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Effects of environmental temperature on oxygen diffusion capacity during post-natal development in the altricial rodent, *Phyllotis darwini*

Efectos de la temperatura ambiental sobre la capacidad de difusión de oxígeno durante el desarrollo postnatal en el roedor altricial *Phyllotis darwini*

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ABSTRACT

In this contribution we studied the developmental phenotypic plasticity of respiratory structures involved in the exchange of gases in an altricial rodent, the leaf-eared mouse (*Phyllotis darwini*). We studied morphological pulmonary parameters of warm (30 °C) and cold acclimated animals 15 °C) at different developmental stages. We found that acclimation treatments did not affect neither lung volume, the alveolo-capillary barrier thickness nor the respiratory surface density. In consequence the oxygen diffusion capacity was not affected. Although *Phyllotis darwini* exhibited structural respiratory changes expected by its ontogenetic development, these structures were not affected by thermal acclimation during the post-natal development.

Key words: phenotypic plasticity, lung, altricial rodents.

RESUMEN

Estudiamos la plasticidad fenotípica del desarrollo de las estructuras respiratorias responsables del intercambio de oxígeno en el roedor altricial *Phyllotis darwini*. Investigamos los parámetros morfológicos del pulmón en animales aclimatados a alta temperatura (bajo requerimiento energético de termorregulación: 30 °C) y a baja temperatura (alto requerimiento: 15 °C) en diferentes etapas del desarrollo. Encontramos que la temperatura ambiental no afectó el volumen pulmonar, el grosor de la barrera alvéolo-capilar ni la densidad de superficie alveolar. Y en consecuencia, tampoco la capacidad de difusión de oxígeno. Aun cuando *Phyllotis darwini* mostró los cambios respiratorios esperados de acuerdo a su desarrollo ontogénico, las estructuras evaluadas no fueron afectadas por la aclimatación térmica durante el desarrollo postnatal.

Palabras clave: plasticidad fenotípica, pulmones, roedores altriciales.

INTRODUCTION

Phenotypic plasticity of physiological features, or physiological flexibility, which has been visualized as an adaptive response, it is very common among vertebrate species (Pigluicci 1996, Sabat & Bozinovic 2000, Pigluicci et al. 2006). By contrast, some morphological features appear to be more rigid and rarely are modified by the environmental cues (Sabat et al. 1998). In addition, some authors have reported the existence of phenotypic plasticity of morphology and physiology during ontogeny but absence of response when animals are adults, i.e., exhibiting an inflexible norm of reaction (Toloza & Diamond 1990, Biviano et al. 1993, Bozinovic 1993, Zhao et al. 1996). Thus the plasticity of some morphological traits may be dependent on the ontogenetic stage at which acclimation acts (Sabat & Bozinovic 2000), whereas for others it does not (McKechnie et al. 2007).

Accordingly, studies of phenotypic variability induced by the environment may underestimate the potential plastic response if these studies use only adult animals

(Schlichting & Pigliucci 1998). Several studies have demonstrated the existence of structural variation during the development of the respiratory organs in mammals (Collins et al. 1986, Winkler & Cheville 1987, Castleman & Lay 1990, Massaro & Massaro 2002) and birds (Seymour et al. 2004, Runciman et al. 2005). Additionally, the structures linked with aerobic capacities have been reported to undergo dramatic ontogenetic changes, and also it appears to be more plastic in altricial than in precocial mammals during development (Tenney & Remmers 1966, Burri 1974, Blanco et al. 1991, Burri et al. 1991, Hammond et al. 1999, 2001, Canals et al. 2000, McMutry 2002). For example, alveolar septation in precocial mammals is developed in utero, while in altricial animals the septation is post-natal (Collins et al. 1986, Winkler & Cheville 1984, Castleman & Lay 1990, Massaro & Massaro 2002); alveoli of the rat (*Rattus* sp.) do not develop adequately until the day 7 after birth; its alveolar-capillary barrier begins to be slimed between the second and third weeks (Burri 1974, Burri et al. 1991), and ramification in the aerial pathways of this species varies during post-natal development (Canals et al. 2000).

The influence of climate on metabolic capacities of rodents has been well documented (Rosenmann & Morrison 1974, Bozinovic & Rosenmann 1989, Tieleman et al. 2002, 2003, Novoa et al. 2005). Nevertheless the results appear to be contradictory with regard to environmentally-induced respiratory challenges. For example, while high altitude does not seem to affect alveolar septation in *Cavia porcellus* (von Gesner, 1554) and *Ovis aries* (Linnaeus, 1758) (Tenney & Remmers 1966), hypoxia increases alveolar size and surface in rats (*Rattus* sp.) (Blanco et al. 1991). Hammond et al. (1999, 2001) reported phenotypic changes in the rodent *Peromyscus maniculatus* (Wagner, 1845) acclimatized to high and low altitudes; these authors demonstrated that rodents acclimatized to lower oxygen partial pressures have higher hematocrit and larger lung and heart masses than those acclimatized to high oxygen partial pressures. Regarding energetic demands imposed by the style of locomotion, associations between erythrocyte size and oxygen diffusion capacity and variations in the morphology of the bronchial tree have been also reported (Canals et al. 2007, 2008).

Considering the strict association between oxygen consumption and structural oxygen diffusion capacity (oxygen conductance), and the proposition suggesting that the state of structural design is commensurate to functional needs from regulated morphogenesis (hypothesis of symmorphosis) (Weibel et al. 1991, 1992, Seymour et al. 2004, 2005, Canals et al. 2005, 2007, Runciman et al. 2005, Figueroa et al. 2007) we expected that the variables involved in the interchange and distribution of gases have the ability to change during the post-natal development according to the different metabolic demands imposed by the thermal environment. Hence, in this contribution we studied the environmentally induced developmental phenotypic plasticity in the respiratory structures involved in the exchange of gases in the leaf-eared mouse *Phyllotis darwini* (Waterhouse 1837) (Rodentia: Muridae), an altricial rodent which inhabits central Chile.

MATERIAL AND METHODS

Animal models and sample size

Fifteen adult males (mean [± SD] body mass = 54.4 ± 17.3 g) and 15 adult females (37.7 ± 7.6 g) of *P. darwini*, a rodent dwelling in grasslands, scrub, open forest and rocky areas, were captured in Quebrada de la Plata, Maipú in central Chile, (33°27' S, 70°42' W), between September and December, 2005. The animals were taken to the laboratory, where they were kept in pairs at room temperature with food (sunflower seeds and rabbit food Champion®) and water ad lib. After mating, pregnant females were maintained in individual cages (40 x 40 x 20 cm) in thermoregulated climate chambers and randomly assigned to one of two temperature treatments. One group was maintained at low energetic requirements for thermoregulation at a constant temperature of 30 ± 2 ºC (warm acclimated group) and a second group maintained at high energetic requirements for thermoregulation below the inferior limit of thermoneutrality for this species (Bozinovic et al. 1988) at 15 ± 2 ºC (cold-acclimated group). The animals were observed daily, and were not allowed to build nest. The offspring were kept

with the mother until the experimentation day or until weaning. After offspring were born, three unrelated individuals were selected for each environmental condition at days: one (newborn), seven, 14, 21 and 60 (adult). The entire sample collection was between December 2005 and December 2007. In the newborn group at 30 ºC, four individuals were analyzed. The individuals had to be sacrificed in order to perform the structural study. Thus, except for one group with four individuals, three independent individuals were studied for each age group and environmental condition, leading to a total sample size of n = 31.

Pulmonary structure

After the metabolic experiments, individuals were euthanized using CO_2, complying with the current laws of Chile and the standards of the ethical committee of the Facultad de Ciencias, Universidad de Chile, where the experiments were performed. A small tracheotomy was performed and the lungs instilled with a 2.5 % glutaraldehyde in a 0.01 mol L^{-1} phosphate buffer solution (pH = 7.4, 350 mOsm) using a plastic catheter with the reservoir located 20 cm above the level of the sternum. The trachea was ligated to maintain the intrapulmonary fixative volume. After 24 h, the lungs were removed by thoracotomy and their volumes (V_L) were estimated by means of a water displacement method (Scherle 1970).

Immediately following this procedure, the lungs were removed and immersed in the same fixative at 4 °C for a minimum of two hours. Next, tissues (right and left lungs) were processed for routine light microscopy (LM) and transmission electron microscopy (TEM). Briefly, two pieces of 1-2 mm thickness (one for LM and one for TEM) were obtained from each one of three zones (upper, middle and basal) in each lung. The pieces were washed with buffer and post-fixed with 1 % osmium tetraoxide for 1 h at 4 °C. For light microscopy slices were dehydrated in ascending series of ethanol and infiltrated and embedded in epoxy resin constructing cubes of 2-3 mm^3, obtaining semi-thin randomly oriented sections of 1 μm. Tissue samples were stained with 1 % toluidine blue and viewed with a microscope. For TEM, the pieces were stained with 1 % osmium tetroxide, en bloc stained with 2 % uranyl acetate, and dehydrated in ascending series of alcohol. Ultrathin sections of 60-90 nm of thickness were made, contrasted with Pb-citrate and mounted on copper mesh grids and viewed in a JOEL/JEM 100SX transmission electron microscope. Sections were photographed and digitalized, and twelve semi-thin and twelve ultra-thin sections per individual were analyzed using Scion Image Software.

The respiratory surface density (AS_d) was estimated by means of the line-intersection stereological method (Weibel 1970-1971) in the semi thin sections with light microscopy at 10X magnification:

$$AS_d = \frac{2I}{1/2 \cdot P_T \cdot Z},$$

where I is the number of intersections between line probes of length Z with the respiratory surface and P_T is the number of testing points. The number of line segments was $^1/_2$ the number of points.

The harmonic mean thickness of the air-blood barrier (τ_h) was estimated by a stereological method in a square lattice grid as suggested by Weibel (1970-1971) and Maina (2002):

$$\frac{1}{\tau_h} = \frac{3}{2} \cdot \frac{\sum_{j=1}^{m} f_j \cdot \frac{1}{l_j}}{\sum_{j=1}^{m} f_j},$$

where l_j is the mid-value of the intercept length of linear probes (on the direction of the line), f_j the frequency of class j and m is the number of classes.

The thickness of the alveolo-capillary barrier, the density of the respiratory surface and the lung volume allowed the estimation of the oxygen diffusion capacity. The parenchymal lung volume was estimated as $Vp = 0.9 \bullet V_L$ (Maina 2002). From these structural measurements the morphometric oxygen diffusion capacity (tissue) was estimated using:

$$D_tO_2 = \kappa \frac{AS_d \cdot Vp}{\tau_h},$$

where D_tO_2 is the oxygen diffusion capacity of the alveolo-capillary barrier (tissue) and κ is the Krogh's diffusion coefficient $\kappa = 4.1 \cdot 10^{-10}$ $cm^2\ s^{-1}\ mbar^{-1} = 4.1 \cdot 10^{-12}\ cm^2 \cdot s^{-1} \cdot Pa^{-1}$ (Gehr et al. 1981). AS_d is the alveolar surface density, Vp is the parenchymal lung volume and τ_h is

the harmonic mean thickness of the blood-gas barrier. The total air-erythrocyte oxygen diffusion capacity was estimated with $D_LO_2 \approx D_tO_2/10$, a reasonable estimator derived from the ratio D_LO_2/D_tO_2. From data of Maina et al. (1991) and Maina (2002) we observed that D_LO_2 is about 1/10 of D_tO_2: $D_LO_2/D_tO_2 = 0.092 \pm 0.070$ in 32 birds and 0.100 ± 0.031 in 10 bats (Canals et al. 2005).

The body mass response and the two structural measures (AS_d and τ_h) were analyzed with a two-way analysis of variance (ANOVA), using acclimation temperature and age groups as factors. The lung volume of the two groups was compared with an analysis of covariance, using the age as a co-variable.

RESULTS

Lung volume

Body mass increased at a similar rate in the two groups ($F_{1,21} = 0.176$, $P = 0.68$) (Table 1). Because some tissues had variable degrees of lung collapse or partial rupture, we only obtained useful measurements of lung volume in 16 individuals (Table 2). Since this produced an imbalance in the model with combinations of age groups and experimental conditions, we compared lung volume between the two temperatures using an analysis of covariance, considering age as a covariate. Lung volume increased with age ($F_{2,14} = 23.74$, $P < 0.001$), while mass-specific lung volume was similar among groups ($F_{2,14} = 0.347$, $P > 0.05$). No differences were found neither for absolute nor for mass-specific lung volume between experimental groups ($F_{1,14} = 0.038$, $P = 0.83$ and $F_{1,14} = 0.347$, $P = 0.79$, respectively).

Blood-gas barrier

The thickness of the alveolo-capillary barrier diminished with age in both groups ($F_{4,21} = 2.75$, $P = 0.055$), from 0.434 ± 0.046 μm to 0.376 ± 0.134 μm in the groups acclimated to 30 ºC and from 0.419 ± 0.024 μm to 0.322 ± 0.042 μm in the group acclimated to 15 ºC, but there were no differences among the temperature groups ($F_{1,21} = 0.227$, $P = 0.64$) (Fig. 1 and 2).

TABLE 1

Body mass (Mb) of *Phyllotis darwini* during the post-natal development at two environmental temperatures 15 and 30 ºC Average ± 1 standard deviation

Masa corporal (Mb) de *Phyllotis darwini* a través del desarrollo postnatal bajo dos temperaturas ambientales: 15 y 30 °C. Promedio ± 1 desviación estándar

Age (days)	Body mass (Mb) (g)	
	15 °C	30 °C
1	3.63 ± 0.63	3.95 ± 0.82
7	9.57 ± 0.94	8.23 ± 1.04
14	14.63 ± 0.91	11.53 ± 2.05
21	18.43 ± 6.31	16.73 ± 1.15
60	38.0 ± 8.23	41.26 ± 1.47

TABLE 2

Lung volume (V_L) and mass-specific lung volume (V_L/Mb) of individuals of different ages of *Phyllotis darwini* at two environmental temperatures 15 and 30 °C. Sample size in brackets

Volumen pulmonar (V_L) y volumen pulmonar masa-específico (V_L/Mb) de individuos de diferentes edades del roedor *Phyllotis darwini* bajo dos temperaturas ambientales: 15 y 30 °C. Número de pulmones en paréntesis

Age (days)	Lung volume (V_L)(cm^3)		Mass-specific lung volume V_L/Mb($cm^3\ g^{-1}$)	
	15 °C	30 °C	15 °C	30 °C
1	0.128 ± 0.030 (3)	0.124 ± 0.042 (3)	0.037 ± 0.016	0.033 ± 0.013
7	0.275 ± 0.058 (3)	0.342 ± 0.219 (3)	0.029 ± 0.009	0.039 ± 0.029
14	-	0.330 ± 0.067 (2)	-	0.027 ± 0.010
21	0.600 (1)	-	0.025	-
60	-	1.04 (1)	-	0.024

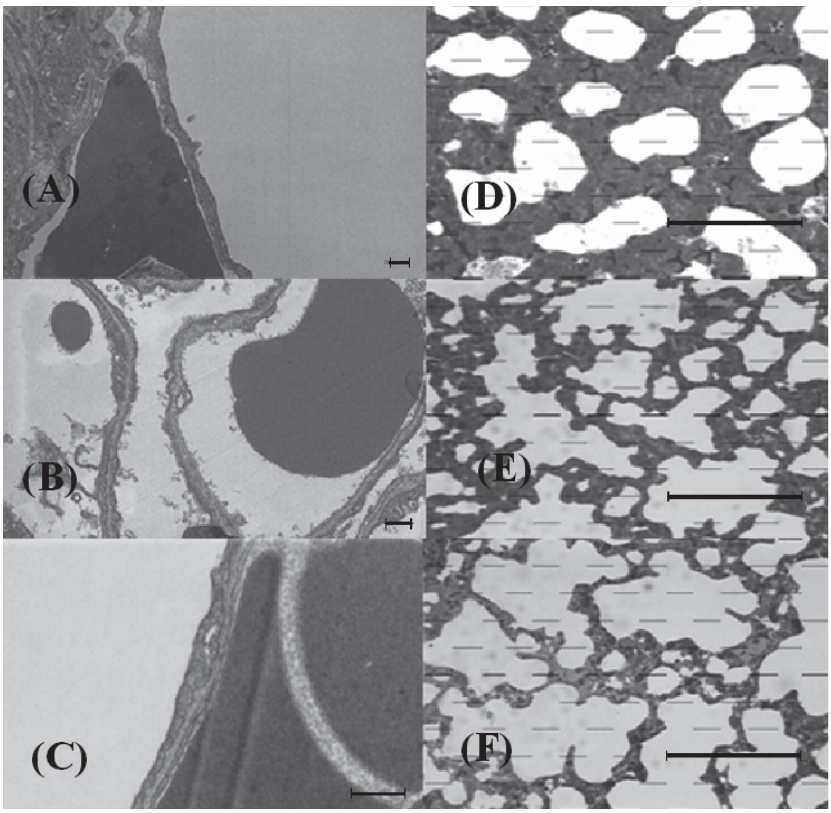

Fig. 1: Optic and transmission electronic microscopy of the lung tissue in the species *Phyllotis darwini* at different developmental stages: (A) one day old, 8000X, scale bar = 1 μm, (B) fourteen days old, 8000X, scale bar = 1 μm, (C) sixty days old, 10000X, scale bar = 1 μm, (D) one day old, 10X, scale bar = 100 μm, (E) fourteen days old, 10X, scale bar = 100 μm, (F) sixty day old, 10X, scale bar = 100 μm.

Microscopía óptica y electrónica del tejido pulmonar en *Phyllotis darwini* en diferentes estados de desarrollo: (A) un día de edad, 8000X, barra = 1 μm, (B) catorce días de edad, 8000X, barra = 1 μm, (C) sesenta días de edad, 10000X, barra = 1 μm, (D) un día de edad, 10X, barra = 100 μm, (E) catorce días de edad, 10X, barra = 100 μm, (F) sesenta días de edad, 10X, barra = 100 μm.

Alveolar surface density

The density of the alveolar surface increased with age in both groups ($F_{4,21}$ = 3.49, P = 0.024), from 902.75 ± 194.62 cm^{-1} to 1,802.20 ± 450.37 cm^{-1} in the group acclimated to 30 ºC and from 973.70 ± 363.21 cm^{-1} to 1,392.06 ± 311.78 cm^{-1} in the group acclimated to 15º C, with no difference between temperature groups ($F_{1,21}$ = 0.323, P = 0.576) (Fig. 1 and 3).

Oxygen diffusion capacity

Oxygen diffusion capacity was obtained based upon the density of alveolar surface, the thickness of the alveolo-capillary barrier, and lung volumes estimations, by the Scherle method when possible and by the allometric relationship $V_L = 0.046Mb^{1.06}$ (body mass in kg and V_L in L) in other cases (Schmidt-Nielsen 1987). Both, the oxygen diffusion capacity and the mass specific oxygen diffusion capacity increase with age ($F_{4,21}$ = 25.22 and $F_{4,21}$ = 3.62; P < 0.01 respectively; P = 0.021); however, no difference was found between warm and cold acclimated groups ($F_{1,21}$ = 0.25 and $F_{1,21}$ = 0.13 for total and mass specific oxygen diffusion capacity; P > 0.62 and 0.72 respectively) (Table 3).

DISCUSSION

We expected that the altricial rodent *Phyllotis darwini*, exposed to high energetic

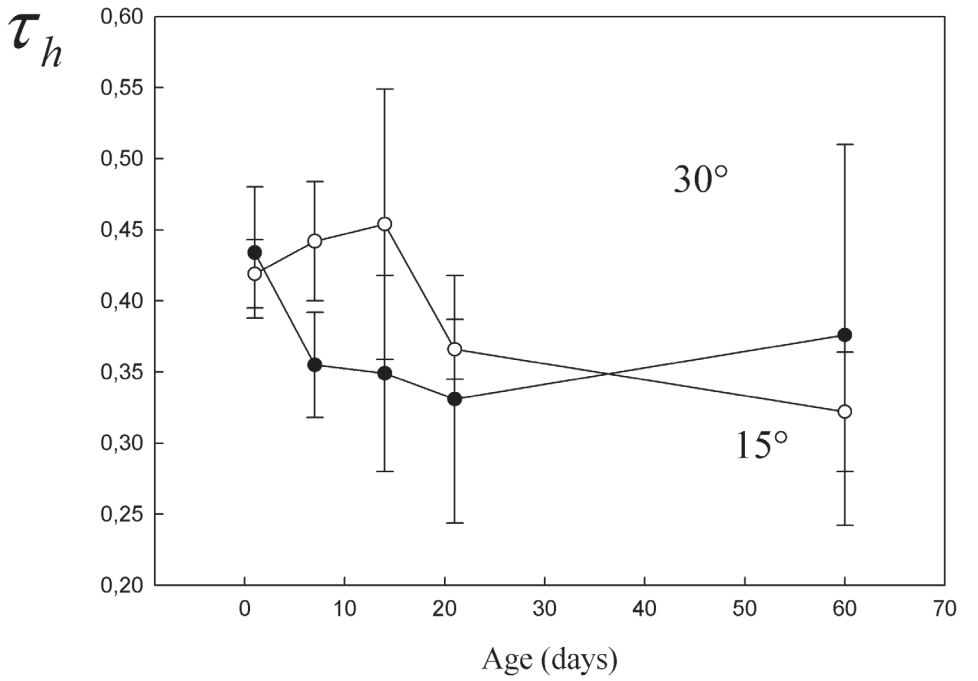

Fig. 2: Alveolo-capillary barrier thickness (τ_h) of warm acclimated animals (30 °C; bold circles) and cold acclimated animals (15 °C; open circles) of the species *Phyllotis darwini*, at different ages.

Grosor de la barrera alvéolo-capilar (τ_h) de animales de la especie *Phyllotis darwini* aclimatados a alta (30 °C; círculos negros) y baja temperatura (15 °C; círculos blancos), a diferentes edades.

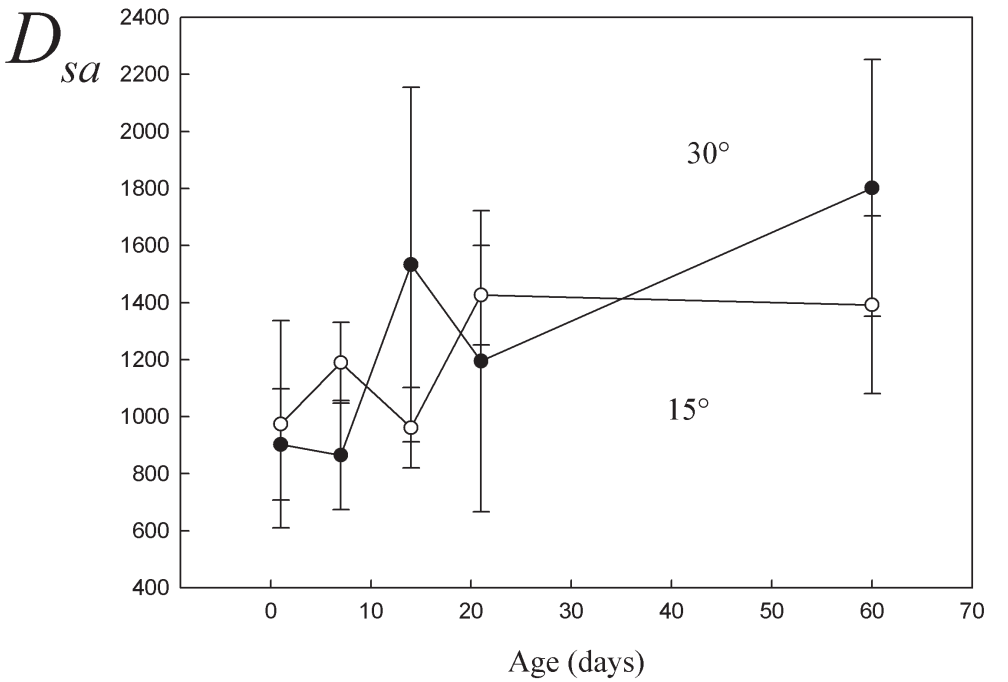

Fig. 3: Respiratory surface density (AS_d) of warm acclimated animals (30 °C; bold circles) and cold acclimated animals (15 °C; open circles) of the species *Phyllotis darwini*, at different ages.

Densidad de superficie respiratoria (AS_d) de animales de la especie *Phyllotis darwini* aclimatados a alta (30 °C; círculos negros) y baja temperatura (15 °C; círculos blancos), a diferentes edades.

TABLE 3

Estimations of the oxygen diffusion capacity (D_LO_2) and the mass specific-D_LO_2 in individuals of different ages of *Phyllotis darwini* at two environmental temperatures 15 and 30 °C

Estimaciones de la capacidad de difusión de oxígeno total (D_LO_2) y masa-específica (D_LO_2/g) en individuos de diferentes edades del roedor *Phyllotis darwini* bajo dos temperaturas ambientales: 15 y 30 °C

Age	Temperature (°C)	Oxygen diffusion capacity, D_LO_2 ($mLO_2\ s^{-1}Pa^{-1}$) x 10^{-5}	Mass specific oxygen diffusion capacity, D_LO_2/g($mLO_2\ s^{-1}Pa^{-1}g^{-1}$) x 10^{-6}
1	30	1.02 ± 0.52	2.66 ± 1.48
7	30	2.35 ± 1.74	2.74 ± 1.73
14	30	5.68 ± 1.96	5.20 ± 2.37
21	30	6.74 ± 0.43	3.89 ± 0.48
60	30	28.77 ± 13.4	6.99 ± 3.29
1	15	1.15 ± 0.71	3.48 ± 2.77
7	15	2.79 ± 0.97	3.01 ± 1.35
14	15	4.21 ± 1.04	2.86 ± 0.56
21	15	8.62 ± 3.68	4.68 ± 1.24
60	15	23.01 ± 7.4	6.20 ± 2.12

thermoregulatory requirements would reach higher aerobic capacity as adults and that such modification would be accompanied by changes in the structural lung parameters that facilitate oxygen diffusion. However, the two experimental groups exhibited similar curves of increase of body mass, development of the alveolo-capillary membrane, lung volume and alveolar surface density. In spite of the difference in the thermal environment experienced by the animals, the oxygen diffusion capacity of the alveolo-capillary membrane was similar in the two groups.

Individuals showed only the changes expected by their ontogenetic development. Values of D_LO_2 increased with the age and size of animals, reaching their maximum in the adult stage in both experimental groups. This occurs mainly as a consequence of the reduction of the alveolo-capillary membrane and the increase of the alveolar surface density, since the mass-specific lung volume was practically constant. Regarding the two dominant factors τ_h and AS_d, τ_h reduced its thickness by 23.8 %, in the warm acclimated group and by 24.9 % in the cold acclimated group, while the density of the alveolar surface increased by 99.6 % in the warm acclimated group, and by 43.7 % in the cold acclimated group. Thus, the alveolar surface density appears to be the main factor responsible for the change in D_tO_2 and as a consequence in D_LO_2. This is consistent with the reported pattern of postnatal development of alveolar septation in altricial mammals such as *Rattus rattus* (Burri 1974, Burri et al. 1991, McMutry 2002).

One possible explanation for the lack of plasticity is the existence of maternal effects that could reduce the effects of the environmental conditions. In this sense, Nespolo (2003) reported that in *P. darwini* there is a strong correlation between growth rates before weaning and adult body mass. In our case although we can not deny the existence of maternal effects, it is probable that those were randomly distributed and paired in both experimental traits. If maternal effects were different between experimental groups, differences in the structural variables at early developmental stages would be expected. However we did not find differences in pulmonary variables nor in body mass. In contrast, differences in metabolism and in the thermoregulatory capacity of newborn offspring of this species can be obtained by exposing pregnant mothers to different environmental temperatures (M. Canals unpublished results).

In our experimental setup, offspring were kept with the mother until weaning, otherwise they might have died. Nevertheless, individuals could also save energy by huddling, which allows them to reduce their overall surface structure by masking out the effect of thermal stress (Canals et al. 1989, 1997, 1998, Canals & Bozinovic 2009). For example, in *Mus musculus* the energy saving during huddling (huddling effectiveness), varies depending on the developmental stage from 65.0 % in juveniles to 49.8 % in sub-adults and 42.4 % in adults, probably as a result from variations in the capacity to change form or shape when huddling (Canals et al. 1998). However, huddling in newborn was present in both experimental conditions even though one experimental temperature was within the thermoneutral zone (Bozinovic 1988) whilst the

other one was at least 13 °C below the minimal critical temperature of the thermoneutral zone for adults of this species. Huddling is a usual behavior in newborns at any environmental temperature because the thermoregulatory capacity is not well developed and the thermoneutral zone is displaced toward high temperatures in early stages of development (Canals et al. 1998). In more advanced developmental stages, huddling behavior, although present, may be insufficient to mask thermal stress all the time. Assessing the effect of 15 °C environmental temperature in this species (using Bozinovic et al. 1988) we estimate increments of about 36 % of the energetic requirements to maintain homeothermy in always grouped individuals of this species. When individuals are not grouped this increase in the energetic requirements reaches 121 %. Except for newborns it is improbable that individuals were always grouped. Huddling behavior dynamics change in time, and individuals can move from one place to another. For example, in *M. musculus* individuals sometimes form groups and sometimes are isolated and the critical temperature below which huddling really reaches high frequency is about 15 °C (Canals & Bozinovic in press). Thus despite huddling behavior, we are confident that an environmental temperature of 15 °C, is stressful for the animals.

On the other hand, lack of plasticity may be a consequence of the high Andean origin of *P. darwini* (Engel et al. 1998). Higher energetic requirements for thermoregulation maintained for long time might have conditioned at an evolutionary scale, the possession of an optimized air-blood barrier (Figueroa et al. 2007) independent of the present requirements experienced by the animals. In this study, adults of *P. darwini* showed a very thin alveolo-capillary barrier: 0.322-0.367 μm, and also an oxygen diffusion capacity value between 3.2 and 16.3 % higher than the expected value for a mammal of its body size: 6.01 x 10^{-6} mLO_2 s^{-1} $Pa^{-1}g^{-1}$ (Figueroa et al. 2007) and also higher than other small murids such as *Mus musculus* (Linnaeus, 1758), *Mus wagneri* (Linnaeus, 1758), *Rattus rattus* (Linnaeus, 1758), (Gehr et al. 1981), *Abrothrix andinus* (Philippi, 1858) and *Abrothrix olivaceus* Waterhouse, 1837 (Canals et al. 2005).

In this vein, at our knowledge there are not studies of the heritability of oxygen diffusion capacity, which could estimate the extent to which a trait has been under selection in the past (Nespolo et al. 2003). Thus how quickly those traits will respond to selection remains unknown.

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