

## Bioenergetics and inter-individual variation in physiological capacities in a relict mammal – the Monito del Monte (*Dromiciops gliroides*)

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### SUMMARY

In evolutionary physiology, studies of inter-individual variation (i.e. repeatability) in functional capacities are valuable as they indicate – within populations – what attributes could respond to natural selection. Although repeatability and quantitative genetics of physiological traits in energy metabolism of eutherian mammals have been well characterized, few or no studies have been performed on marsupials. We studied the repeatability (i.e. intraclass correlation coefficient,  $\tau$ ) of bioenergetics for Monito del Monte (*Dromiciops gliroides*), the sole living representative of an otherwise extinct marsupial order (Microbiotheria). We measured resting metabolic rate as CO<sub>2</sub> production ( $\dot{V}_{\text{CO}_2}$ ) and O<sub>2</sub> consumption ( $\dot{V}_{\text{O}_2}$ ) simultaneously, together with minimum thermal conductance (C), evaporative water loss (EWL) and respiratory quotient (RQ), in a sample of ca. 20 individuals. Our results suggest that *D. gliroides* exhibits poor control of body temperature ( $T_b$ ), with a thermal amplitude of ca. 10°C in normothermia. As a consequence, repeatability of  $T_b$  and metabolic rate (either as  $\dot{V}_{\text{CO}_2}$  or  $\dot{V}_{\text{O}_2}$ ) were relatively low ( $\tau_{T_b}=0.25\pm 0.04$ ,  $\tau_{\dot{V}_{\text{CO}_2}}=0.14\pm 0.03$ ,  $\tau_{\dot{V}_{\text{O}_2}}=0.24\pm 0.02$ , jackknife estimations of standard errors). Thermal conductance exhibited near-zero or negative repeatability and was lower than expected for marsupials. However, we found significant repeatability for RQ and EWL ( $\tau=0.32\pm 0.03$  and  $0.49\pm 0.09$ , respectively). In general, these results suggest that Monito del Monte exhibits some ‘reptilian’ physiological characteristics. The relatively low repeatability of physiological variables, which otherwise exhibit large inter-individual and genetic variance in eutherian mammals, suggests that these capacities do not exhibit evolutionary potential in the ancient order Microbiotheria.

Key words: whole-animal metabolism, respiratory quotient, *Dromiciops*, Australasian fauna, repeatability, evolution of endothermy.

### INTRODUCTION

Approximately two decades ago, in a seminar paper, Bennett and colleagues (Bennett et al., 1987) called for more studies dealing with inter-individual variability. Since then, many researchers have focused on characterizing the variation of physiological capacities in natural populations (Berteaux et al., 1996; Chappell, 1993; Chappell et al., 1995; Friedman et al., 1992; Garland and Bennett, 1990; Garland et al., 1990; Garland and Else, 1987; Hayes et al., 1998; Hayes and Chappell, 1990; Hayes et al., 1992; Hayes and Jenkins, 1997; Huey and Dunham, 1987; Speakman et al., 1994). In the case of mammalian physiology, it would be fair to say that this new focus of research has culminated in a good understanding of the genetic and environmental sources of variation of energy metabolism (see below).

The literature suggests that energy metabolism is a repeatable trait, and most of its consistency is due to additive genetic effects that vary depending on the kind of variable being studied [e.g. locomotory, thermoregulatory, maximum, basal (Dohm et al., 2001; Hayes et al., 1992; Konarzewski and Diamond, 1994; Ksiazek et al., 2004; Labocha et al., 2004; Nespolo et al., 2003; Nespolo et al., 2005; Nespolo and Franco, 2007; Sadowska et al., 2005)]. These findings, however, were restricted to eutherian mammals. As far as we are aware, there is not a single study of repeatability in any aspect of physiology in other groups of mammals (i.e. monotremes and marsupials) (Luo, 2007; Warren et al., 2008). In addition, inter-individual variation in several whole-animal physiological capacities besides energy metabolism, such as respiratory quotient, minimum thermal conductance and evaporative water loss, remain almost unexplored in these groups.

One important individual capacity is the respiratory exchange ratio, or respiratory quotient (RQ, the instantaneous ratio between CO<sub>2</sub> production and O<sub>2</sub> consumption). In animals, an RQ of near 1.0 indicates that most of the energy metabolism is utilizing carbohydrate catabolism, and a RQ near 0.7 indicates that energy metabolism is occurring by fat catabolism (Andrews, 2004; Schmidt-Nielsen, 1995). Thus, RQ might be an instantaneous indicator of the type of nutrients that are being metabolized in a living animal. Not surprisingly, the use of RQ has proven to be a valuable technique in, for instance, studies of human obesity (Valtueña et al., 1997; Wielinga et al., 2007), as an indicator of metabolic switches in hibernating animals (Buck and Barnes, 2000) and hypoxia adaptation in invertebrates (Nielsen and Christian, 2007). However, whether RQ is a consistent property of individuals or depends totally on a response to an environmental factor (i.e. fasting, nutrient type) is open to question.

Another important variable derived from respirometry measures is ‘wet’ thermal conductance (*sensu* McNab, 1980) – the rate of heat loss from the body (the inverse of insulation). Thermal conductance (C) is of key importance for survival in small endotherms, especially those living in cold and/or seasonal environments, as changes in the properties of fur or feathers can significantly reduce heat loss (Bozinovic and Merritt, 1992; Klaasen et al., 2002; Luna-Jorquera et al., 1997; McNab, 1980; Novoa et al., 1994; Scholander, 1955). However, there have been few attempts to determine the source of phenotypic variation for this trait in mammals, which has exhibited significant heritability (Nespolo et al., 2003).

Evaporative water loss (EWL) – the rate of water loss from the body due to evaporation – is an important variable related to the

resistance to dehydration in vertebrates, which in small endotherms could be of crucial relevance to survival (Anderson et al., 1997; Hayes et al., 1998; Maloney and Dawson, 1998; Munn and Dawson, 2001; Wang and Wang, 2000; Williams and Tieleman, 2000). The repeatability of this trait was studied by Hayes and colleagues (Hayes et al., 1998; see also Hayes and Jenkins, 1997), who found up to 65% repeatable variation.

A response to natural selection needs genetic variation (or, in general terms, high heritability) in a given trait within a population (Roff, 2007). In the absence of genetic-by-environmental interaction, and other confounding factors such as non-additive genetic variation, when a trait exhibits high genetic variation, it also exhibits high repeatability (Falconer and Mackay, 1997; Nespolo and Franco, 2007). In such situations, the following assumptions can be made: (1) mutation–selection balance maintains genetic variation in populations [i.e. the increase in variation due to mutation/recombination is compensated by its reduction due to directional/stabilizing selection (see Roff, 2002; Turelli, 1988)]; and (2) the actual physiological features of a group have not changed much compared with its ancestors [a basic assumption in studies of the evolution of endothermy and the aerobic capacity model (see Bennett et al., 2000; Crompton et al., 1978; Dawson et al., 1979; Hayes and Garland, 1995; Koteja, 2000; Sadowska et al., 2005)]. With these assumptions, the existence of low repeatability in an actual species of an ancient lineage would suggest that its ancestors did exhibit low genetic variation (and hence the trait did not exhibit potential to respond to selection). Obviously, low genetic variation could also arise because of fixation of all genes related to the trait after strong and persistent directional selection (i.e. disrupting the mutation–selection balance). But in this case, further descendants of this group (e.g. rodents, in the case of mammals) would also exhibit low genetic and inter-individual variation, which is not supported by empirical evidence in energy metabolism and related traits (Bacigalupe et al., 2004; Berteaux et al., 1996; Dohm et al., 2001; Hayes et al., 1998; Hayes and Jenkins, 1997; Hayes and O'Connor, 1999; Konarzewski and Diamond, 1995; Konarzewski et al., 2005; Książek et al., 2004; Labocha et al., 2004; Nespolo et al., 2003; Nespolo et al., 2005; Nespolo and Franco, 2007; Sadowska et al., 2005). In other words, what we are proposing is that repeatability studies performed on living representatives of ancient groups could provide an insight into questions regarding whether the trait had the potential to evolve in these ancient lineages.

The study of mammalian evolution experienced a breakthrough after the identification of the South American marsupial *Monito del Monte* (*Dromiciops gliroides*) as the sole living representative of the mammalian order (Microbiotheria), of Australasian origin, previously thought to be extinct (Asher et al., 2004; Palma and Spotorno, 1999; Spotorno et al., 1997). A handful of studies have been conducted on this 'living fossil' (Amico and Aizen, 2000; Asher et al., 2004; Bozinovic et al., 2004; Brugini and Flores, 2007; Guglielmone et al., 2004; Kirsch et al., 1991; Lobos et al., 2005; Marin-Vial et al., 2007; Navone and Suriano, 1992; Palma and Spotorno, 1999; Pridmore, 1994; Saavedra and Simonetti, 2001; Silva, 2005; Westerman and Edwards, 1991), which, in addition to its phylogenetic relationships, describe a few basic aspects of its biology. In fact, the sole study that addressed a physiological feature of *D. gliroides* described it as a hibernator and reported that its basal metabolic rate was below the expected value for marsupials (Bozinovic et al., 2004).

In this study, our aims were: (1) to perform a wide screening of the bioenergetic traits of *D. gliroides*; (2) to determine the inter-

individual variation in physiological capacities of *D. gliroides*; and (3) to use this information to provide insight into the ancestral physiological features of mammals.

## MATERIALS AND METHODS

### Study animals

Twenty-one individuals of *Dromiciops gliroides* were captured near Valdivia, Chile (39 deg. 48'S, 73 deg. 14'W; 9 m) during the austral summer, with intensive trapping using modified tomahawk traps located in trees and shrubs, 1 m above ground, and baited with bananas and yeast. Animals were transported to the laboratory the day of capture, placed in plastic cages of 45×30×20 cm with 2 cm of bedding, maintained in a climatic chamber (PiTec Instruments, Chile) at 20±1°C (mean ± s.d.) and with a 12h:12h photoperiod for two weeks, and fed with water and a mix of mealworms and blackberries. Metabolic measurements were then performed on each animal during daylight (i.e. their resting period) and after 6 h of fasting. Each period of metabolic measurement lasted 3 h (see below). Because of an unfortunate mistake during animal maintenance, we lost six individuals that escaped from the climatic chamber. Thus, sample sizes varied between repetitions.

### Respirometry measurements

All measurements were performed with a respirometry system consisting of a Li-Cor 6262 H<sub>2</sub>O/CO<sub>2</sub> analyzer (LiCor, USA) and an Oxzilla II (Sable Systems International, USA) dual oxygen analyzer, in a series configuration. The H<sub>2</sub>O/CO<sub>2</sub> analyzer was calibrated periodically against a known gas sample of 291 p.p.m. for CO<sub>2</sub> and against air saturated with water vapor at 20°C for H<sub>2</sub>O. We used cylindrical metabolic chambers of 200 ml, and a flow rate of 1000±1 ml min<sup>-1</sup> controlled by a Sierra mass-flow controller (Sierra Instruments, USA), located upstream of the metabolic chamber and after two columns with H<sub>2</sub>O and CO<sub>2</sub> scrubbers (Drierite and Baralyme, respectively). The metabolic chamber was located in an incubator, and ambient temperature ( $T_a$ ) was set to 20°C and continuously recorded by a Cole Parmer (USA) thermocouple located inside the incubator. Where small variations in  $T_a$  due to the incubator inertia could inflate the residual error of further repeatability analyses, then we included  $T_a$  as a covariable in all the analyses, but it was never significant, and the results were unchanged when not including it. As we used a test temperature (i.e. 20°C) below thermoneutrality, technically what we obtained was resting metabolic rate (RMR). This  $T_a$  was chosen in order to minimize the amount of heat lost by evaporation (McNab, 1980), without increasing metabolism because of cold. Dry and CO<sub>2</sub>-free air passed through the mass flowmeter, then to the metabolic chamber and then through a Gast (Gast Manufacturing, USA) pump (i.e. negative pressure). After that, the air was injected into the LiCor 6262 by a subsampler (Intelligent Subsamplers, Sable Systems, USA), at a flow rate of 200 ml min<sup>-1</sup>. Then, the air was passed again through the scrubbers and finally it was injected into the Oxzilla II. With this system, we recorded simultaneously: (1) carbon dioxide production ( $\dot{V}_{CO_2}$ ); (2) oxygen consumption ( $\dot{V}_{O_2}$ ) and (3) evaporative water loss (EWL).

Each record was corrected (1) for drift deviations, especially for the O<sub>2</sub> signal (the LiCor 6262 did not experience drift), (2) for negative values in the O<sub>2</sub> record (reciprocal transformation) and (3) to align both  $\dot{V}_{CO_2}$  and  $\dot{V}_{O_2}$  record for a ca. 10 s lag between them. Finally, to calculate  $\dot{V}_{CO_2}$  and  $\dot{V}_{O_2}$ , we computed (1) the average of the entire record; (2) the average of the minimum steady-state 10 min of recording and (3) the average of the last 10 min of recording.

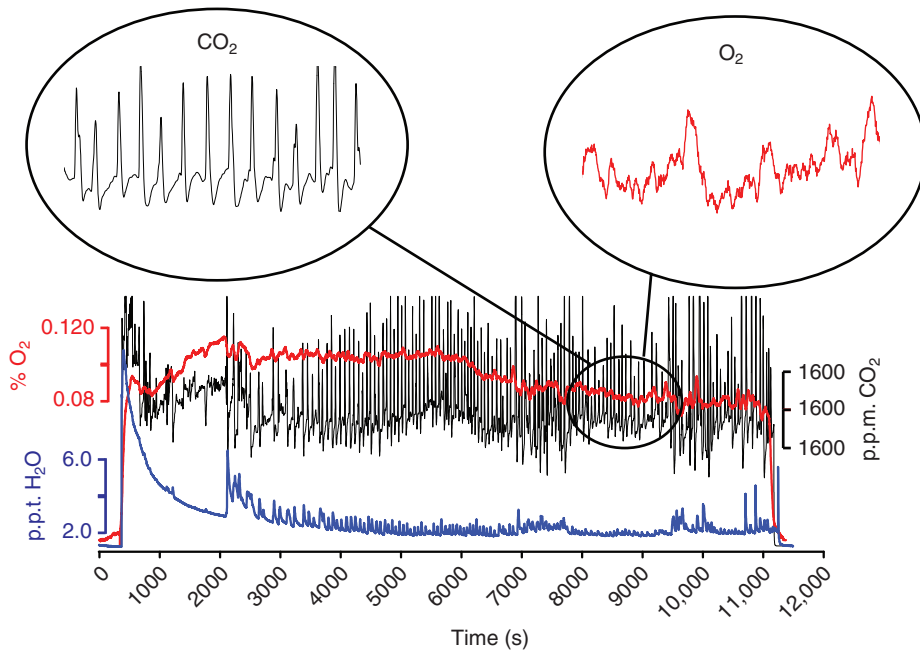


Fig. 1. A representative respirometric recording showing the measured variables as obtained from the analyzers: CO<sub>2</sub> production, in parts per million (p.p.m., in black), O<sub>2</sub> consumption, in fractional scale (in red) and H<sub>2</sub>O production, in parts per thousand (p.p.t., in blue). A detailed trace of CO<sub>2</sub> and O<sub>2</sub> is shown in the zoom. The CO<sub>2</sub> analyzer is accurate enough to show, in some cases, the changes in gas concentration arising from respiratory movements.

The basic statistics (coefficient of variation, normality and correlation with body mass) indicated that the average of the last 10 min was the parameter that gave by far the best statistical properties, probably because animals were calmer having acclimatized to the conditions. We then used this procedure for the general analyses. For EWL, we computed the overall mean across the complete record.

From the respirometric records and according to the configuration of the system (i.e. flowmeter was upstream from the chamber, both CO<sub>2</sub> and water were scrubbed, and use of flow-mass controllers), we computed the following variables (see Withers, 1977):

1. Rate of CO<sub>2</sub> production ( $\dot{V}_{\text{CO}_2}$ ), as:

$$\dot{V}_{\text{CO}_2} = \text{FeCO}_2 \times \text{FR} - [\text{FeCO}_2 \times (\text{FiO}_2 - \text{FeO}_2)] / (1 - \text{FeCO}_2), \quad (1)$$

where  $\dot{V}_{\text{CO}_2}$  is in mlCO<sub>2</sub> min<sup>-1</sup>; FiCO<sub>2</sub> is the input fractional concentration of CO<sub>2</sub>; FeCO<sub>2</sub> is the excurrent fractional concentration of CO<sub>2</sub>; FR is the flow rate (ml min<sup>-1</sup>); FiO<sub>2</sub> is the input fractional concentration of O<sub>2</sub>; and FeO<sub>2</sub> is the excurrent fractional concentration of O<sub>2</sub>.

The fractional concentration of CO<sub>2</sub> was corrected before calculation for water dilution as:

$$\text{CO}_2 = \text{UCO}_2 \times \text{bp} / (\text{BP} - \text{WVP}), \quad (2)$$

where UCO<sub>2</sub> is the uncorrected CO<sub>2</sub> signal; bp is the barometric pressure (kPa); and WVP is the water vapor partial pressure (kPa).

2. Evaporative water loss (EWL) as:

$$\text{EWL} = \text{bp} (\text{p.p.t.} / 1000), \quad (3)$$

where EWL is in kPa; and p.p.t. is H<sub>2</sub>O provided, in parts per thousand.

3. Rate of O<sub>2</sub> consumption ( $\dot{V}_{\text{O}_2}$ ) as:

$$\dot{V}_{\text{O}_2} = (\text{FiO}_2 - \text{FeO}_2) \times \text{FR} / (1 + \text{FiO}_2 - \text{FeO}_2 - 0.2094), \quad (4)$$

where  $\dot{V}_{\text{O}_2}$  is in ml O<sub>2</sub> min<sup>-1</sup>.

4. Minimum thermal conductance (C) (McNab, 1980):

$$(a) C_{\dot{V}_{\text{O}_2}} = \dot{V}_{\text{O}_2} / (T_b - T_a), \text{ and } (b) C_{\dot{V}_{\text{CO}_2}} = \dot{V}_{\text{CO}_2} / (T_b - T_a), \quad (5)$$

where  $T_b$  is the body temperature and  $T_a$  is the ambient temperature.

Physiological variables were measured three times in most individuals, with a three-week interval between measurements (i.e. a total period of nine weeks).

### Statistics

All data were analyzed with Statistica 6.1 (StatSoft, www.statsoft.com). Repeatabilities were computed as the intraclass-correlation coefficient ( $\tau$ ), which is the ratio between inter-individual variance and total variance (inter-individual plus residual variance). Both variances were computed from one-way ANOVAs and expected mean squares in a variance component analysis, using body mass ( $M_b$ ) as covariable when the dependent variable was correlated with  $M_b$ . Standard errors of  $\tau$  were computed by jackknife, by deleting an individual each time and computing the (N-1)  $\tau$  (i.e. pseudovalues) and then by calculating the standard deviation of this sample of pseudovalues (Quinn and Keough, 2002; Roff, 2006).

### RESULTS

In all cases, during metabolic measurements, individuals fell asleep (indicated by the characteristic 'spherical' posture of the sleeping animals) after ca. 20 min in the chamber, and records rapidly attained steady-state values (Fig. 1). In spite of the fact that all individuals were normothermic (which is clearly evident by eye, because they were awake and active), individuals displayed a great deal of variation in body temperature ( $T_b$ ), ranging from 25 to 36°C (Table 1). We excluded two individuals that became torpid during the recording period. Torpor was indicated by a sudden drop in  $\dot{V}_{\text{O}_2}$  and  $\dot{V}_{\text{CO}_2}$  to 5% of the common resting values. Furthermore, torpid *D. gliroides* exhibit unambiguous behavioral characteristics in addition to low metabolism and  $T_b$  (R.F.N., C. Verdugo and P.C., unpublished) (see also Bozinovic et al., 2004). Torpid individuals are characterized by their low respiratory frequency and the position of ears and nose, which can be easily seen by eye during metabolic trials.

The large variation in  $T_b$  was also reflected in the physiological measures (Table 1, Fig. 1). There was a significant increase in body mass ( $M_b$ ) between trials ( $F_{2,14}=33.8$ ;  $P<0.001$ ; repeated-measures ANOVA; Table 1), and a reduction in  $T_b$  ( $F_{2,14}=7.8$ ;  $P=0.005$ ;

Table 1. Descriptive statistics of the studied variables

Trait	First measurement						Second measurement						Third measurement					
	N	Mean	Min	Max	s.e.	c.v.%	N	Mean	Min	Max	s.e.	c.v.%	N	Mean	Min	Max	s.e.	c.v.%
Body mass (g)	20	27.35	14.60	39.56	1.65	27.0	18	36.95	14.10	59.20	3.46	39.7	13	51.52	25.80	68.60	4.06	28.4
Body temperature (°C)	20	32.70	25.70	36.40	0.62	8.5	18	30.99	27.30	36.00	0.52	7.1	13	29.19	25.10	33.20	0.60	7.4
$C_{\dot{V}_{CO_2}}$ (ml min <sup>-1</sup> °C)	19	0.069	0.042	0.137	0.004	27.5	17	0.075	0.038	0.098	0.004	25.4	13	0.090	0.071	0.13	0.005	18.5
$C_{\dot{V}_{O_2}}$ (ml min <sup>-1</sup> °C)	20	0.064	0.017	0.913	0.047	34.5	18	0.074	0.032	0.110	0.005	27.9	13	0.099	0.073	0.12	0.004	13.1
$\dot{V}_{CO_2}$ (ml min <sup>-1</sup> )	19	0.784	0.408	0.981	0.032	17.7	17	0.831	0.397	1.32	0.059	28.9	13	0.996	0.650	1.614	0.078	28.1
$\dot{V}_{O_2}$ (ml min <sup>-1</sup> )	20	0.703	0.173	0.909	0.047	29.7	18	0.815	0.336	1.09	0.051	27.0	13	1.059	0.845	1.468	0.060	20.5
RQ	19	1.07	0.84	1.77	0.05	21.9	17	0.98	0.68	1.35	0.04	16.3	13	0.94	0.72	1.26	0.05	18.2
EWL (kPa)	19	162.07	67.31	330.44	14.01	36.7	16	105.69	69.63	164.15	7.76	29.4	13	116.7	43.43	406.84	26.2	81.0

$C_{\dot{V}_{CO_2}}$  is the minimum thermal conductance computed as  $C_{\dot{V}_{CO_2}} = \dot{V}_{CO_2} / (T_b - T_a)$ ;  $C_{\dot{V}_{O_2}}$  is the minimum thermal conductance computed as  $C_{\dot{V}_{O_2}} = \dot{V}_{O_2} / (T_b - T_a)$ ;  $\dot{V}_{CO_2}$  is the CO<sub>2</sub> production rate;  $\dot{V}_{O_2}$  is the O<sub>2</sub> consumption rate; RQ is the mean respiratory quotient; EWL is the evaporative water loss. N, number of samples; s.e., standard error; c.v., coefficient of variation.

repeated-measures ANOVA; Table 1). This decrease in  $T_b$  was observed as a negative correlation between  $M_b$  and  $T_b$  ( $R = -0.59$ ;  $F_{1,18} = 9.51$ ;  $P = 0.006$ , data from the first repetition), which suggests that larger individuals exhibited lower  $T_b$  values. Thermal conductance increased across repetitions as computed from  $\dot{V}_{CO_2}$  ( $F_{2,14} = 7.02$ ;  $P = 0.008$ ; repeated-measures ANOVA; Table 1) and from  $\dot{V}_{O_2}$  ( $F_{2,14} = 21.5$ ;  $P < 0.001$ ; repeated-measures ANOVA; Table 1). Although  $\dot{V}_{O_2}$  showed a significant increase across repetitions ( $F_{2,14} = 5.7$ ;  $P = 0.02$ ; repeated-measures ANOVA; Table 1),  $\dot{V}_{CO_2}$  did not change significantly ( $F_{2,14} = 0.54$ ;  $P = 0.60$ ; repeated-measures ANOVA; Table 1).

The magnitude of the respiratory quotient (RQ) was reduced across repetitions, but this trend was nonsignificant ( $F_{2,14} = 1.41$ ;  $P = 0.28$ ; repeated-measures ANOVA; Table 1). Within records (in each repetition), comparing the first, second and third hour of measurements, there was a consistent increase in RQ (Fig. 2). Respiratory water loss (RWL) did not change across repetitions ( $F_{2,12} = 3.16$ ;  $P = 0.08$ ; repeated-measures ANOVA; Table 1). Repeatability was near zero and nonsignificant for thermal conductance, measured both from  $\dot{V}_{CO_2}$  and  $\dot{V}_{O_2}$ , which suggests that this variable does not exhibit inter-individual variation (Table 2). However, with the exception of EWL, we found moderate repeatability in the remainder of the traits ( $M_b$ ,  $T_b$ ,  $\dot{V}_{CO_2}$ ,  $\dot{V}_{O_2}$  and RQ) (Table 2).

## DISCUSSION

In a population, the evolutionary potential, restrictions and trade-offs between a suite of traits are encapsulated in the structure of genetic variances and covariances between them, also known as the G-matrix (Steppan et al., 2002). Since its proposition, the powerful tools of multivariate quantitative genetics have been successfully applied to predict adaptive evolution in an enormous variety of organisms and traits (Roff, 2007). This, together with advances in phenotypic selection studies (Brodie et al., 1995; Hoekstra et al., 2001; Kingsolver et al., 2001), has produced a considerable improvement in the knowledge of the processes and mechanisms of adaptive evolution in the field. Most of these studies, however, have been restricted to short-lived animals and plants and conducted on morphological traits. The study of the sources of phenotypic variation in mammalian bioenergetics has received some attention (see Introduction), but mostly in rodents and other eutherians. It is surprising that marsupials and monotremes, which have been studied in considerable detail by physiological ecologists, are not known from this perspective. The first step in characterizing inter-individual variation in a trait or a group of traits is to determine its repeatability, which for energy metabolism appears to be high (ca.

60%), irrespective of species (Nespolo and Franco, 2007). Repeatability, computed as the intraclass correlation coefficient, represents the upper limit of heritability and hence could provide insight into whether a trait would present heritable variation without the need to perform pedigree-based analyses (Falconer and Mackay, 1997; Lessells and Boag, 1987). In this study, we performed a wide screening of the repeatability of bioenergetics in a little-studied marsupial, *D. gliroides*, and we found rather unexpected results, namely poor thermoregulatory abilities and low inter-individual variation in most bioenergetic traits. We will discuss each case separately.

### Body temperature

In eutherians, body temperature ( $T_b$ ) is one of the most precisely controlled physiological variables, which usually has a circadian amplitude of less than 2°C and is maintained over a wide range of ambient temperatures (Arend and McNab, 2001; Haim, 1996; Refinetti, 1999). Also (and probably as a consequence),  $T_b$  appears in eutherians as a repeatable trait that exhibits high heritability (Gordon and Rezvani, 2001; Nespolo et al., 2003; Rhodes et al., 2000). By contrast, non-hibernating marsupials and monotremes exhibit, in general, larger short-term variations in  $T_b$ , whose daily range is up to 6°C (Gemmell et al., 1997). In fact, in several marsupial species,  $T_b$  is correlated with metabolic rate (Kinnear and Shield, 1975; Opazo et al., 1999), reflecting their somewhat imperfect control of  $T_b$ . Our results suggest that awake (i.e. non-torpid) *D. gliroides* exhibits an unusually large thermal variation in  $T_b$  (up to 10°C). These results are supported by  $T_b$  measurements using implanted data loggers (R.F.N., C. Verdugo and P.C., unpublished) and by the negative correlation between body mass and  $T_b$ . This poor control of  $T_b$  indicates high residual variation in this trait, which is translated into low time-consistency, as its repeatability of 25% suggests.

### Thermal conductance

According to McNab (McNab, 1980), minimum thermal conductance, as measured in this study and below thermoneutrality, reflects the ease with which heat is exchanged between the body and the environment. Thus, it is a measure of the ability of the animal to maintain heat. The magnitudes of minimum thermal conductance computed from oxygen consumption ( $\dot{V}_{O_2}$ ) and CO<sub>2</sub> production ( $\dot{V}_{CO_2}$ ) (i.e.  $C_{\dot{V}_{O_2}}$  and  $C_{\dot{V}_{CO_2}}$ , respectively) that we found are in agreement with what Bozinovic and colleagues (Bozinovic et al., 2004) found for this species and are lower than those expected for marsupials. Both in small mammals (eutherians and marsupials) and birds (Novoa et al., 1994), thermal conductance

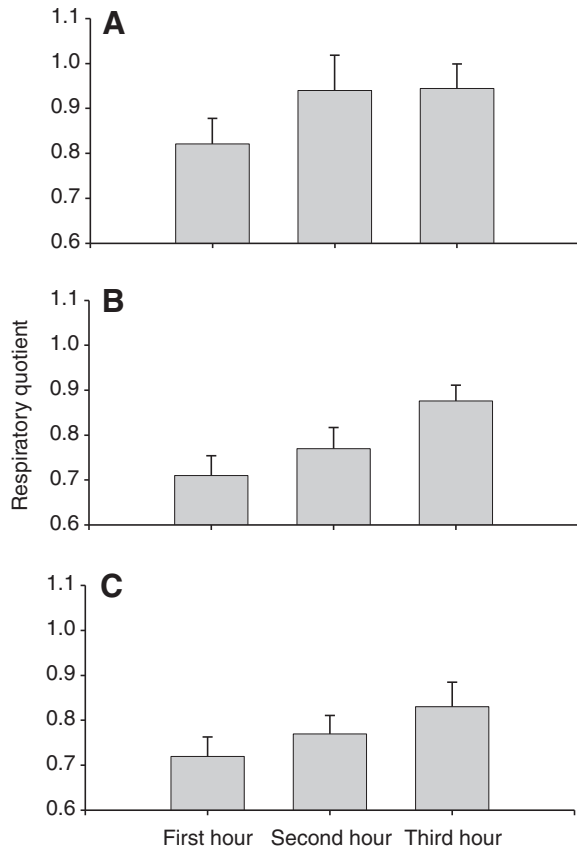


Fig. 2. Within-record comparisons of respiratory quotients (averages of the first, second and third hour of recording) in the first repetition (A), second repetition (B) and third repetition (C) (means  $\pm$  s.e.m.; A,  $N=19$ ; B,  $N=17$ ; C,  $N=13$ ). Differences were marginally significant in the first measurement ( $F_{2,36}=2.9$ ;  $P=0.068$ ; repeated-measures ANOVA), significant in the second measurement ( $F_{2,34}=8.10$ ;  $P=0.0013$ ; repeated-measures ANOVA) and non significant in the third measurement ( $F_{2,24}=2.61$ ;  $P=0.094$ ; repeated-measures ANOVA). After pooling repetitions, the comparison became highly significant ( $F_{2,98}=10.48$ ;  $P=0.0001$ ; repeated-measures ANOVA).

can exhibit changes on a seasonal basis and also after thermal acclimation (Bozinovic et al., 1990; Dawson and Olson, 1988; Holloway and Geiser, 2001; Smith and Dawson, 1984). This phenotypic plasticity suggests an adaptive value for thermal

conductance. However, this contrasts with the almost absolute absence of inter-individual variation in this trait in *D. gliroides* as only negative repeatability estimations were found (i.e. the actual value of repeatability was near zero). In other words, thermal conductance in *D. gliroides* would not respond to selection.

**Respiratory quotient**

During a single trial, animals exhibited a consistent increase in respiratory quotient, which could be interpreted as a shift from fat-based metabolism to carbohydrate-based metabolism (Buck and Barnes, 2000). This finding appears contradictory to the 6h fasting state that animals experienced as they should gradually use fat reserves after a period of fasting. However, it should be noted that, in carnivorous mammals, 6h of fasting could be enough for gut emptiness to occur but not for the complete metabolizing of nutrients in circulating blood (Hume et al., 1993). These nutrients were the main fuel for individuals during the first hour of metabolic recording, which explains the low RQ at the beginning of the trials. Then, metabolism shifted into carbohydrate-based metabolism as the first line of energy reserves are liver and muscle glycogen (Willmer et al., 2005). Similarly, Walsberg and Wolf (Walsberg and Wolf, 1995) found in Verdins (insectivorous birds) fed with mealworms (as our *Dromiciops*) that RQ increased during the first six hours of fasting, from 0.71 to near 0.80, and showed that House Sparrows (a granivorous bird) fed with grains actually reduced their RQ during the same period, an exactly inverse result (from 0.81 to 0.70). In other words, insectivorous animals appear to exhibit similar trends in the use of energy reserves, irrespective of species.

Our repeatability estimation of RQ was a value of 30% and was significant. This suggests that, to some extent, the capacity to metabolize different nutrient types is not completely determined by the type of diet and the physiological status of the animal. That is to say, some individuals would be better suited to carbohydrate-based metabolism and others to fat-based metabolism. Whether this inter-individual variation could be of adaptive significance will depend on the pressures imposed by their environment. The environment of *D. gliroides* is characterized by seasonal and unpredictable food availability. In fact, in summer and autumn, this species is frugivorous, shifting to insectivory during autumn and winter (Amico and Aizen, 2000). In this sense, inter-individual variation in RQ could benefit *D. gliroides* as it would permit it to respond to changes in food availability, both in type and magnitude.

Table 2. Repeatability of metabolic variables (intraclass correlation coefficient,  $\tau$ ), computed as the ratio between inter-individual variance and total variance from one-way ANOVAs or ANCOVAs using body mass ( $M_b$ ) as covariable when correlated with the variable being measured

Variable	Covariable	Between-individual variance	Within-individual variance	$\tau \pm$ s.e.
$T_b$ ( $^{\circ}$ C)	$M_b$ $F_{1,37.4}=12.2$ $P=0.0013$	1.55	4.73	$0.25 \pm 0.036$ $F_{20,29}=1.78$ $P=0.078$
Body mass (g)	—	42.8	196.1	$0.18 \pm 0.026$ $F_{20,30}=1.53$ $P=0.14$
$C_{\dot{V}CO_2}$ (ml $min^{-1}^{\circ}$ C)	$M_b$ $F_{1,46.9}=24.4$ $P<0.0001$	-0.37	2.37	$-0.18$ $F_{19,28}=0.63$ $P=0.85$
$C_{\dot{V}O_2}$ (ml $min^{-1}^{\circ}$ C)	$M_b$ $F_{1,46.6}=33.6$ $P<0.0001$	-0.16	3.97	$-0.04$ $F_{20,29}=0.63$ $P=0.85$
$\dot{V}_{CO_2}$	$M_b$ $F_{1,39.2}=15.3$ $P=0.0004$	58.66	355.53	$0.14 \pm 0.03$ $F_{19,28}=1.39$ $P=0.21$
$\dot{V}_{O_2}$	$M_b$ $F_{1,37.8}=26.9$ $P<0.0001$	79.92	249.98	$0.24 \pm 0.02$ $F_{20,29}=1.70$ $P=0.09$
RQ	—	0.013	0.027	$0.32 \pm 0.03$ $F_{20,30}=2.13$ $P=0.031$
EWL (kPa)	—	2281.7	2397.8	$0.49 \pm 0.09$ $F_{19,27}=3.23$ $P=0.003$

Degrees of freedom for the covariable were computed by the Satterthwaite approximation.

$C_{\dot{V}CO_2}$  is the minimum thermal conductance computed as  $C_{\dot{V}CO_2} = \dot{V}_{CO_2} / (T_b - T_a)$ ;  $C_{\dot{V}O_2}$  is the minimum thermal conductance computed as  $C_{\dot{V}O_2} = \dot{V}_{O_2} / (T_b - T_a)$ ;  $\dot{V}_{CO_2}$  is the  $CO_2$  production rate;  $\dot{V}_{O_2}$  is the  $O_2$  production rate; RQ is the respiratory quotient averaged over the whole record (three hours); EWL is the evaporative water loss.

### Evaporative water loss

Our results suggest that evaporative water loss (EWL) exhibits high and significant repeatability in *D. gliroides*. This is in accordance with a previous determination of EWL repeatability in a rodent (Hayes et al., 1998), suggesting that inter-individual variation in this trait exists. The adaptive value of EWL as a trait has been suggested several times as it exhibits physiological flexibility after laboratory acclimation (Williams and Tieleman, 2000) and is reduced in marsupial species of desert environments (Dawson et al., 2000; Gibson and Hume, 2000). In fact, Diaz and colleagues (Diaz et al., 2001) reported a high capacity for urine concentration in a didelphid, comparable to values in desert rodents. Thus, it is plausible that the interspecific variation in EWL that we found could present evolutionary potential.

### Energy metabolism

According to Bozinovic and colleagues (Bozinovic et al., 2004), *D. gliroides* exhibits lower values of energy metabolism than what is expected for a marsupial of its size. Our  $\dot{V}O_2$  and  $\dot{V}CO_2$  (corrected by RQ) measurements at 20°C are in accordance with those reported by these authors. However, metabolic rate appears to present low repeatability, which could be explained by the poor thermoregulatory control that this species exhibits. The repeatability of energy metabolism in *D. gliroides* is not in accordance with what is known for mammals (see Nespolo and Franco, 2007 and references therein), although we do not know of another repeatability study performed on marsupials. Furthermore, although marsupials in general are considered to exhibit 'mammalian' physiological features (Crompton et al., 1978; Hinds and MacMillen, 1984), our results unambiguously suggest that *D. gliroides* (and hence Microbiotheria) furnishes an exception. In terms of thermoregulatory abilities, and with the exception of evaporative water loss, *D. gliroides* exhibits 'reptilian' features (e.g. large amplitude in  $T_b$ , correlation between  $T_b$  and  $M_b$ , low time-consistency of energy metabolism, low thermal conductance).

### Did microbiotherians have a reptile-like physiology?

The order Microbiotheria, with its seven described species, belongs to the Australidelphia magnorder, of Australasian origin (McKenna and Bell, 1997). The oldest fossil is dated ca. 40 million years of age, when Australia, South America and the Antarctic continent were merged into Gondwana. The extinction of Microbiotheria coincided with the invasion of the North American eutherian fauna, after the formation of the Panama isthmus (ca. 3.5 million years ago), with *D. gliroides* being the sole surviving species (McKenna and Bell, 1997). According to Ruben (Ruben, 1995) (see also Warren et al., 2008), extant mammals (monotrematas, marsupials and eutherians) probably shared a common ancestor approximately 160 million years ago, and mammals probably evolved from mammal-like reptiles (therapsids) 200 million years ago. Thus, the evolution of endothermy could be a process that occurred during the past 100 million years, which includes the rise and supposed extinction of Microbiotheria.

Several competing hypotheses have been proposed to explain the evolution of endothermy in birds and mammals (see Hayes and Garland, 1995). All of them propose that, at some point, natural selection acted either directly or indirectly on body temperature, energy metabolism and/or thermal conductance. Any response to selection would have needed genetic variation in these traits. As repeatability represents the upper limit of heritability, low repeatabilities would be indicative of low genetic variation, and hence a low potential to respond to selection in a given trait. Thus,

our results suggesting low repeatability in bioenergetic traits in the 'relict mammal' *D. gliroides* support the idea of a low potential to respond to selection on these traits in Microbiotheria. The low thermoregulatory abilities of this species, together with the recent evidence of the high repeatability and genetic variation in energy metabolism of eutherian mammals (see Introduction), suggests that the adaptive shifts towards controlled endothermy should have happened recently. This is of course an ambitious conclusion that needs confirmation by further research on other basal species such as insectivores and monotrematas. However, this would not be the first time that an author has inferred past evolutionary processes from experimental data obtained from an extant species (e.g. Bennett et al., 2000; Crompton et al., 1978; Dohm et al., 2001; Sadowska et al., 2005).

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