4a of Withers (1977a), and $\dot{V}_{O_2max}$ was determined as the highest continuous average value of $\dot{V}_{O_2}$ over a 60 s period.

During $\dot{V}_{O_2max}$ trials we measured breathing frequency ($f$; Hz) and tidal volume ($V_T$; ml) using whole-body plethysmography (Withers, 1977a,b; Bucher, 1981; Chappell, 1985). Chamber pressure changes due to warming and humidification of tidal air were recorded with a pressure

transducer (Omega PX 164-010; Omega Engineering, Inc., Stanford, USA) connected to the computer and sampled at 125 Hz. The system was calibrated after each trial by injecting a known volume of heliox (1.0 ml) into the chamber at rates matching the kinetics of inhalation cycles. $V_T$ was calculated from calibration data and pressure changes during inspiration according to Malan (1973); we assumed lung temperature was 37°C (based on post-measurement $T_b$ data) and that air in the respiratory tract was 100% saturated with water vapor. Oxygen extraction efficiency (OEE, %) was calculated as $100\times\dot{V}_{O_2max}/(0.2095V_{MIN})$, where $V_{MIN}$ (minute volume) is $fV_T$.

### *Analysis of acclimation effects*

In most animal taxa the relationship between body mass and metabolism is best fit by the power equation $\dot{V}_{O_2}=aM_b^b$ (Darveau et al., 2002). Hence, all statistical tests, with the exception of repeatability analyses, were performed with log-transformed values of $\dot{V}_{O_2max}$ and $M_b$ (for simplicity, we refer to log-transformed data as $\dot{V}_{O_2max}$ and $M_b$). Sequential Bonferroni adjustments (Rice, 1989) were employed to control for Type I errors in multiple simultaneous tests. All analyses were performed using SPSS for Windows.

Analyses of variance (ANOVA) and covariance (ANCOVA) were performed to ensure that there were no differences between groups during the first 3 weeks of measurements ($T_a$=23°C). Comparisons were done between all six groups within a given week, and $M_b$ was included as a covariate for other traits. No between-group differences were observed in $M_b$ during the first 3 weeks ($P>0.640$ in all weeks). However, $\dot{V}_{O_2max}$ was highly variable during the first week, and significantly different among groups (ANCOVA, $F_{5,34}$= 3.452, $P$=0.013; Fig. 1), whereas no differences were observed during the second and third weeks (ANCOVA, $F_{5,34}$= 0.902, $P$=0.491 and $F_{5,34}$= 0.814, $P$=0.548, respectively). We believe that variation in the first week was probably related to non-standardized procedures and initial adjustment of animals to the experimental conditions. Therefore, data from the first week of measurements was not included in other analyses.

Temporal changes in $\dot{V}_{O_2max}$ and ventilatory variables during acclimation were analyzed in two ways. First, we used general linear mixed models for repeated measures (GLM), in which individuals were experimental units with time as a within-subjects factor. We employed Mauchly's sphericity test to determine if the variance–covariance matrix of the repeated measure variables is circular in form ['sphericity' or Huynh–Felt (H–F) condition; i.e. whether orthonormalized contrasts are independent and have equal variances. This can be thought of as an extension of the homogeneity of variance assumption in independent measures ANOVA]. Where the sphericity condition did not hold, $P$-values of within-subject effects were reported with H–F adjustments, which basically consist of discounting degrees of freedom by a factor proportional to the H–F condition to be met (Littell et al., 1996). Sex and treatment were included as between-subject factors; this allowed us to quantify the effects of cold-acclimation controlling for sex (time × acclimation effect). To determine when physiological changes occurred, contrasts (differences between successive weekly values for individuals) were compared with multivariate ANOVAs (test of within-subjects contrasts). Comparisons among contrasts were performed separately for each treatment (cold-acclimated and control), with sex included as a fixed factor.

Second, we assessed the effects of cold acclimation with separate ANCOVAs similar to the preliminary analyses described above. We pooled data of all subgroups in each treatment within each week of measurement (9 weeks in total), and compared pooled weekly values between the two treatments. Acclimation and sex were included as fixed factors and $M_b$ was included as a covariate. To study the relationship between $\dot{V}_{O_2max}$ and ventilatory traits, we performed a similar analysis with $\dot{V}_{O_2max}$ as an additional covariate.

### *Repeatability*

We performed one-tailed Pearson product–moment correlations between values measured at different weeks to determine repeatability. We used this approach instead of the intraclass correlation coefficient to assess repeatability, because we expected cold acclimation to change the mean values for many of the traits measured (see Hayes and Jenkins, 1997). A drawback of this method is that only pair-wise comparisons can be performed, necessitating adjustment of $\alpha$ if repeatability is estimated over several intervals (Rice, 1989).

Pearson correlations were performed at 2, 6 and 10 weeks. At 2 and 10 weeks the cold-acclimated group was fully acclimated, whereas at 6 weeks cold-acclimated individuals were still increasing $\dot{V}_{O_2max}$ (Fig. 1). Because different groups were measured at different times in the course of acclimation, analyses were initially performed by group (6 groups in total). However, sample sizes were small within groups ($N$=6 or 7), decreasing statistical power, and interpretation of results was complex (see Discussion). Therefore, correlations were performed with values pooled per week (weeks 2, 6 and 10; 2 treatments in total, $N$=19 or 20 per treatment), on both non-transformed traits ($M_b$ differences are intrinsic in this case) and mass-independent traits (residuals from least-square mass regressions carried out separately for both initial and final measurements), and a sequential Bonferroni was employed to control Type I errors.

As a second method to estimate inter-individual variation through acclimation, product–moment correlations were performed between the average values of traits measured during 2 weeks prior to acclimation (weeks 2 and 3) and during the last 2 weeks of acclimation (weeks 9 and 10). Assessing repeatability of average values increases the robustness of analyses because potential effects of measurement errors are minimized (Falconer, 1989; Hayes and Jenkins, 1997).

## Results

### *Measurement artifacts in control mice*

Because the measurement protocol (repeated brief but acute cold exposures) could have induced thermal acclimation

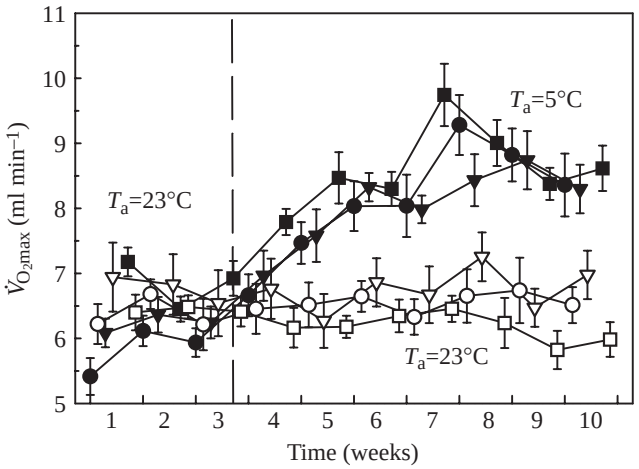


Fig. 1. $\dot{V}_{O_2max}$ values throughout acclimation in *P. maniculatus* submitted to cold-acclimation (closed symbols) and control individuals (open symbols). The broken vertical line indicates the beginning of the cold-acclimation. Values are means ± S.E.M., and different symbols are used for the three different subgroups measured in each treatment (see Materials and methods for details and *N* values).

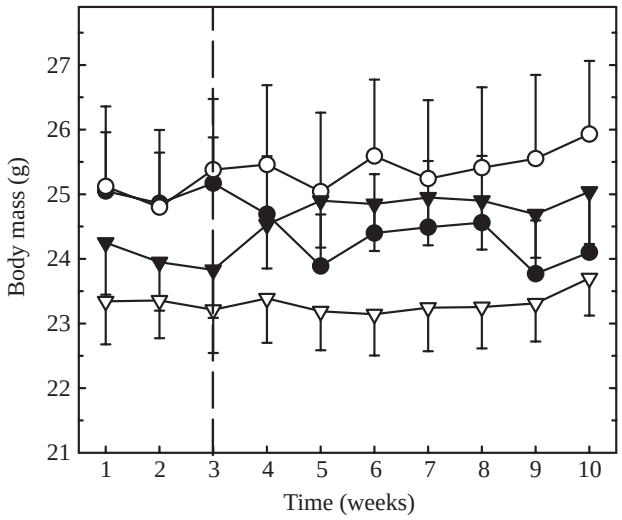


Fig. 2. Body mass values throughout acclimation for control (open symbols) and cold-acclimated individuals (closed symbols). Circles, females; triangles, males. The broken vertical line indicates the beginning of the cold-acclimation. Values are means ± S.E.M.

Table 1. F-*values for within-subjects effects from repeated measures on body mass (*$M_b$*), maximal* $O_2$ *consumption (*$\dot{V}_{O_2max}$*) and ventilation traits in the control group*

| | $M_b$[a] | $\dot{V}_{O_2max}$ | $f$[a] | $V_T$ | $V_{MIN}$ | OEE |
|---|---|---|---|---|---|---|
| d.f. | 8,136 | 8,136 | 8,136 | 8,136 | 8,136 | 8,136 |
| H–F epsilon | 0.747 | – | 0.655 | – | – | – |
| Time | 1.977 | **2.625*** | 1.857 | **17.958***** | **11.121***** | **12.251***** |
| Time × sex | 0.873 | 1.481 | 0.379 | 0.428 | 0.411 | 0.500 |

*f*, breathing frequency; $V_T$, tidal volume; $V_{MIN}$, minute volume; OEE, oxygen extraction efficiency; d.f., degrees of freedom.

[a]Non-spherical covariance matrix of contrasts.

Data include the entire 10-week experimental period, except for the first week. Sex was included as a fixed factor. When the sphericity condition was not met (see Materials and methods), *P*-values are reported with H–F corrections (both numerator and denominator d.f. were multiplied by H–F epsilon to obtain corrected d.f.).

Values in bold indicate statistical significance ($\alpha$=0.05); *$P$<0.05; ***$P$<0.001.

Table 2. F-*values obtained from tests of within-subjects effects from repeated measures on* $M_b$, $\dot{V}_{O_2max}$ *and ventilatory traits during the course of acclimation (first week not included)*

| | $M_b$[a] | $\dot{V}_{O_2max}$ | $f$[a] | $V_T$ | $V_{MIN}$ | OEE |
|---|---|---|---|---|---|---|
| d.f. | 8,280 | 8,280 | 8,280 | 8,280 | 8,280 | 8,280 |
| H–F epsilon | 0.591 | – | 0.711 | – | – | – |
| Time | 1.234 | **36.040***** | **7.650***** | **19.693***** | **13.328***** | **37.718***** |
| Time × sex | **2.564*** | 0.821 | 0.320 | 0.329 | 0.363 | 0.489 |
| Time × acclimation | 0.978 | **33.265***** | 1.281 | **2.291*** | **2.671**** | **3.124**** |
| Time × acclimation × sex | **3.771**** | 1.023 | 0.749 | 0.906 | 1.081 | 1.058 |

*f*, breathing frequency; $V_T$, tidal volume; $V_{MIN}$, minute volume; OEE, oxygen extraction efficiency; d.f., degrees of freedom.

[a]Non-spherical covariance matrix of contrasts.

Sex and acclimation temperature were included as fixed factors. When the sphericity condition was not met (see Materials and methods), *P*-values were calculated employing H–F corrections (both numerator and denominator d.f. were multiplied by H–F epsilon to obtain corrected d.f.).

Values in bold indicate statistical significance ($\alpha$=0.05); * $P$<0.05; **$P$<0.01; ***$P$<0.001.

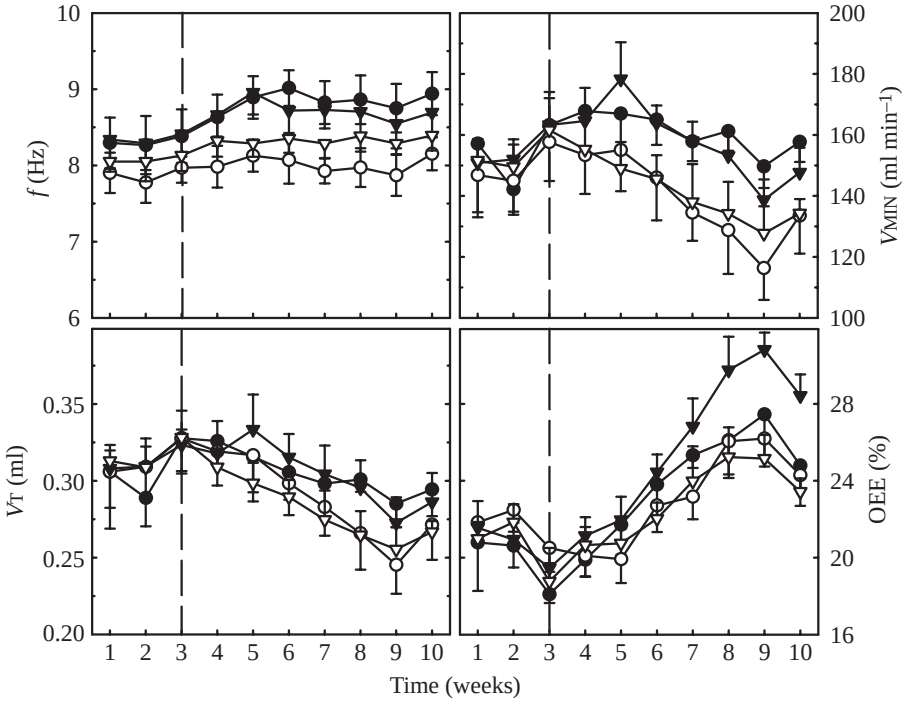


Fig. 3. Changes in respiratory frequency (*f*), tidal volume ($V_T$), minute volume ($V_{MIN}$) and oxygen extraction (OEE) throughout acclimation in control (open symbols) and cold-acclimated individuals (closed symbols). Circles, females; triangles, males. The broken vertical line indicates the beginning of the cold-acclimation. Values are means ± S.E.M.

(Heimer and Morrison, 1978), we looked for changes in mass, $\dot{V}_{O_2max}$ and ventilation traits in the control mice over the 10-week course of the experiment. There was no change in $M_b$ and no effect of gender in control mice (Table 1, Fig. 1), but $\dot{V}_{O_2max}$ and all ventilatory traits changed significantly (Table 1, Fig. 1). Both $V_T$ and $V_{MIN}$ decreased over time, while *f* increased slightly and OEE increased substantially (from approx. 22% to approx. 26%). These changes could be interpreted as training effects of repeated trials. However, the week-to-week changes in $\dot{V}_{O_2max}$ were small and showed no overall trend: there was no difference in mean $\dot{V}_{O_2max}$ between the start (week 2) and end (week 10) of the experiment in the control mice (paired-samples *t*-test; $t_{18}$=1.437, *P*=0.168).

### *Acclimation effects and temporal dynamics*

There were no sex differences in response to cold-acclimation in any of the physiological traits. However, $M_b$ responded differently to acclimation depending on both sex and acclimation temperatures (time × acclimation × sex effect, Table 2). In the cold-acclimation treatment, males increased $M_b$ while females reduced $M_b$. In control mice there were no significant differences in $M_b$ between sexes (Fig. 2).

Prior to acclimation, there were no changes in $\dot{V}_{O_2max}$ in the cold-acclimated group (contrasts of week 2 *vs.* week 3; $F_{1,18}$=0.521, *P*=0.480), and no differences between the $\dot{V}_{O_2max}$ of the cold-acclimation and control groups (Table 3). Cold-acclimation had a strong and significant effect on $\dot{V}_{O_2max}$, with final values about 34% higher than for warm-acclimated mice (Fig. 1, Table 2). Within-subject contrasts showed that cold-acclimation significantly affected $\dot{V}_{O_2max}$ from weeks 3 to 10 ($F_{1,18}$>29.579, *P*<0.0001 in all cases), and that the 'overshoot' of $\dot{V}_{O_2max}$ during week 8 (Fig. 1) was statistically significant. Results from ANCOVA confirm that acclimatory responses in $\dot{V}_{O_2max}$ were significant by the first week of acclimation. $M_b$ remained a significant predictor of $\dot{V}_{O_2max}$ during each week of the acclimation regime (Table 3).

As for control mice, cold-acclimated mice showed a general trend of decreasing $V_T$ and $V_{MIN}$ over time (with a concomitant increase in OEE; Fig. 3). Tests of between-subject effects in the repeated-measures analyses (i.e. testing the overall effect of acclimation in each of the traits by comparing the two treatments) showed that cold acclimation significantly increased *f* compared to controls ($F_{1,35}$=4.961, *P*=0.032), but there was no significant effect of acclimation on $V_T$, $V_{MIN}$ and OEE ($F_{1,35}$<3.805, *P*>0.059 in all cases). These results should

Table 3. *Week-by-week effects of mass, acclimation regime, and sex on* $\dot{V}_{O_2max}$*, expressed as* F*-values obtained for independent ANCOVAs performed on weekly means*

| Week | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|
| d.f. | 1,37 | 1,37 | 1,37 | 1,36 | 1,35 | 1,36 | 1,35 | 1,35 | 1,35 |
| $M_b$ | **12.4**** | **12.9***** | **15.2***** | **18.4***** | **17.3***** | **17.6***** | **12.7**** | **12.9***** | **36.5***** |
| Acclimation | 3.10 | 0.001 | **7.01*** | **38.5***** | **60.1***** | **52.2***** | **51.2***** | **80.0***** | **86.8***** |
| Sex | 3.96 | 1.73 | 2.51 | 3.53 | 1.60 | 2.69 | 5.70* | 4.27* | 5.26* |

Acclimation time and sex were fixed factors, and body mass ($M_b$) was included as a covariate. Values in bold indicate that main effects of each independent factor/variable were statistically significant after sequential Bonferroni adjustment for multiple simultaneous tests; *$P$<0.05; **$P$<0.01; ***$P$<0.001.

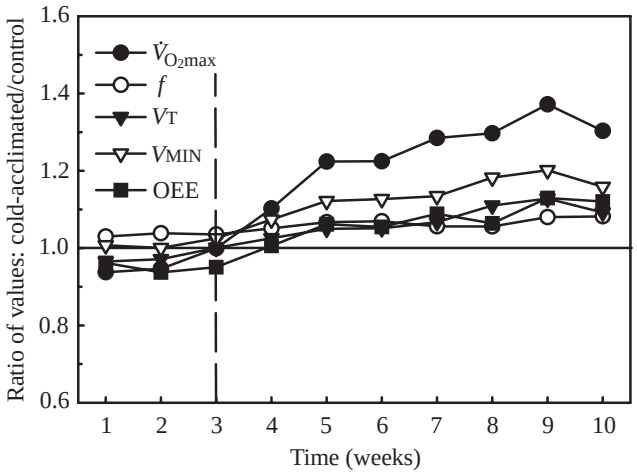


Fig. 4. Ratio of values in cold-acclimated/control animals for maximum metabolic rate ($\dot{V}_{O_2max}$) respiratory frequency ($f$), tidal volume ($V_T$), minute volume ($V_{MIN}$) and oxygen extraction (OEE) throughout acclimation. Ratios were calculated after taking body mass into account (values were expressed on a per gram basis). The straight horizontal line represents a 1:1 ratio and the broken vertical line indicates when cold-acclimation began.

be considered cautiously, however. The between-subject effect of acclimation in $V_{MIN}$ and OEE bordered significance ($P<0.1$ in both cases), and because $M_b$ was not included in the model in this instance, inter-individual differences in $M_b$ could be accounting for part of the between-subject variation. Within-subject effect analyses (where $M_b$ is implicitly included, given the repeated-measures design) largely support this view: cold-acclimated individuals had significantly higher $V_T$, $V_{MIN}$ and OEE values than control mice (Table 2, Figs 3 and 4). As for $\dot{V}_{O_2max}$, the changes in $f$ were apparent shortly after the start of cold acclimation (although these were not statistically significant after Bonferroni adjustment; $P<0.047$ from weeks 4 to 10; Fig. 3).

When $\dot{V}_{O_2max}$ was included as a covariate, we observed a significant positive relationship between $V_T$, $V_{MIN}$ and $\dot{V}_{O_2max}$ (the positive coefficient was apparent in partial plots from multiple regressions analogous to the ANCOVAs reported here). However, there was no relationship between $\dot{V}_{O_2max}$ and either $f$ or OEE (Table 4).

### *Repeatability*

$M_b$ and absolute and mass-independent $\dot{V}_{O_2max}$ were significantly repeatable in both control and cold-acclimated treatments when compared between weeks 2 and 10 (Tables 5

Table 4. *Determinates of ventilation traits, expressed as* F-*values from independent ANCOVAs performed with weekly means*

| | Week | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| | d.f. | 1,36 | 1,36 | 1,36 | 1,35 | 1,34 | 1,35 | 1,34 | 1,34 | 1,34 |
| $f$ | | | | | | | | | | |
| | $M_b$ | 0.011 | 0.681 | 0.003 | 0.188 | 0.224 | 0.558 | 0.292 | 0.230 | 2.15 |
| | $\dot{V}_{O_2max}$ | 2.323 | 0.118 | 2.618 | 0.192 | 1.19 | 0.598 | 0.080 | 3.290 | 3.54 |
| | Acclimation | 1.956 | 1.133 | 0.832 | 1.923 | 0.388 | 4.54* | 2.44 | 0.114 | 0.197 |
| | Sex | 0.065 | 0.613 | 0.387 | 0.396 | 0.063 | 1.19 | 0.306 | 0.044 | 0.532 |
| $V_T$ | | | | | | | | | | |
| | $M_b$ | 0.795 | 1.028 | 3.10 | 2.988 | 6.83* | 7.52** | **10.6**** | **10.5**** | 3.42 |
| | $\dot{V}_{O_2max}$ | **5.87*** | **26.7***** | **14.8***** | **13.0***** | **12.7**** | 4.97* | **9.72**** | **10.0**** | **8.34**** |
| | Acclimation | 0.008 | 0.090 | 0.847 | 3.625 | 4.17* | 0.613 | 0.851 | 0.949 | 1.62 |
| | Sex | 0.020 | 0.002 | 1.138 | 0.698 | 0.048 | 0.047 | 0.519 | 0.468 | 1.19 |
| $V_{MIN}$ | | | | | | | | | | |
| | $M_b$ | 0.894 | 1.978 | 2.883 | 3.50 | 2.34 | 7.50** | 6.33* | 5.62* | 0.657 |
| | $\dot{V}_{O_2max}$ | **11.4**** | **17.1***** | **22.4***** | **14.5***** | **12.8**** | 1.89 | 3.31 | **16.3***** | **17.2***** |
| | Acclimation | 0.764 | 0.092 | 0.080 | 1.12 | 1.82 | 0.222 | 0.037 | 1.45 | 2.62 |
| | Sex | 0.083 | 0.487 | 0.193 | 0.097 | 0.027 | 0.185 | 0.108 | 0.796 | 3.22 |
| OEE | | | | | | | | | | |
| | $M_b$ | 0.580 | 1.579 | 2.403 | 3.52 | 2.01 | 5.87* | 4.93* | 3.58 | 0.062 |
| | $\dot{V}_{O_2max}$ | 0.021 | 0.827 | 1.38 | 0.924 | 0.079 | 7.25* | 2.63 | 0.051 | 0.187 |
| | Acclimation | 1.428 | 0.766 | 0.004 | 0.370 | 0.657 | 0.348 | 0.135 | 1.54 | 2.555 |
| | Sex | 0.115 | 0.515 | 0.113 | 0.001 | 0.094 | 0.007 | 0.001 | 0.412 | 1.582 |

$f$, breathing frequency; $V_T$, tidal volume; $V_{MIN}$, minute volume; OEE, oxygen extraction efficiency.

The fixed factors were sex and acclimation regime, whereas maximal $O_2$ consumption $\dot{V}_{O_2max}$ and body mass $M_b$ were included as covariates.

Values in bold were statistically significant after sequential Bonferroni adjustment for multiple simultaneous tests; *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Table 5. *Repeatability of* $M_b$, $V_{O_2max}$ *and ventilatory traits in control and cold-acclimated mice, expressed as Pearson correlations (*r*)*

| | Control (*N*=19) | | | Cold acclimated (*N*=20) | | |
|---|---|---|---|---|---|---|
| Week | 2–6 | 2–10 | 6–10 | 2–6 | 2–10 | 6–10 |
| $M_b$ | **0.922*** ** | **0.928*** ** | **0.978*** ** | **0.845*** ** | **0.807*** ** | **0.937*** ** |
| $\dot{V}_{O_2max}$ | **0.850*** ** | **0.831*** ** | **0.822*** ** | **0.476*** | **0.637**** | **0.761*** ** |
| *f* | **0.576**** | **0.595**** | **0.762*** ** | **0.877*** ** | **0.850*** ** | **0.880*** ** |
| $V_T$ | **0.593**** | **0.563**** | **0.616**** | 0.368 | **0.463*** | **0.785*** ** |
| $V_{MIN}$ | **0.626**** | **0.540**** | **0.608**** | 0.186 | 0.275 | **0.736*** ** |
| OEE | 0.200 | 0.217 | 0.154 | –0.243 | 0.249 | 0.295 |

$M_b$, body mass; $\dot{V}_{O_2max}$, maximal $O_2$ consumption; *f*, breathing frequency; $V_T$, tidal volume; $V_{MIN}$, minute volume; OEE, oxygen extraction efficiency.

Values were statistically significant (one-tailed test) after a sequential Bonferroni correction for multiple simultaneous tests; *$P$<0.05; **$P$<0.01; ***$P$<0.001.

Table 6. *Repeatability of mass-independent* $V_{O_2max}$ *and ventilatory traits for control and cold-acclimated mice, expressed as Pearson correlations (*r*)*

| | Control (*N*=19) | | | Cold acclimated (*N*=20) | | |
|---|---|---|---|---|---|---|
| Week | 2–6 | 2–10 | 6–10 | 2–6 | 2–10 | 6–10 |
| $\dot{V}_{O_2max}$ | **0.792*** ** | **0.780*** ** | **0.741*** ** | 0.312 | **0.572**** | **0.551**** |
| *f* | **0.589**** | **0.586**** | **0.754*** ** | **0.875*** ** | **0.834*** ** | **0.868*** ** |
| $V_T$ | **0.535**** | 0.410 | 0.296 | 0.191 | 0.214 | **0.616**** |
| $V_{MIN}$ | **0.570**** | 0.391 | 0.373 | –0.20 | 0.070 | **0.640**** |
| OEE | 0.229 | 0.195 | 0.021 | –0.264 | 0.224 | 0.298 |

$\dot{V}_{O_2max}$, maximal $O_2$ consumption; *f*, breathing frequency; $V_T$, tidal volume; $V_{MIN}$, minute volume; OEE, oxygen extraction efficiency.

Values in bold were statistically significant (one-tailed test) after a sequential Bonferroni correction for multiple simultaneous tests; *$P$<0.05; **$P$<0.01; ***$P$<0.001.

and 6). However, mass-independent $\dot{V}_{O_2max}$ was not repeatable between week 2 and the middle of cold acclimation (Table 6). Product–moment correlations on initial and final $M_b$, $\dot{V}_{O_2max}$ and mass-independent $\dot{V}_{O_2max}$ (mean values for weeks 2+3 and 9+10) were consistent with the results obtained when mean initial and mean final values were employed to estimate repeatability throughout acclimation (Fig. 5).

Breathing frequency (*f*) was highly repeatable in all conditions (Tables 5 and 6). Both $V_T$ and $V_{MIN}$ were significantly repeatable in control mice throughout the experiment, but repeatability was abolished by cold-acclimation (there was no significant repeatability in $V_T$ or $V_{MIN}$ between pre- and post-acclimation tests). However, $V_T$ and $V_{MIN}$ were repeatable within the period of cold acclimation (week 6 *vs.* week 10; Tables 5 and 6). Inter-individual differences in OEE were not repeatable in either group (Table 5). However, when consistency of mean initial *vs*. mean final OEE was assessed, repeatability of OEE was significant in control ($N$=19, $r$=0.445, $P$<0.01), but not in cold-acclimated animals.

In general, cold-acclimation tended to decrease individual consistency (with the notable exception of *f*). When we compared all repeatability analyses, excluding *f* (Tables 5 and 6), repeatabilities were significantly higher in control mice than between pre- and post-acclimation in the cold-acclimated group (paired *t*-test, $t_{26}$=2.864, one-tailed $P$<0.004).

## Discussion

We found a mean increase of 33.7% in $\dot{V}_{O_2max}$ after 7 weeks of cold-acclimation, which is consistent with the 30–40% increase reported in previous acclimation and acclimatization studies in *Peromyscus* (Heimer and Morrison, 1978; Hayes and Chappell, 1986). Our $\dot{V}_{O_2max}$ values are higher than previously reported for *Peromyscus* (Heimer and Morrison, 1978; Chappell, 1984; Chappell and Snyder, 1984; Hayes and Chappell, 1986; but see Wickler, 1981). However, $M_b$ in our sample was generally greater than in previous studies, which may account for some of the differences in $\dot{V}_{O_2max}$. Also, there may have been age differences among animals in these the studies, and age has a substantial impact on aerobic capacity in deer mice (Chappell et al., 2003).

There was no change in $\dot{V}_{O_2max}$ in our control mice between the beginning and end of trials. That result contrasts with the findings of Heimer and Morrison (1978), who reported a significant training effect on $\dot{V}_{O_2max}$ in warm-acclimated

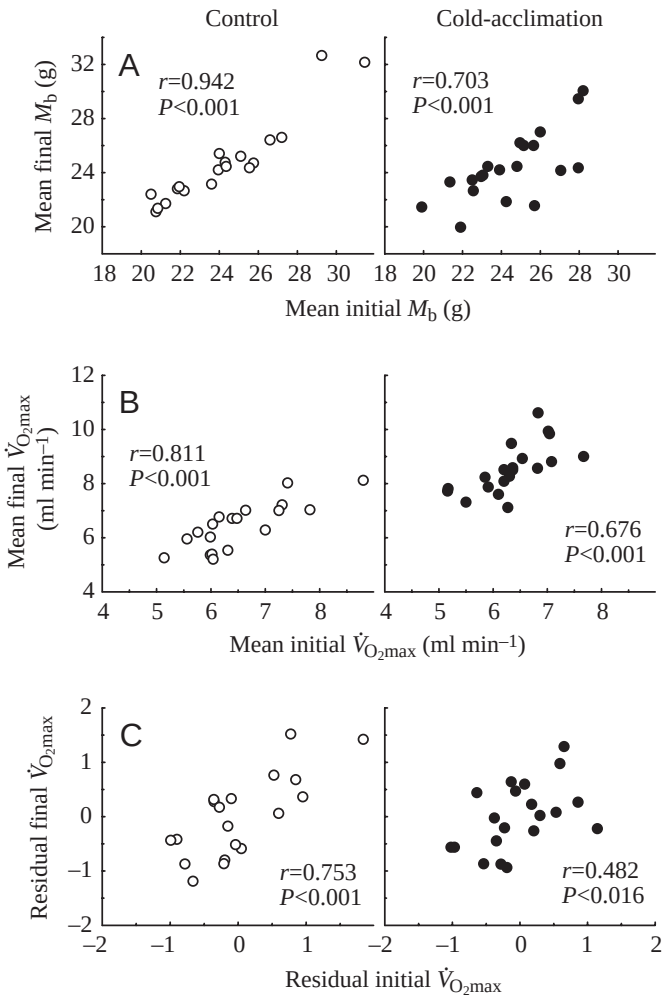


Fig. 5. Individual consistency between initial and final body mass (A), $\dot{V}_{O_2max}$ (B) and mass-independent $\dot{V}_{O_2max}$ (C) in control (left) and cold-acclimated (right) animals. Initial and final values were calculated as the individual mean value from weeks 2+3, and 9+10, respectively (see Materials and methods). The Pearson product–moment coefficient for each correlation is also reported, and its respective probability value.

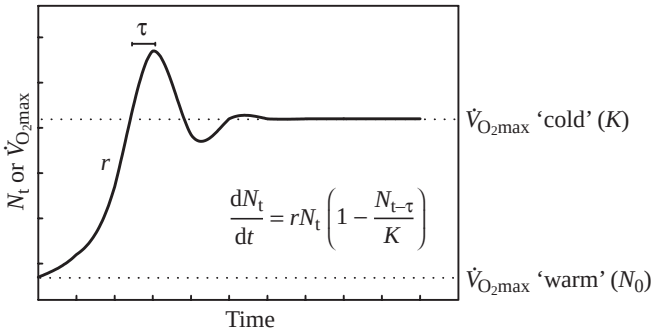


Fig. 6. Diagram showing how the time-lagged logistic growth curve can be analogous to the temporal course of $\dot{V}_{O_2max}$ during acclimation in a negative feedback control system (in this hypothetical case, the animal went from a warm to a cold temperature, as in the present study; see Fig. 1). $N_t$, population size at time $t$; $r$, maximum rate of growth and/or of acclimation; τ, length of the delay between stimulus and response. $\dot{V}_{O_2max}$ 'warm' and $\dot{V}_{O_2max}$ 'cold' (dotted lines) represent the values of $\dot{V}_{O_2max}$ at 'equilibrium' – i.e. when the animal is actually acclimated to warm and cold conditions. These values are analogous to the initial population size at $t$=0 ($N_0$) and the carrying capacity of the habitat ($K$), respectively.

*Peromyscus*. The difference between the studies is most likely due to different measurement protocols: Heimer and Morrison measured $\dot{V}_{O_2max}$ in heliox twice a week (instead of once per week in our protocol), and the duration of their cold-exposure trials were almost twice as long as ours (about 30 min; fig. 1 in their study).

We did find training effects in ventilatory traits, but the magnitude of the changes varied considerably between control and cold-acclimation groups (Fig. 3); for example, $f$ increased by about 3.3% in control and 6.1% in cold-acclimated animals. Mean initial and final $f$ values in control groups (8.00 and 8.27 Hz) were substantially higher than those reported for *Peromyscus* measured in air at –10°C (about 5.5 Hz; fig. 1B in Chappell, 1985). The difference could be that Chappell's mice probably did not attain $\dot{V}_{O_2max}$, and because the physical properties of air and heliox are not identical (Kudukis et al., 1997).

Cold-acclimated mice largely accommodated a 34% elevation in $\dot{V}_{O_2max}$ by increasing OEE, with little change in pulmonary convection. A similar response has been reported in other species (Mortola and Frappell, 2000), but many small mammals use different strategies (i.e. supporting increased metabolism by increasing ventilation; Casey et al., 1979; Chappell, 1992; Chappell and Dawson, 1994). An increase in OEE permits greater aerobic metabolism (and hence heat production) without compromising respiratory heat loss (Chappell, 1985; Mortola and Frappell, 2000). In this context, it was particularly interesting that warm-acclimated individuals reduced ventilation and increased OEE after repeated cold-exposure trials even though they maintained a fairly constant $\dot{V}_{O_2max}$.

### *Temporal changes during acclimation*

Interestingly, our data revealed a significant 'overshoot' of $\dot{V}_{O_2max}$ at weeks 4–5 of cold-acclimation, followed by a decline of about 6% to an apparently stable final value attained in week 7 (Fig. 1). Given that a similar overshoot was found in a distantly related rodent (*Abrothrix andinus*; Nespolo and Rosenmann, 1997), and that most studies of acclimation responses had low temporal resolution (i.e. they measured metabolism only at the beginning and end of the acclimation period), this may be a general response to cold acclimation and not a unique characteristic of *Peromyscus*.

We postulate that this overshoot in metabolism reflects the control of a homeostatic status through negative feedback. Acclimation responses require a continuous perception of a non-homeostatic status due to a new thermal environment (information acquisition cost; *sensu* DeWitt et al., 1998),

responding simultaneously in multiple levels of organization in an integrated fashion (production costs), and finally resetting the set points of all traits involved in order to maintain homeostasis (i.e. negative feedback regulation).

Because of the complexity involved in modulating these responses, and the intrinsic time lag present in any physiological system from the detection of a particular stimulus to the overall response associated with it, we hypothesized that 'over-acclimation' (e.g. higher $\dot{V}_{O_2max}$ values than required for a particular $T_a$) would occur, in an analogous way to predictions of population sizes above carrying capacity when delay is incorporated in the logistic equation (Roughgarden, 1998). Such an equation is physiologically realistic (see Fig. 6) and provides interesting predictions worth testing, such as increased overshoots concomitantly with (i) a higher contrast between acclimating temperatures [hence, whether or not the overshoot is detected in a particular study will depend not only on the frequency of sampling but also on (presumably) the difference between pre and post-acclimation temperatures (18°C in this study)] and (ii) increased acclimatory rates (everything else being equal). The logistic curve also predicts that (iii) animals with low acclimatory rates ($r$) would probably not show any overshoot during acclimation, as the product of acclimatory rates × delay time ($r\tau$; see Fig. 6) is low. In this context, we would expect that species from highly seasonal environments would have higher acclimatory rates than species from thermally stable environments.

### *Individual variation in $\dot{V}_{O_2max}$ and ventilatory traits*

Maximal oxygen consumption was highly repeatable over a period of 8 weeks in both control and cold-acclimated mice, as previously shown for $\dot{V}_{O_2max}$ in *Peromyscus* (Hayes, 1989; Hayes and Chappell, 1990). This means that an individual's relative performance remains consistent even after absolute performance increased dramatically due to acclimation; in other words, the proportional change in performance due to acclimation was roughly the same in all individuals. However, repeatability was lower while animals were still acclimating to cold conditions, suggesting individual variation in rates of acclimation. Two of the three subgroups of cold-acclimated mice showed higher product–moment coefficients between pre- and post-acclimatory $\dot{V}_{O_2max}$, with considerably lower repeatabilities between the pre- and mid-acclimation periods (Table 5).

Contrary to our expectations, the consistency of $f$ was higher in cold-acclimated animals than in controls. The opposite was true for $V_T$ and $V_{MIN}$; they remained repeatable in controls and were not consistent during the initial stages of cold acclimation (Table 5). However, consistency of both $V_T$ and $V_{MIN}$ returned at the end of the acclimation period. In general, these traits are more consistent during stable conditions than during acclimatory change (Tables 5 and 6), as was true for $\dot{V}_{O_2max}$ (Hayes, 1989; Hayes and Chappell, 1990). In contrast, an intermediate level metabolic index – daily energy expenditure (Speakman et al., 1994; Berteaux et al., 1996) – showed substantial and inconsistent intra-individual variation. By studying traits under conditions that maximize metabolic performance, researchers are most likely to detect individual differences that might be under selection (Berteaux et al., 1996), such as Hayes and O'Connor (1999) reported for $\dot{V}_{O_2max}$ in *Peromyscus*.

### *Concluding remarks*

Our results emphasize two important points that physiologists should take into account when designing acclimation experiments. First, responses to acclimation may be either under- or overestimated depending on when animals are measured, and presumably the appropriate measurement time will vary according to the species being studied. Hence, caution is warranted when comparing thermal acclimation responses in different species, and particularly when acclimation times are not consistent. One approach often used in acclimation experiments is to acclimate for a 'standard' period (e.g. Heimer and Morrison, 1978; Nespolo et al., 2001a). That approach (with the implicit assumption of consistent response rates) could generate misleading conclusions if the kinetics of acclimation differed between individuals or among groups or species (see above).

Second, acclimation rates differ among physiological traits. For example, in our deer mice $f$ showed rapid responses to acclimation and was fairly stable by the third week of cold exposure, whereas $\dot{V}_{O_2max}$ did not become stable until week 5 of cold exposure, and OEE continued to change until close to the end of the 7-week acclimatory period; Fig. 3). For many studies, these problems can be ameliorated by allowing a sufficiently long acclimation period for animals to attain a stable acclimated condition (see above) – but the appropriate period can be firmly established only with detailed knowledge of the temporal pattern of acclimation. Considering the high plasticity of *Peromyscus*, our results suggest that an acclimatory period of about 2 months would be enough to ensure that animals are actually 'acclimated' – and not 'acclimating' (see above).

Interestingly, the regulation of physiological plasticity could be under selection and evolving – if this 'trait' has a genetic basis. Different species show highly contrasting responses to thermal acclimation (e.g. this study; Nespolo et al., 2001a,b), and it seems reasonable to expect that these species will vary in their acclimatory responses at a temporal level as well. For instance, the fossorial rodent *Spalacopus cyanus* showed extremely low acclimatory responses in $\dot{V}_{O_2max}$ (11% difference in $\dot{V}_{O_2max}$ at temperatures of 15° and 30°C; Nespolo et al., 2001b). It was impossible, however, to discriminate whether that is because (i) their 'set-points' in $\dot{V}_{O_2max}$ at these temperatures was relatively narrow, (ii) their rates of thermal acclimation ($r$ in Fig. 6) were extremely low, (iii) the delay ($\tau$ in Fig. 6) to respond to thermal changes was high, or (iv) a combination of these factors. Hence, although in both cases the outcome would be the same, it is not clear at which level natural selection could be acting, and correlated responses in underlying physiological traits might be considerably different. Furthermore, so far the genetic background underlying each of

these variables is not known, and it is not clear which traits could evolve in this scenario when selection is acting at the level of physiological plasticity. In summary, the question of how acclimatory responses – and phenotypic plasticity, more generally – evolve still remains poorly understood.

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